Increased CD8 Tumor Infiltrating Lymphocytes in Colorectal Cancer Microenvironment Supports an Adaptive Immune Resistance Mechanism of PD-L1 Expression

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Abstract

Background: Tumor cells express programmed death ligand-1 (PD-L1) through several biological processes, thereby having different clinical significance depending on the underlying mechanism of expression. Currently, mechanisms leading to PD-L1 gene expression in colorectal cancer (CRC) are not fully understood. Methods: We investigated 98 Indonesia CRC patients to determine PD-L1 protein expressions and their correlations with PD-L1 gene copy number status, tumor infiltrating lymphocytes (TILs), tumor mutational profile, as well as clinicopathologic features. Results: Our investigation demonstrated that 18% of patients positively expressed PD-L1. Further analysis on PD-L1 copy number revealed that all PD-L1+ tumors had normal copy number, indicating that the expression of PD-L1 was not a consequence of genetic amplification of PD-L1. From TILs analysis, there was a significant increase of CD8 in all tumor cells expressing PD-L1 (P=0.0051), indicating that the inducible PD-L1 expression was the prominent mechanism occurred in CRC. Furthermore, the expression of PD-L1 in this CRC population was significantly associated with high frequency of MSI compared to the remainder PD-L1- tumors (P=0.0001), suggesting the natural immunogenicity of tumors via MSI status plays role in attracting immune response. On the other hand, p53 mutations which were frequently observed within Indonesian CRCs (76.5%), they were not associated with PD-L1 expression (p=0.1108), as well as KRAS gene (29.6%; p=0.5772) and BRAF gene mutations (5%; p=0.2171). Conclusion: Our study demonstrated that PD-L1 expressions in CRC were predominantly found as a consequence of infiltrating CD8 T lymphocytes that in part arise in the setting of microsatellite instability. Taken together, our findings further support the role of adaptive immune resistance to drive PD-L1 induction in tumor microenvironment and may provide important rationale for strategy implementation of immunotherapy for CRC cases.

Keywords: CD8- Colorectal Cancer- PD-L1
aims to maintain immune homeostasis. In contrast, the same mechanism has been exploited by tumor cells to evade or block host immune surveillance. Currently, inhibition of PD-1/PD-L1 interaction to restore anti-tumor immunity has shown a remarkably durable clinical response rate in patients, notably in melanoma, renal, lung, prostate and bladder carcinoma (Llosa et al., 2015).

Expression of PD-L1 is, in general, associated with response to anti-PD-1/PD-L1 monoclonal antibody. However, the expression of PD-L1 is regulated in different ways, which may affect different and significant responses to therapy. Two mechanisms of PD-L1 expressions have been shown previously. First, constitutive PD-L1 expression on tumor cells are determined by cancer-driving gene alteration. Second, PD-L1 expression is inducible as a response to host adaptive immune resistance (Ribas and Hu-Lieskovan, 2016; Shi, 2018). In this study, we aimed to investigate the tendency of PD-L1 expression mechanism in CRC patients by determining its correlation with PD-L1 gene copy number status, tumor infiltrating lymphocytes (TILs), tumor mutational profile, as well as clinicopathologic features.

Materials and Methods

Sample collection and slide preparation

Ninety eight formalin fixed paraffin embedded (FFPE) tumor tissue blocks from 98 CRC patients along with paired haematoxylin-eosin (HE) stained tissue slides were obtained from Medistra Hospital upon approval by Medistra ethic committee. All blocks were sectioned into 4 μm thin specimens and mounted on coated sample slides for further assessment which were conducted in Stem Cell and Cancer Institute (SCI) laboratory.

Isolation of genomic DNA from FFPE tissue

Tumor enriched areas were marked by senior pathologist on HE slides and corresponding areas from the unstained slide were manually scraped using sterile needle. The paraffin flakes were then transferred into 1.5 ml tube, deparaffinised with 1 ml of xylene, vortexed and centrifuged to pellet the tissue. The tissue pellet was then washed with 70% alcohol twice and then proceeded to nuclease free water (Qiagen, Germany) and stored at -20°C until further used.

PD-L1 Copy number assays

Copy number analyses of PD-L1 were performed on 18 positive tumor tissue specimens using TaqMan copy number assay kit specific for PD-L1 and RNase P, (Hs01477451_cn, Applied Biosystems) on a StepOnePlus Real Time PCR System (Life Technologies) according to manufacturers’ instructions and described elsewhere (Ikeda et al., 2016). Briefly, polymerase chain reaction (PCR) was performed with TaqMan Genotyping Master Mix (Life Technologies). Each single-well reaction contained 20 ng of genomic DNA and was run for PD-L1 gene (FAM dye-labeled probe) and Rnase P gene (VIC dye-labeled probe) simultaneously. Copy number were then calculated with CopyCaller v2.0 Software (Life Technologies) using ΔΔ Ct relative quantification method. The results were then calculated as relative copy number of PD-L1 gene normalized to RNase P. Blood samples from healthy subjects were used as reference. A PD-L1 copy number of 3 or greater was defined as amplification positive, whereas a PD-L1 gene copy number less than 3 was defined as negative.

PD-L1 expression, evaluation of TILs and immunoscore calculation

Immunohistochemistry (IHC) was performed using the following antibodies: Rabbit anti-PD-L1 XP® mAb at dilution of 1:300 (EIL3N), Rabbit anti-CD3-epsilon mAb at dilution of 1:300 (D7A6E) XP® mAb, and Rabbit anti-CD8-alpha at dilution of 1:300 (D8A8Y) mAb (all were manufactured by Cell Signaling Technology, USA). Briefly, sections were deparaffinized with serial xylene dipping followed by an ethanol gradient for rehydration. Sections were then incubated in 3% hydrogen peroxide to block endogenous peroxidase to block endogenous peroxidase activity followed with antigen retrieval using EDTA buffer. After washing with Tris Bufferd Saline with Tween® 20, sections were then incubated overnight at 4°C with primary antibody. The expression of PD-L1, CD3 and C8 were detected using ChemMate EnVision Detection Kit with DAB substrate (Dako, USA) following the manufacturer’s instruction. Panoramic Scanner (3DHistech, Hungary) was then used for image acquisition and processing.

PD-L1 positivity was defined as PD-L1 expression on ≥ 5% of membranous positive cell staining of any intensity. Immunoscore (IS) assessment was carried out based on density of each CD3+ and CD8+ TILs in two tumor areas, namely centre of tumor (CT) and invasive margin (IM) using digital pathology software (QuPath, UK; Bankhead et al., 2017). TIL densities were further being classified as high (valued as 1) or low (valued as 0) according to the density cut off (Anitei et al., 2014) and summed up to generate numerical IS scales ranging from 0-4.

We used anti-human PD-L1 antibody clone EIL3N® from Cell Signalling Technology (USA), in ratio of 1:300 with overnight incubation time. Rabbit XP® was then used as secondary antibody followed by counterstaining with haematoxylin and slides dehydration. Expression of PD-L1 by cellsPD-L1 expression was identified based on stained cell within the cytoplasm or on the cell surface. PD-L1 positivity was determined by using the 5% cut off. Tumor infiltrating lymphocytes (TIL) intensity was assessed by immunohistochemistry assay. We used CD3-epsilon (D7A6E) XP (R) Rabbit mAb and CD8-alpha (D8A8Y) Rabbit mAb (Cell Signaling Technology, USA). The antibody ratio and incubation time was equal to previous PD-L1 staining process. Stained slides for PD-L1 and TILs were then scanned using Panoramic Scanner (3DHistech, Hungary) for digital imaging process. Only TILs slide that was further analyzed using digital pathology software (QuPath, UK; Bankhead et al., 2017) for calculation of positive cells densityto calculate density of stained cells. Immunoscore (IS) was determined based on density of
each CD3+ and CD8+ TILs in two tumor areas, namely centre of tumor (CT) and invasive margin (IM). TIL densities were further being classified as high (valued as 1) or low (valued as 0) according to the density cut off (Anitei et al., 2014) and summed up to generate numerical the IS scales ranging from 0-4 which comprised of IS 0 to IS 4.

**Microsatellite status and p53 mutation analysis**

Microsatellite and p53 mutation status were determined by IHC as described above. Microsatellite status were determined using Rabbit monoclonal antibodies against four mismatch repair proteins (MMR); MLH1 (ES05), MSH2 (FE11), MSH6 (EP49), and PMS2 (EP51) (all were manufactured by Agilent, USA). Each antibody was used at ratio of 1:100 prior to primary antibody incubation for one hour. Adjacent normal tissue served as an internal control for positive staining and a negative control staining was carried out without the primary antibody. Specimens with deficient nuclear expression of MMR proteins in the tumour cells but proficient in normal cells were identified as microsatellite unstable (MSI).

The presence of p53 mutation was detected using monoclonal antibody against p53 (DO-7, Agilent, USA) at ratio of 1:500 prior to primary antibody incubation for one hour. Specimens displayed restricted overexpression pattern were identified as p53 wild type, while specimens showed diffused or complete negative expression were identified as mutated p53 (Nyiraneza et al., 2012).

**KRAS and BRAF genotyping**

Mutations on KRAS and BRAF genes were assessed using high resolution melting (HRM) method. The protocols from previous publications were adopted for the mutation analysis of KRAS (Levi et al., 2017) and BRAF (V600E) (Kristensen et al., 2011). Assessment using ABI 3500 Genetic Analyzer was performed for further validation of KRAS mutation in samples that exhibited split peak pattern of PCR product melt-curves.

**Statistical Analysis**

The Fisher-Exact test for categorical variables was used to determine the significance between clinicopathological and molecular data. P<0.05 was considered statistically significant.

**Results**

**The characteristics of CRC patients**

This study investigated 98 CRC specimens that were collected from 2010 – 2017. The median age of patients was 69, ranging from 10 to 93 years old, whereas 57.1% of them were females. About 55.1% (54/98) had metastasis to distant organs, and histologically, 80.6% were well or moderately differentiated. Most tumours were located at the left side of the colon (68.4%; 67/98). Table 1 described the clinicopathological and molecular characteristics of patients stratified by PD-L1 status.

| PD-L1 expression and copy number variation |

We began our investigation on PD-L1 expression profiles. As seen in Table 1, we identified 18.37% (18/98) of patients expressed PD-L1 in their tumours (Figure 1.A). These PD-L1+ positive tumours consisted of equal percentage between the left-sided and the right-sided CRC cases (50.0%; 8/16 for each side). On the other hand, PD-L1- negative tumours were found more often on left side of colon (81.9%; 59/72; P=0.0190) since more than half of Indonesian CRC population suffered from left-sided CRC (Figure 1.B). No further significant of difference was observed between these two subsets based on age and gender, as well as grade of tumour differentiation.

In order to confirm whether PD-L1 amplification...
contributes to PD-L1 expression, analysis on PD-L1 gene copy number was then performed. In the present study, we used DNA isolated from healthy blood donor as our control. None of our CRC’s PD-L1 DNA copy number exceeded more than two copies. Our result showed that PD-L1 amplification was not commonly occurred in CRC population.

Patterns of tumor infiltrating lymphocytes (TIL), CD8^+ density and correlation with PD-L1 expression

Our immunostaining analysis demonstrated that TILs were present in both PD-L1^+ and PD-L1^- tumor samples (Supplement and Supporting Data/SSD 1). From 18 positive PD-L1 specimens, 9 specimens were examined for immunoscore (IS) value due to the availability of center of tumor (CT) and invasive margin (IM) areas. Majority of PD-L1^+ tumors (55.6%) had high IS of 4 in their tumor microenvironment (5/9), while the other 11.1% of patients had IS 3 (1/9) and 33.3% had IS 2 (3/9). On the other hand, IS 3 was frequently found in our PD-L1^- subset (57.1%; 8/14), followed by IS 2 (28.6%; 4/14) and IS 4 (14.3%; 2/14).

Regarding the subset of TILs, further analysis on CD8^- density in center of tumor area (tCD8^-) demonstrated that (77.7%;14/18) PD-L1^+ tumors contained a high number of tCD8^- . On the other hand, representative samples of PD-L1^- subset of patients (25.0%; 4/16) demonstrated a significantly lower tCD8^- density (Figure 1C) (P=0.0051).

Microsatellite instability and mutational status

Table 1 summarized several molecular characteristics, that known to contribute to CRC development, stratified by PD-L1 status. We found that 89.8% (88/98) of CRC patients were determined to be microsatellite stable (MSS) while 10.2% (10/98) were MSI. There was a significant association between PD-L1 expression with MSI frequency, as shown by 80% (8/10) of MSI tumor also expressed PD-L1 compared to MSS tumors which only 11.3% (10/88) expressed PD-L1 (Figure 1D) (P=0.0001).

Based on our p53 immunostaining, 76.5% (75/98) of patients harboured mutation on their p53. Regarding to PD-L1 status, 12 of 18 PD-L1^+ cases (66.7%) had p53 mutation while the rest (6/18) remains wild type. In addition to microsatellite status, MSS subset of patients tended to harbour p53 mutation (83.3%; 70/84) compared to MSI patients (55.6%; 5/9; P=0.0671).

Meanwhile, we found 29.6% (29/98) of CRC patients harboured KRAS mutations, and 5.1% (5/98) had BRAF mutation V600E. Neither KRAS nor BRAF mutational status were associated with PD-L1 expression.

Overall, based on molecular characteristics, 82 of our CRC patients exhibited one or more mutations, with 59 harbouring one mutation and 23 harbouring two mutations (SSD 2). These included 8 (8.2%) with KRAS/BRAF gene mutations, 50 (51.0%) with p53 mutations and 23 (23.5%)
with KRAS/BRAF in addition to p53 mutations.

Discussion

PD-L1 is expressed on tumour cells in many type of malignancies and implies a weakened host immune response and consequent poor prognosis (Anitei et al., 2014). With regard to CRC, PD-L1 expression is found in small subset of patients, ranging from 9% to 15% which is similar with our result (18.4%) (Rosenbaum et al., 2016; Valentini et al., 2018). To date, two mechanisms underlying this upregulation of PD-L1 have been reported (Ribas and Hu-Lieskovan, 2016; Shi, 2018). Firstly, amplification in PD-L1 locus is correlated significantly to constitutive PD-L1 expression in malignant cells (Ikeda et al., 2016; Ribas and Hu-Lieskovan, 2016). Secondly, Inducible PD-L1 expression mechanism refers to adaptive immune resistance in response to local inflammatory signals (e.g. IFN-γ) which are produced by active anti-tumor immune response (cytotoxic T-cell and/or Th1 pathway activation) (Sammaded and Chen, 2014).

One of the prominent factors in inducing PD-L1 expression is the interferon-γ (IFN-γ) acting mainly via the JAK/STAT1/interferon regulatory factor (IRF) 1 pathway in multiple types of cancers (Ikeda et al., 2016; Moon et al., 2017). As a consequence of immune response to tumor antigens, attracted cytotoxic T-cells produce IFN-γ which are produced by active anti-tumor immune response (cytotoxic T-cell and/or Th1 pathway activation) (Sanmamed and Chen, 2014).

The majority of CRCs developed via a chromosomal instability pathway, and approximately 12-15% have deficiency in the mismatch repair (MMR) gene which is responsible for MSI (Ahn et al., 2016). The defect in MMR gene facilitates production of aberrant proteins acting as neo-antigens burdens (Xiao and Freeman, 2015). Some of these neo-antigens will be processed, presented on MHC, and recognized as foreign by T-cells thus attracting immune response within tumor microenvironment. In our small retrospective study, which showed small percentage of MSI patients (10.2%), there was a significant correlation between PD-L1 upregulation and MSI status (P=0.0001). This intimate association between MSI and PD-L1 expression has also been reviewed previously (Gatalica 2016). Presumably, this high neo-antigen burden might be one explanation for the high level of TIL-related induced PD-L1 expression in CRC as described previously (Hodges et al., 2017; Yi et al., 2018). Nevertheless, adaptive immune resistance caused by tumor’s MSI status was not the main factor in inducing the expression of PD-L1 as more than half of our PD-L1+ tumors were MSS. Previous study by Llosa et al., (2015) reported similar result using cell lines model. They explored PD-L1 expression in MSI and MSS CRC cell lines and found that in response to IFN-γ both cell lines were modestly upregulated PD-L1 and HLA-DR (Cooks et al., 2013).

Recent advances in CRC immunotherapy suggest MSI tumors will benefit from checkpoint inhibitors therapy (Birendra et al., 2017; Spallanzani et al., 2018). As reported in CRC phase II clinical trials setting, by Lee et al., (2015) after the administration of Pembrolizumab...
(an anti PD-L1 mAb), a partial objective response rate of 40% were observed in MSI compared to 0% MSS CRC patients. Moreover, a CRC phase II trial by Overman et al., (2017) highlighted a partial response to Nivolumab alone or in association with Ipilimumab in 31% of MSI patients versus 10% of MSS patients.

In addition to RAS pathway, although other study reported there were correlations between PD-L1 expression with BRAF mutation in CRC (Rosenbaum et al., 2016), so far we could not find any relationship of RAS/BRAF mutation with PD-L1 expression.

Regarding to our clinicopathological data, although it did not reach significant, tumors that did not express PD-L1 were most likely left-sided CRC (81.9%), whereas tumors expressing PD-L1 shared equal proportion (50.0%) (P=0.0190) (Figure 1B). There is evidence of different response to treatment regarding tumor localization in CRC patients (Ulivi et al., 2017). Interesting result was found when we stratified tumor location based on PD-L1 expression and then correlated it with microsatellite status. Our study showed that PD-L1+/MSI subset was found mostly at the right side of colon (71.4%), which was commonly found in CRC and it was predicted to have more favourable prognosis compare to other types of CRCs (Sugai et al., 2006; Ulivi et al., 2017; Valentini et al., 2018).

In conclusion, our study demonstrated that PD-L1 expression in CRC was predominantly found as a consequence of infiltrating CD8 T lymphocytes that in part arise in the setting of microsatellite instability and high neo-antigen load, or in the setting of chronic inflammation pathway. This finding supports the role of adaptive immune resistance to drive PD-L1 induction in tumor microenvironment and may provide important rationale for strategy implementation of immunotherapy for CRC.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Supplement and Supporting Data (SSD)

SSD 1. Figure CD3 and CD8 presence in various IS within PD-L1 positive and negative subset
SSD 2. Table Mutation Burden in CRC Subset Patients
SSD 3. Quantitative PCR-based DNA copy number analysis of PD-L1 in 18 positive samples
SSD 4. Gel showing qPCR products of DNA copy number analysis of PD-L1 from 18 positive samples

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