Potential prognostic value of PD-L1 and NKG2A expression in Indonesian patients with skin nodular melanoma

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Research note

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Abstract

Objective

Biomarker mRNA levels have been suggested to be predictors of patient survival and therapy response in melanoma cases. This study aimed to investigate the correlations between the mRNA expression levels of *PD-L1* and *NKG2A* in melanoma tissue and clinicopathologic characteristics and survival in Indonesian patients with primary nodular melanoma.

Results

Thirty-two tissue samples were analyzed. Upregulated *PD-L1* was associated with shorter overall survival (hazard ratio: 2.930; 95% confidence interval: 1.011–8.489, $p = 0.048$) compared with patients with normoregulated *PD-L1*. A significant positive correlation was found between the expression levels of *PD-L1* and *NKG2A* (r_s: 0.768, $p < 0.001$). However, no clinicopathologic associations with *PD-L1* and *NKG2A* mRNA levels were statistically proven. Comparison with other studies suggested that the choice of adjuvant therapy and the presence of TILs affect the prognostic role of *PD-L1* expression. *NKG2A* was not proven to be an independent predictive factor but may become an adjunct target for therapy. The strong correlation between *PD-L1* and *NKG2A* suggests that anti-*PD-1* and anti-*NKG2A* agents could be effective in patients with *PD-L1* upregulation. The combination of the mRNA levels of these two target genes may provide a novel prognostic and therapeutic direction for immunotherapy.

Introduction

Among cutaneous malignancies, melanoma results in the highest mortality; statistics revealed 287,723 new cases and 60,712 deaths from this disease worldwide in 2018 [1]. These numbers are expected to increase, with an estimated 340,721 new cases projected in 2025 [2]. Despite trials of several combinations of systemic chemotherapeutic regimens, significant improvements in survival have not been achieved. Thus, current research is focused on new therapeutic agents, such as targeted therapy and immune checkpoint blockers.

Agents that block immune checkpoints, such as *PD-1*/*PD-L1*, help improve the immune response to cancerous cells. Nivolumab, an anti-*PD-1* antibody, has been approved by the FDA for the treatment of advanced melanomas [3]. Other immunotherapies, such as monalizumab, a humanized anti-*NKG2A* antibody that enhances NK cell and CD8 + T cell activity [4], are also under development. These drugs show great promise and more durable responses compared with targeted therapy agents.

Before starting anti-*PD-1* immunotherapy, clinicians commonly test tumor tissues for *PD-L1* expression by using immunohistochemistry (IHC). Tumors expressing *PD-L1* respond better to anti-*PD-1*/*PD-L1* agents compared with non-expressers [5]. However, the results of recent studies on the effect of *PD-L1* expression on survival are conflicting [6]. Detection using IHC also presents several limitations, such as the varying performance of different antibodies, nonstandard cut-off values, and operator dependence [7, 8].

Researchers are exploring new methods to predict therapy responses and survival in melanoma patients. Several studies have investigated the use of biomarker mRNA levels as an alternative parameter [9–11]. Gupta *et al.* reported that mRNA levels of *PD-L1*/2 show potential in predicting survival and response toward immunotherapy in metastatic melanoma [12]. Given the emergence of monalizumab, the potential prognostic and therapeutic roles of *NKG2A* should also be investigated.

Immuno-oncological research is fairly rare in Indonesia. Most Indonesian patients are treated with surgical resection and adjuvant treatment with dacarbazine chemotherapy and radiotherapy. Information on the expression of immune-checkpoint molecules is needed to gauge the potential efficacy of using immunotherapy agents in Indonesia. Thus, this study aimed to investigate the prognostic role of mRNA levels of *PD-L1* and *NKG2A*, as well as the associated clinicopathologic characteristics.

Materials And Methods

Formalin-fixed paraffin-embedded (FFPE) tissue samples from patients diagnosed in 2012–2019 with primary cutaneous nodular melanoma were collected from the archives of the Department of Anatomical Pathology, Dr. Sardjito Hospital, which is the main cancer referral center in Yogyakarta, Indonesia. Cases with prior chemotherapy or radiotherapy, incomplete clinical data, and degraded specimens were excluded. Thirty-two samples were analyzed in this retrospective cohort study, and all patients were of Javanese ethnicity.

RNA was extracted from FFPE tissues using GeneAll→ Ribospin™ II (GeneAll Biotechnology, Seoul, South Korea). Real-time polymerase chain reaction (RT-PCR) for *PD-L1* and *NKG2A* expression quantification was conducted using AccuPower® GreenStar™ RT-qPCR PreMix on an Exicycler™ 96 (Bioneer Corp., Daejeon, South Korea) with primer pairs and thermocycler conditions as previously described by Vassilakopoulou *et al.* and Meckawy *et al.* [13, 14]. The expressions of *PD-L1* and *NKG2A* were calculated from the quantification cycle (Cq) values of the gene targets and normalized against *GAPDH* as an internal control. Subsequent normalization was performed using the ΔΔCq values of RNA derived from healthy skin tissues. Age, sex, tumor location, Breslow thickness, greatest diameter, lymph node involvement, and stage were retrieved from medical records. Pathological data, including the presence of necrosis, lymphovascular invasion, tumor-infiltrating lymphocytes (TILs), and mitotic index, were obtained from hematoxylin–eosin and Ki67 IHC stained slides. Survival status (living or deceased) was determined through telephone calls at the point of follow-up of the study (until April 2020).

Samples were classified as normoregulated if the expression was lower than or equal to the mean of the *PD-L1* and *NKG2A* levels; conversely, samples were classified as upregulated if the expression was above the mean of the *PD-L1* and *NKG2A* levels. Comparison of mRNA level averages based on categorical

clinicopathologic characteristics was performed using Mann–Whitney U tests. Spearman correlation was used to analyze associations between the expression of *PD-L1* and *NKG2A* and continuous clinicopathologic features. Kaplan–Meier analysis and log-rank tests with Cox regression were used to determine hazard ratios (HRs) for survival analysis.

Results

The characteristics of the subjects are presented in Table 1. Most tumors were located on the extremities (71.88%) and thicker than 4 mm (81.25%). Necrosis and TILs were present in 71.88% and 75% of the samples, respectively. The clinical stages were evenly distributed among stages II (31.25%), III (34.38%), and IV (34.38%).

The expression of *PD-L1* and *NKG2A* was not significantly associated with the patients' clinicopathologic characteristics (Additional file Table S1). Spearman correlation showed that *NKG2A* and *PD-L1* mRNA levels were strongly correlated (Additional file Table S2).

Table 1
Clinicopathologic characteristics of the subjects

Age (years), mean ± SD*	**61.75 ± 16.28**
Sex, n (%)	
Male	9 (28.12)
Female	23 (71.88)
Tumor location, n (%)	
Trunk	2 (6.24)
Head and neck	7 (21.88)
Extremity	23 (71.88)
Lymph node metastases, n (%)	
Present	21 (65.63)
Absent	11 (34.37)
Breslow thickness	
≤ 1 mm	0 (0.00)
1.01–2.00 mm	3 (9.38)
2.01–4.00 mm	0 (0.00)
> 4.00 mm	29 (90.62)
Necrosis, n (%)	
Present	23 (71.88)
Absent	9 (28.12)
Tumor diameter (mm), mean ± SD*	29.13 ± 24.20
Ulceration	
Present	17 (53.13)
Absent	15 (46.87)
Tumor-infiltrating lymphocyte	
Present (brisk and non-brisk)	24 (75.00)
Absent	8 (25.00)
Clinical stage	
I	0 (0.00)
II	10 (31.25)
III	11 (34.38)
IV	11 (34.38)
Survival status	
Alive	8 (25.00)
Deceased	24 (75.00)
Overall survival (months), mean ± SD*	22.81 ± 15.50
*SD: standard deviation	

In the Cox univariate regression analysis, upregulated *PD-L1* had an HR of 2.930 (95% CI: 1.011–1.963) for mortality compared with normoregulated cases (Additional file Table S3). Higher stage and *NKG2A* upregulation also increased HRs, but the differences observed were not statistically significant. In multivariate analysis, all three variables were weakly correlated.

Figure 1 shows that patients with normoregulated *PD-L1* expression had significantly longer median survival time (27.000 ± 3.093 months) compared with subjects with upregulated expression (15.000 ± 3.657 months; $p = 0.036$). No significant difference in survival time was observed for the normoregulated (27.000 ± 3.221 months) and upregulated *NKG2A* subjects (19.000 ± 3.811 months; $p = 0.483$) (Fig. 2).

The survival curves of patients with and without TILs, which did not differ significantly ($p = 0.662$) (Additional file Fig. 1). The survival curves of the upregulated and normoregulated groups for *PD-L1* and *NKG2A* did not differ significantly when divided based on the presence of TILs (Additional files Figs. 2 and 3).

Discussion

In this study, we compared the clinicopathologic characteristics and overall survival of Indonesian primary nodular melanoma cases with different mRNA levels of *PD-L1* and *NKG2A*. Two important findings were observed in our study: (1) melanoma cases with *PD-L1* overexpression had significantly lower survival rates compared with those with normal *PD-L1* expression and (2) *PD-L1* and *NKG2A* levels were strongly correlated.

We observed that patients with tumors showing upregulated *PD-L1* had significantly lower overall survival, with an approximately threefold higher HR, compared with those with tumors showing normal *PD-L1*. The *PD-L1* molecule interacts with *PD-1* receptors on T cells, causing anergy, exhaustion, and even apoptosis [15]. Melanoma cells can thus escape the immune system by increasing their *PD-L1* expression. Experiments using melanoma cell lines showed that cells with upregulated *PD-L1* demonstrate highly invasive and aggressive behavior [16]. In a study on melanoma patients treated with surgery and dacarbazine adjuvant chemotherapy, patients with positive *PD-L1* on IHC staining had lower median survival time compared with the subgroup with negative or indeterminate *PD-L1* status (9.7 months vs. 11.6 months) [17].

Our results seem to contradict those of Gupta *et al*., who observed that higher *PD-L1* mRNA levels reflect a better prognosis for patients with melanoma treated with anti-*PD-1* agents [12]. This incongruity may have stemmed from differences in the treatments administered to the subjects. Patients with high expression of *PD-L1* respond well to anti-*PD-1* antibodies, thus explaining the increase in progression-free and overall survival [5]. The results suggest that *PD-L1* expression is a negative prognostic factor in patients with melanoma in general. However, when treated with anti-*PD-1* antibodies, patients with high levels of *PD-L1* respond well and have good outcomes. Therefore, the choice of therapy also affects the performance of *PD-L1* as a prognostic factor.

PD-L1 expression can occur as an independent phenomenon or a secondary reaction to the presence of TILs. TILs can secrete interferon-gamma, which induces the expression of *PD-L1* in tumor cells [15]. Studies have reported the reactive pathway as the predominant background for *PD-L1* expression in melanomas because of the strongly positive correlation between *PD-L1* and TILs [18]. However, in our study, the group without TILs had higher average *PD-L1* mRNA levels than the group with TILs. Combined with the lower survival of *PD-L1* overexpressers, this finding suggests that the cases with increased *PD-L1* in our study likely have no TILs and are independent (constitutive) expressers.

Constitutive and reactive expression of *PD-L1* may have different prognostic implications. When previous studies divided patients based on *PD-L1* expression and the presence of TILs, patients with constitutive *PD-L1* expression without lymphocyte infiltrates showed the poorest outcomes, followed by reactive *PD-L1* expressers, those with *PD-L1*(-) without TILs, and, finally, those with *PD-L1*(-) and TILs [16, 19]. In our results, both groups with upregulated *PD-L1* showed poorer prognoses than the groups with normoregulated *PD-L1*. However, survival did not differ significantly when the cases were divided further based on TIL status, likely because none of the patients were treated using immunotherapy, in which the presence of TILs predicts improved response and outcomes [20]. Another factor that could explain this lack of significance is the limited sample size.

Patients with upregulated *NKG2A* mRNA did not differ significantly from normo-regulated patients in terms of survival. *NKG2A* is an inhibitory receptor found on NK cells that plays a major role in the immune response against tumors [21]. Cancer cells can attempt to evade the immune system by upregulating HLA self-molecules that activate *NKG2A* receptors and impair the function of NK cells. Trials in mouse models indicate that monalizumab is ineffective as a single therapy but highly effective when used together with other immunotherapy agents that promote activated TILs, such as anti-*PD-1* or cancer vaccines [22]. One escape strategy used by cells to escape cytotoxic TILs is downregulation of MHC I expression, which renders them targets for NK cells [23]. This finding may explain the role of anti-*NKG2A* as an adjunct treatment for other immunotherapies. Our results reinforce the idea that *NKG2A* may not be an independent therapeutic and prognostic factor but may play a role when combined with other factors.

The mRNA expressions of *NKG2A* and *PD-L1* were strongly correlated. This finding indicates that tumors with high *PD-L1* expression would also likely express *NKG2A* strongly and, thus, respond well to anti-*NKG2A* agents. When *NKG2A* expression was combined with the TIL parameter, the distribution of survival curves obtained resembled the curves for *PD-L1* combined with TILs. *NKG2A* upregulation with and without the presence of TILs may have different pathogeneses and prognostic implications, similar to *PD-L1*.

The lack of correlation between the expression of *NKG2A* and *PD-L1* and clinicopathologic characteristics in this work resembles the findings of several previous studies [6, 18]. The small sample size of this study may have contributed to the low statistical significance found.

The findings of this study must be interpreted with caution because of the small sample size of patients who did not receive adjuvant immunotherapy. However, our results support the findings of several studies that show that mRNA profiles may serve as a prognostic factor in melanoma cases [12,24]. Our interesting results suggest that further research and clinical trials are warranted to ascertain the roles of *PD-L1* and *NKG2A* in the prognosis and therapy of Asian patients who had not previously received immune checkpoint inhibitors.

Conclusions

We investigated the correlations between *PD-L1* and *NKG2A* expression levels and clinicopathologic characteristics and survival in patients with primary nodular melanoma in Yogyakarta, Indonesia. Patients with upregulated *PD-L1* expression had significantly shorter overall survival than those with normoregulated expression. *PD-L1* and *NKG2A* mRNA levels were positively correlated.

Our findings suggest that the choice of therapy and presence of TILs may affect the prognostic role of *PD-L1* expression. *NKG2A* was not proven to be an independent predictive factor but may serve as an adjunct target for therapy. The strong correlation between *PD-L1* and *NKG2A* suggests that anti-*PD1* and anti-*NKG2A* agents may be effective in patients with *PD-L1* upregulation. Studies with larger subject groups are needed to confirm the patterns of *PD-L1* expression in Asian cases.

Limitations

Our study was limited by its small sample size and homogenous ethnic population. Results among diverse Indonesian and Asian populations may differ. Examination of TILs did not discriminate between lymphocyte subtypes.

Abbreviations

CD
Cluster of differentiation
Cq
Quantification cycle
DNA
Deoxyribonucleic acid
FFPE
Formalin-fixed paraffin-embedded
GADPH
Glyceraldehyde 3-phosphate dehydrogenase
HLA
Human leukocyte antigen
IHC
Immunohistochemistry
MHC
Major histocompatibility complex
mRNA
Messenger ribonucleic acid
NK
Natural killer
NKG2A
Natural killer group 2A
PD-1
Programmed death-1
PD-L1
Programmed death-ligand 1
RT-PCR
Real-time polymerase chain reaction
TILs
Tumor-infiltrating lymphocytes

Declarations

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Availability of data and materials

This submission contains all of the data analyzed during the study. Unprocessed data can be requested from the corresponding author.

Authors' contributions

RDS designed the study and performed the analysis. HTR, MFP, SLA, KS, and TA wrote the manuscript. YI and MRR contributed to the data collection. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study complied with the Declaration of Helsinki and the Belmont Report. The protocol for this study was approved by the Medical and Health Research Ethics Committee of the Faculty of Medicine, Public Health, and Nursing, Universitas Gadjah Mada (KE/FK/0599/EC/2020). The study was conducted with formal permission from the appropriate hospital officials after explaining the research objectives and procedures. Patients were informed preoperatively of the use of data and tissue samples for research. All patients have consented in written forms.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

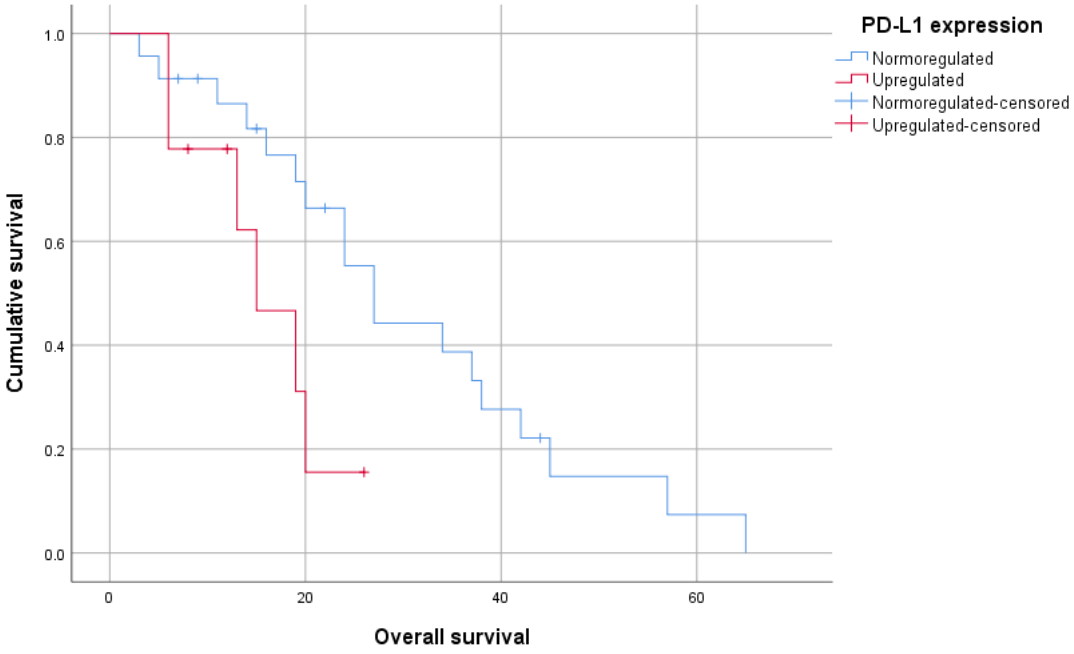

Figure 1

Kaplan–Meier survival curves comparing the survival of patients with primary nodular melanoma with upregulated or normoregulated PD-L1 expression (p = 0.036)

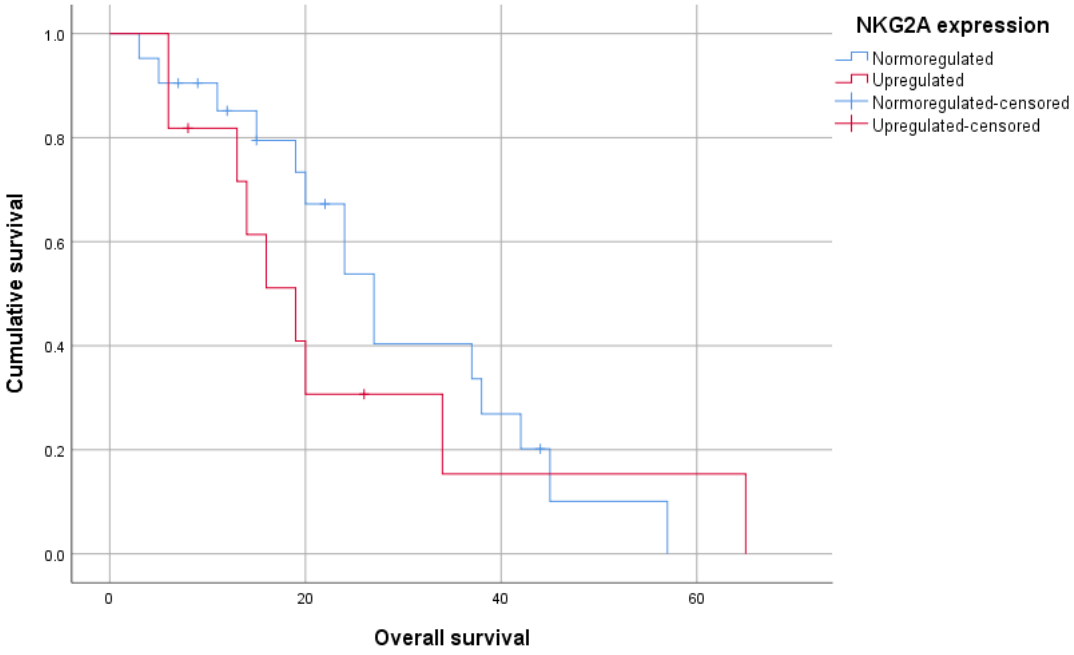

Figure 2

Kaplan–Meier survival curves comparing the survival of patients with primary nodular melanoma with upregulated or normoregulated NKG2A expression (p = 0.483)

Supplementary Files

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