**Whole-Genome Genomics Correlates of Response To Anti-PD1 Therapy in Relapsed/Refractory Natural Killer/T Cell Lymphoma**

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Running title: Genomic correlates of response to anti-PD1 therapy

Key point: Whole-genome sequencing reveals correlates of response in patients to anti-PD1 therapy in Natural killer/T-cell lymphoma

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**Abstract**

Natural killer/ T-cell lymphoma (NKTL) patients failing L-asparaginase regimens have extremely poor treatment outcomes. Previous case series showed promising activity when relapsed or refractory NKTL patients were treated with anti-programmed death 1 (PD1) inhibitors. Here, we continue to unravel the molecular profiles with whole-genome sequencing (WGS) on an extended cohort of 11 pembrolizumab-treated patients (median age at diagnosis, 42 years; range, 27-66 years) with a median follow-up of 11 months (range, 2 - 25 months) since starting anti-PD1 therapy. Seven patients achieved complete response (CR) and four patients had progressive disease (PD). Using WGS, we found structural rearrangements of the *PD-L1* gene, *JAK3*-activating mutations and *ARID1B* homozygous insertion in four, two and one of the CR patients' tumors, respectively. Interesting, these alterations, especially PD-L1 rearrangements, were absent in the four PD cases. Expression of PD1 ligand (PD-L1) was strong in nine patients (5 CR and 4 PD cases) and weak in two patients (both CR). PD1 blockade with pembrolizumab was a potent strategy for NKTL patients and genomic screening could potentially accompany PD-L1 immunohistochemical screening to better select patients for anti-PD1 therapy.

## Introduction

Natural killer/T-cell nasal-type lymphoma (NKTL) is an uncommon and aggressive malignancy with a predilection for Asian, Mexican and South American populations [1]. With the exception of Japan, it is the most common mature T-cell lymphoma in Asia [2]. NKTL often presents as an extra nodal disease and mostly affects the upper aerodigestive tract. Neoplastic cells are invariably infected by the Epstein Barr virus (EBV) and are characterized by a cytotoxic phenotype [3]. The genetic landscape of NKTL has been recently unraveled by discoveries describing recurring mutations altering the JAK-STAT pathway, epigenetic modifiers, the *DDX3X* gene and genetic predisposition in the *HLA-DPB1* gene but none has employed whole-genome sequencing [4-9].

Recently, immune checkpoint (ICP) inhibitors have revolutionized the treatment of many cancers including some hematologic malignancies [10]. Of note, the most impressive results are observed with the use of programmed death-1 (PD1 or CD279) inhibitors in relapsed/refractory (RR) Hodgkin lymphoma (HL) [11,12] and non-small-cell lung cancer [13]. Investigations on several solid tumors, including non-small-cell lung carcinoma, melanoma and bladder cancer, have generally concluded that immunohistochemistry (IHC) PD-L1 positivity coincides with greater likelihood of response to PD1/PD-L1 blockade. However, there was also a lower but definite response rate in patients with PD-L1-negative tumors [14]. These observations suggested that PD-L1 IHC might not be the optimal selection criterion for PD1 blockade therapy [15].

Here we report retrospectively, for the first time, the genomic mutational profiles of anti-PD1 blockade in 11 RR NKTL patients using WGS data, which provide proof-of-concept data that the response to anti-PD1 is relevant and correlates with *PD-L1* and *JAK3* genomic alterations in this malignancy.

## Study Design/Method

### Patients

Our study cohort consists of 11 patients with RR NKTL failing L-asparaginase regimens from Singapore, China and Hong Kong. Patients were diagnosed with NKTL according to the 2008 World Health Organization classification with cytotoxic, CD3ε+ and EBER+ phenotypes [3]. Response assessment was performed using a combination of PET/CT or CT/MRI, EBV PCR, and in one patient, histological assessment of a resected lesion that was fluorodeoxyglucose avid on PET/CT scan. The duration of response (DoR) was calculated from the date of starting pembrolizumab to the date of progression or death. The median DoR was estimated using the Kaplan-Meier method. Institutional Review Boards from SingHealth (2004/407/F), National University of Singapore (NUS-IRB-10-250) and Sun Yat-sen University Cancer Center (YB2015-015-01) approved the study. All subjects in this study provided written informed consent.

### Whole-genome sequencing and variant-calling

Whole-genome sequencing was performed for all the tumors and their matching whole blood with Illumina TruSeq Nano DNA Library Prep Kit. Sequencing reads were aligned using BWA-MEM [16] to the hs37d5 human reference genome. Strelka [17] and MuSE [18] were used to detect somatic short variants. Variants were subsequently annotated by wAnnovar [19] on 12th March 2018. The genic regions of *PD-L1* and *PD-L2* were manually inspected for somatic structural rearrangements (SR) with IGV. All validation and cloning primers used in this study are described in Supplementary Table 1-2, respectively.

### Data Availability

The WGS data of 11 ENKL-normal/blood pairs have been deposited in European Genome-phenome Archive (EGA) under the study accession code: EGAS00001002420.

## Results and discussion

### Clinical descriptions of the 11 RR NKTL patients and their responses to pembrolizumab

Eleven NKTL patients who, relapsed or were refractory to L-asparaginase containing

chemotherapy regimens, and who, had tumor tissue for analysis, were included in this study. They were all males with a median age of 42 (range 27-66). Seven patients (63.6%) achieved a CR with pembrolizumab treatment and four patients (36%) had PD (Table 1). The median duration of response to pembrolizumab (for responding patients) was 14 months (95% CI 6-UD). Table 1 also describes how each patient has been treated and the PD-L1 IHC level of their respective tumor before pembrolizumab treatment.

**Sequence analysis of the 11 RR NKTL pembrolizumab-treated patients**

Whole-genome sequencing was performed for 11 pairs of tumor-blood samples to study the association between somatic mutations and response to pembrolizumab. The NKTL tumors and whole blood were sequenced to an average depth of 66.6x and 37.5x, respectively (Supplementary Table 3). Somatic variant calling yielded an average of 47 (range: 8 – 127) non-silent variants per sample (Supplementary Table 4). Interestingly, recurrent *PD-L1* SRs (Figure 1A) were validated in four of the seven CR cases and similar *PD-L1* alterations have been reported in other malignancies, such as Adult T-cell leukemia/lymphoma and diffuse large B-cell lymphoma [20]. *JAK3*-activating (p.A573V) mutations (Figure 1B) were also validated in another two pembrolizumab-treated patients who have achieved CR (Supplementary Figure 1). Lastly, we also found a homozygous 3 bp insertion (p.Q131_H132insQ) in the *ARID1B* gene, a chromatin remodeler gene and a subunit in the SWI/SNF complex in the last remaining CR case. A recent study has also reported PBRM1-deficient and ARID2-deficient tumors correlated with better response to anti-PD1/PD-L1 therapy renal cell carcinoma [21]. There seems to be a relationship between truncating alterations in the subunits of the SWI/SNF complex and response to PD1/PD-L1 therapy. However, the exact mechanisms behind these associations remain to be elucidated for NKTL.

Analysis of the WGS data from the four PD patients' tumors did not reveal similar alterations in the *PD-L1* and *JAK3* genes (Figure 1C). A careful inspection was also carried out on the genes associated with major histocompatibility complex and interferon gamma pathways, which are known to associate with resistance to immune checkpoint blockade in melanoma,

but no further mutation in these groups of genes was found in our cohort. However, a *TP53* (p.W14X) stop-gain mutation, a hallmark tumor suppressor gene, was detected in a patient (NKTL27) who had progressive disease (PD) after given pembrolizumab.

**PD-L1 IHC membranous staining of the pretreated NKTL tumors**

We went on to check if PD-L1 IHC staining could explain the response of RR NKTL patients to pembrolizumab. In this study, tumors were stained and assessed for PD-L1 positivity by the same pathologist. All cases, except NKTL29 and NKTL31, have greater than 20% of tumor cells stained positive for PD-L1 (Supplementary Table 5). Interesting, both NKTL29 and NKTL31 are CR cases. In addition, all four PD cases were strongly stained for PD-L1 with an average of 69% PD-L1 positive cells (range, 50% - 90%) but their outcomes were dismal. This suggests that there could be a companion biomarker that could be added to PD-L1 IHC positivity for better predictive power of response to PD1 blockade therapy.

**Regulatory activity of the smallest *PD-L1* structural rearrangement identified**

Four *PD-L1* 3'UTR SRs were identified and three of them were validated to result in the disruptions of *PD-L1* 3'UTR. The last remaining SR is a 206 bp inversion within the 3'UTR of *PD-L1*, which to our knowledge, is also the smallest *PD-L1* SR ever identified. To determine the functional significance of this inversion in regulating PD-L1 expression, the wild type and mutant (with 206 bp inversion) *PD-L1* 3'UTR were cloned into a luciferase reporter assay system and transfected into lymphoma and leukemia cell lines. Our results show that the wild type *PD-L1* 3'UTR could effectively suppress the expression of the reporter protein and the smallest identified SR could relieve this suppression in NK-S1, K-562 and Jurkat cell lines ($P = 0.01$, $P = 0.01$ and $P = 0.03$; Supplementary Figure 2). With this, we characterized the 3'UTR of *PD-L1* to be generally suppressive and the *PD-L1* SRs would probably offer a direct explanation to how some NKTL tumors have evaded immune surveillance.

**Clonality of *PD-L1* structural rearrangements, *JAK3*-activating and *ARID1B*-insertion mutations**

Although the mechanisms of response remain to be elucidated, we were interested in the clonality of each genomic alterations identified in Figure 1C. From the sequencing of the 11 cases, we were able to obtain solutions for the clonal architectures of 10 cases (1 CR case had no solvable clonality with SciClone [22]). Seven cases, five CR cases and two PD cases, had a clonal architecture (Supplementary Table 6). Although, there are tumors that had a non-clonal architecture, all the somatic *PD-L1*, *JAK3* and *ARID1B* alterations identified in this study resided in the founding clone of their corresponding pretreated tumors. This suggests that these alterations were already present during early onset of the disease. Although not definitive, the clonal residencies of these genomic alterations do support the observations of CR in these patients.

**Discussion**

From our analysis, *JAK3*-activating mutations and *PD-L1* 3'UTR disruptions, but not PD-L1 expression, were found to be more associated with response to pembrolizumab in NKTL. Within the responders, the range of PD-L1 IHC scores varied greatly (6%, 2+ to 100%, 3+) and was not predictive of the efficacy of PD-1 blockade on NKTL patients. On the contrary, both NKTL25 and NKTL27 had strong PD-L1 IHC scores but suffered dismal results from PD-1 blockade treatment. This questions the reliability of PD-L1 expression as biomarker for selecting responders to immune checkpoint inhibitors for NKTL.

The identification of two CRs to pembrolizumab harboring *JAK3*-activating mutations in our study also coincides with a recent report showing the long-term benefit of PD-1 blockade in a single lung cancer patient with *JAK3*-activating mutations [23]. Moreover, Zaretsky *et al* recently reported that loss of *JAK1*, *JAK2* or *B2M*, leading to a lack of response to interferon gamma or antigen presentation confers resistance to pembrolizumab in metastatic melanoma [24]. These data led us to speculate that *JAK3*-activating mutations could play an important role in patient's response to immune checkpoint inhibitors.

Here, in a setting of off-label use of pembrolizumab on a group of uncommon RR NKTL

patients, we do acknowledge that the size of the study cohort is small and the results will have to be validated further with an extended cohort of patients.

In summary, PD-1 blockade in ENKL is promising and we hope that our results could pave the way to a proper trial that would have our reported correlates of response as positive selectors for PD-1 blockade.

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**Authorship Contributions**

J.Q.L., T.T., M.-L.N., Y.L., B.K.H.C., J.I., D.H., T.S., W.L.P., C.C.Y.N., V.R., O.R., C.L.C., S.R. and C.K.O. analyzed the data. J.Q.L., M.-L.N., D.T., P.T., B.T.T., C.C.K., J.-X.B, T.Y.L., S.T.L. and C.K.O. were involved with the conception of the study. T.T., Q.-Q.C.,

D.T., R.-J.P., D.M.Z.C., H.M.H., E.T., B.M., Q.C.C., L.-M.P., J.T., N.F.G., Y.T.G., C.P., Y.S.L., Y.L., M.T., M.F., R.A.-Y., T.S.-Y.C, S.-B.N., Y.-L.K., W.H., W.-J.C., and C.K.O. were involved with tissue and data acquisition, and logistics needs for the study. J.Q.L., T.T., M.-L.N., S.T.L. and C.K.O. wrote the manuscript. All authors discussed the results and approved the manuscript.

**Disclosure of Conflicts of Interest**

The authors declare no competing financial interests.

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**Tables** Table 1. Clinical information and outcomes before and after pembrolizumab treatment of 11 NK/T-cell lymphoma patients.

| | | | | | | | | Treatments prior to Pembrolizumab | | | | Pembrolizumab treatment | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Case | Sex | Age, yr | Stage | ECOG | OS, mth | PFS, mth | CTx (cycles) | RT | TP | PD-L1+, % (H-score) | Outcome | DOR[1], mth |
| Complete Responders (CR) | NKTL1 | M | 49 | IV | 1 | 42+ | 19 | GELOX (4),<br>SMILE (5),<br>Romidepsin+Bortezomib,<br>BV+Benda,<br>Lenalidomide+Dara | Nil | Nil | 100% (250) | **CR**: PET/CT<br>**EBV DNA:** negative then became positive and remained stable | 20+ |
| | NKTL26 | M | 32 | I | 1 | 42+ | 2 | SMILE (2),<br>Vinc+DXM+Lasp (1),<br>GELOX (6) | Yes | Nil | 40% (35) | **CR**: PET/CT<br>**EBV DNA:** ND | 24+ |
| | NKTL28 | M | 46 | IV | 3 | 6+ | 0 | SMILE (2),<br>P-GEMOX (1) | Nil | Nil | 70% (190) | **CR**: PET/CT<br>**EBV DNA**: negative | 6+ |
| | NKTL29 | M | 48 | I | 0 | 9+ | 4 | Ifos+MTX+VP-16+DXM+Pasp (4) | Nil | Nil | 6% (7) | **CR**: PET/CT<br>**EBV DNA**: negative | 9+ |
| | NKTL30 | M | 38 | IV | 3 | 15+ | 6 | SMILE (5) | Nil | Nil | 60% (120) | **CR**: PET/CT<br>**EBV DNA**: negative | 11+ |
| | NKTL31 | M | 27 | IV | 0 | 64+ | 17 | Lasp+DXM+Vinc+AraC (4),<br>CHOP (2),<br>P-GEMOX (2),<br>DXM+Pasp+mitoxantrone+VP-16 (4)<br>P-GEMOX+VP-16 (2) | Nil | Auto-HSCT with BEAM + Thalidomide (maintenance) | 20% (20) | **CR:** CT & MRI<br>**EBV DNA:** negative | 24+ |
| | NKTL43 | M | 29 | IV | 2 | 116 | 73 | m-BACOD (4),<br>PIGLET (5),<br>SMILE (3) | Yes | Nil | 90% (190) | **CR**: PET/CT<br>**EBV DNA**: negative<br><br>Patient subsequently underwent MUD BMT and died from GVHD. | 14 |
| Non-CR | NKTL25 | M | 30 | IV | 0 | 14 | 10 | SMILE (6),<br>GEMOX (1) | Yes | Allo-HSCT | 72% (126) | **PD**: DOD | NA |
| | NKTL27 | M | 59 | IV | 0 | 19 | 2 | SMILE (3),<br>GIFOX (4) | Nil | Nil | 50% (85) | **PD**: DOD | NA |
| | NKTL44 | M | 66 | IV | 1 | 37 | 21 | SIMPLE (6) | Nil | Nil | 90% (170) | **PD**: DOD | NA |
| | NKTL45 | M | 42 | IV | 1 | 94 | 87 | SMILE (6),<br>GEMOX (1) | Nil | Allo-HSCT | 65% (70) | **PD**: DOD | NA |

[1]**DOR**: Durability of response as of Jan 2018; + indicates ongoing survival

BV, bretuximab vedotin; Benda, bendamustine; Dara, daratumumab; Vinc, vincristine; DXM, dexamethasone; Lasp, L-asparaginase; Ifos, ifosfamide; MTX, methotrexate; VP-16, etoposide; Pasp, Peg-Lasparaginase; AraC, cytarabine; ND, not done; RT, radiotherapy; TP, transplant

**Figure Legends**

Figure 1. Genomic profiles of 11 patients with relapsed/refractory NKTL lymphomas treated with pembrolizumab. (A) Schematics of the *PD-L1* structural rearrangements that were validated in our study and an instance of the Sanger sequence of the validation done on the rearranged *PD-L1* found in sample NKTL28. (B) A lollipop plot showing the recurrent *JAK3*-activating single nucleotide variants (SNV) found in our cohort and an instance of the Sanger sequence of the validation done on the SNV found in sample NKTL29. (C) Staircase plot of genomic alterations found in the 11 NKTL-blood pairs of whole-genome sequencing data. The top bar of the staircase plot denotes the number of non-silent mutations. The left and right bars denote the recurrence rates of the altered genes in the cohort. The bottom contains the legend to the main matrix of the staircase plot. SG: Singapore.

**Figures**
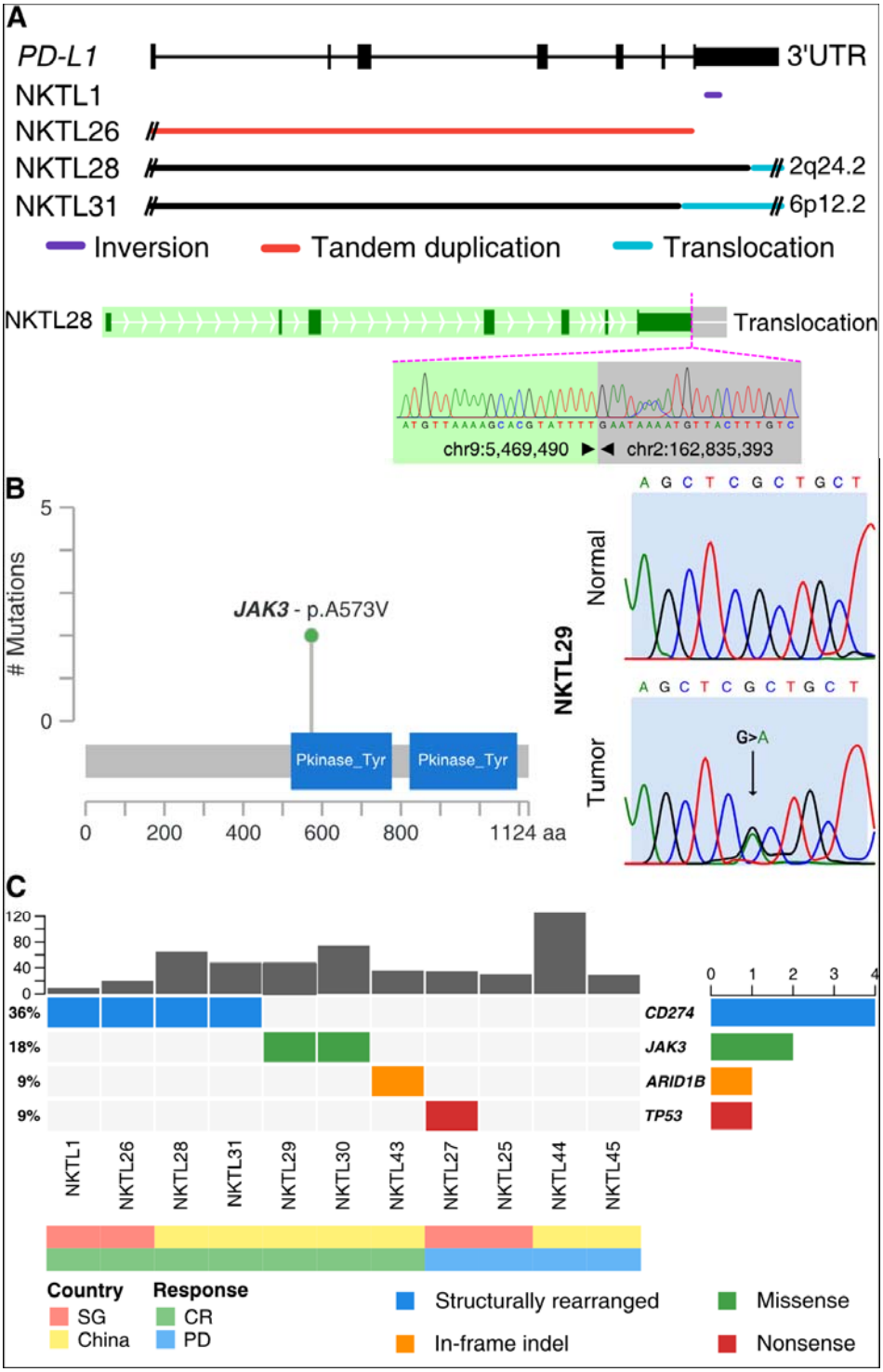
Figure 1.