

## PASD1 Expression in Malaysian Hematological Malignancies Patients

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### ABSTRACT

Per ARNT SIM domain containing 1 (PASD1) protein belongs to the Cancer Testis Antigen (CTA) family. It shows restricted expression in normal tissues but are highly expressed in cancer tissues. This study aims to further investigate PASD1 expression in Malaysian hematological malignancies patients as a potential biomarker for disease progression and vaccine development. Formalin-fixed Paraffin-Embedded (FFPE) tissue specimens from hematological malignancies patients were labeled with anti-PASD1-1 and anti-PASD1-2 antibodies using immunohistochemistry method. Our results show that among DLBCL patients, 7 samples were positive for PASD1-1, 2 samples were positive for PASD1-2 while 3 samples were positive for both of the antibodies. In addition, only 1 sample from T-cell Lymphoblastic Lymphoma (TLL) showed positive staining with PASD1-1. Other classes of hematological malignancies did not show any positive staining with either antibody. Antibody PASD1-1 stained the membrane and cytoplasm of the tumor cells strongly. Moderate nuclear labeling was also observed in some cases of DLBCL with PASD1-2. PASD1-1 staining was more frequent compared to PASD1-2 in most of the samples. Positive PASD1 staining was observed in patients with age range between 36-71 years old, higher in male cases than female by 54% and higher in Malay patients compared to Chinese by 77%. Due to its frequency, the PASD1\_v1 protein may play a role in the DLBCL initiation and progression. A higher number of PASD1 staining in DLBCL samples compared to other lymphoma subtypes may suggest that PASD1 may represent a potential for DLBCL subtyping marker.

### INTRODUCTION

In the past decade, significant advances have been made in the treatment of hematological malignancies. However, the non-solid malignancies remain as one of the major causes of cancer deaths worldwide including Malaysia (1-3). There is a great urgency for a more precise identification and classification of high-risk patients in combination with improved treatment modalities. One of the treatment approaches involves the identification of specific tumor-associated antigens (TAA) on the tumor cells. These molecules can be exploited to alert and boost patients' own immune system to kill the cancer cells.

Cancer testis antigens (CTAs) is one of the TAAs (4). To date, there are approximately 250 genes encoding CTAs have been identified in the CT database [<http://www.cta.lncc.br>]. CTAs can be divided into two groups: CT-X antigens located on the X chromosome and non-X CTAs located on various autosomes (5). Several types of advanced cancers with poorer outcomes are associated with members of the CT-X antigens (5). One of the CT-X antigens that is of our particular interest is Per ARNT SIM (PAS) domain containing 1 (PASD1) protein. It was first identified as a CTA in diffuse large B-cell lymphoma (DLBCL) and acute myeloid leukemia (AML) using SEREX technology (6, 7).

The *PASD1* gene is located at chromosome Xq28, with genomic coordinates (GRCh38): [X: 151,563,183-151,676,738](#) (National Center for Biotechnology Information), a locus containing many CTA genes and encodes two *PASD1* transcripts, *PASD1\_v1* and *PASD1\_v2* (6). *PASD1\_v1* cDNA encodes a 639 amino acid protein while an alternatively spliced variant *PASD1\_v2* (lacking intron 14) encoded a 773 amino acid protein (8). Alternative splice variants have been reported for other CT-X antigens and it is notable that specific isoforms have been linked to various tumor types (4, 5).

In healthy individuals, the *PASD1* expression is limited to germline tissues such as the testis and fetal human tissue (9). However, upon oncogenic transformation, *PASD1* are expressed in somatic tissues (6, 8, 10). The restricted expression properties of *PASD1* makes them attractive candidates as a potential biomarker and therapeutic targets in carcinogenesis. Their use will allow specific targeting to tumor cells while reducing the possibility of autoimmune problems. This is of particular importance especially in hematological malignancies such as in lymphomas where non-specific treatment often leads to great toxicity and side effects.

The aim of this study is to further investigate the expression profile of *PASD1* expression in Malaysian hematological malignancies patients in order to explore the future potential vaccine candidates. In the previous study, Lymphoma Research Group in Nuffield Division of Clinical Laboratory Sciences, University of Oxford have reported that *PASD1* expression was significant in Caucasian population and might have clinical relevant in vaccine development for DLBCL patients (6, 8). No study was done in this part of the region involving *PASD1* in lymphoma. Thus, this study represents the first investigation of *PASD1* in hematological malignancies within Malaysian patient population. We would like to investigate whether *PASD1* expression correlates with a geographical variation or could it be that certain *PASD1* isoforms may be more apparent in certain populations due to many factors. In cancer including hematological malignancies, multiple isoforms exist due to alternative splicing and this has been reported with many other CTAs including *MAGE* (4, 5). Two of the *PASD1* isoforms used in this study were previously reported as CTAs in DLBCL and AML patients among the Caucasian population (6-8). Thus it is intriguing to investigate whether our Malaysian hematologic patients may show similar *PASD1* expression and could be the candidate as a biomarker in diagnosis and immunotherapy.

## MATERIALS AND METHODS

### Samples

Ethics approval was obtained from the UKM Research Ethics Committee (reference number UKM PPI/111/8/JEP-2016-063). The samples were collected from all lymphoma patients who were admitted to UKM Medical Center, Kuala Lumpur from 2016-2017. The cases were histologically confirmed and diagnosed. Patients' data including age, gender, disease classification and tumor staging are summarized in Table 1.

### Antibodies

Two *PASD1* monoclonal antibodies were a kind gift from Dr. Karen Pulford and Prof Alison Banham, Nuffield Division of Clinical Laboratory Sciences, University of Oxford and were described previously (8, 11). Antibody *PASD1*-1 (clone 2ALCC136) recognized the recombinant protein (aa 195-474) present in both *PASD1\_v1* and *PASD1\_v2* proteins, whereas antibody *PASD1*-2 (clone 2ALCC128) was specific for the

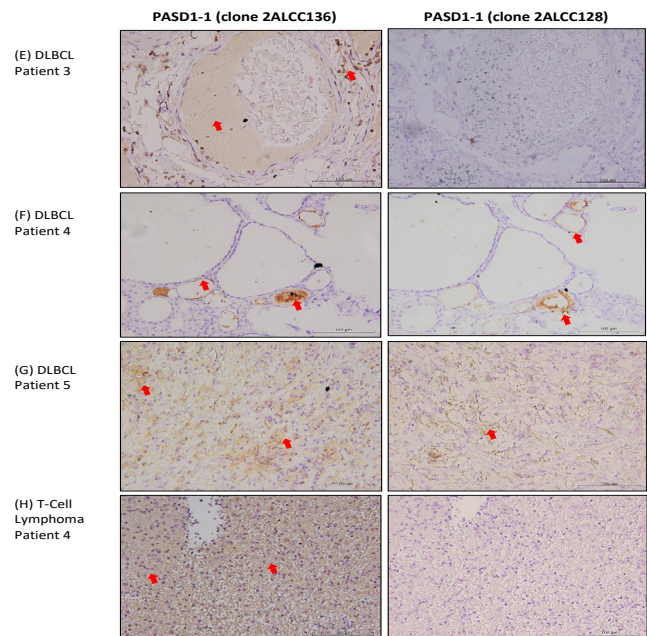
recombinant protein (aa 540-773) present only in the longer *PASD1\_v2* protein.

### Tissues

Formalin-fixed paraffin-embedded sections from lymphoma patients' biopsies were provided by the Department of Pathology, UKKMC. The paraffin blocks were 5-mm cut, dewaxed and rehydrated overnight. Antigen retrieval was carried out for 30 min in 50 mM Tris; 1mM EDTA (pH 9) and then cooled for 10 min at room temperature. Immunohistochemistry (IHC) staining was performed as per manufacturer's instruction (EnVision FLEX mini Kit, high pH -Dako autostainer/autostainer plus). The tissue sections were further incubated for 20 minutes with the antibodies *PASD1*-1 and *PASD1*-2 at a dilution 1:50. Anti-*PASD1* antibodies binding were detected with HRP-anti-mouse IgG and Diaminobenzidine (DAB) substrate. Tissue sections were rinsed with deionized water before counter stained with hematoxylin. Then, tissue sections were dehydrated with graded ethanol concentration and xylene before mounted. The tissue sections were visualized and analyzed under a light microscope.

## RESULTS AND DISCUSSION

Immunohistochemistry (IHC) studies using both antibodies *PASD1*-1 and *PASD1*-2 on human normal testis tissue showed *PASD1* protein expression to be restricted to nuclei of a subpopulation of spermatogonia near the basal membrane in testicular tubules (Figure 1A). Our results showed that *PASD1* protein expression was detected mostly in DLBCL and in one T-cell lymphoblastic lymphoma (TLL) but not in other types hematological malignancies (Fig. 1).



**Fig. 1.** Immunoperoxidase labeling of formalin fixed sections for the detection of *PASD1* protein expression in normal and malignant tissues. (A) Human normal testis tissue (positive control) showed *PASD1* distribution was restricted to the nuclei of primary spermatogonia (arrowed). The intensity of labeling decreased on maturity, being present as nuclear dots only (arrowed) or absent in spermatogonia near to the lumen of the tubule. Comparable patterns of labeling in testis were observed with both *PASD1*-1 and *PASD1*-2 antibodies. (B) No staining was observed in any of the normal tonsil tissues (negative controls) for both antibodies. (C-H) Immunoperoxidase labeling demonstrating heterogeneity of *PASD1* protein expression in sections from DLBL biopsy. (G) *PASD1* protein expression in sections from T-cell lymphoma. Photos were taken under 40x magnification. Arrows show the dark brown staining indicating *PASD1* expression.

In DLBCL samples, IHC staining showed 7 samples stained positive only for PASD1-1, 2 samples were positive only for PASD1-2 while 3 samples were positive for both of the antibodies (Table 1). Furthermore, 1 sample from TLL showed positive staining with PASD1-1. No PASD1-2 staining detected in this sample. Other hematological malignancies did not show any staining with either of the antibodies (Table 1).

**Table 1.** Summary of PASD1 expression in hematological malignancies tested with PASD-1 and PASD1-2. Diffuse large B-cell lymphoma = DLBCL, Extranodal natural killer (NK)/T-cell lymphoma = NKT cell lymphoma.

| Sample biopsy                       | PASD1-1<br>(ALCC136) | PASD1-2<br>(ALCC128) |
|-------------------------------------|----------------------|----------------------|
| DLBCL                               | 10/21                | 5/21                 |
| Mantle cell lymphoma                | 0/2                  | 0/2                  |
| T-Cell lymphoblastic lymphoma       | 1/2                  | 0/2                  |
| Anaplastic T-Cell lymphoma          | 0/1                  | 0/1                  |
| B-Cell Lymphoma                     | 0/1                  | 0/1                  |
| B-Cell acute lymphoblastic leukemia | 0/1                  | 0/1                  |
| NKT Cell lymphoma                   | 0/1                  | 0/1                  |
| Classical Hodgkin lymphoma          | 0/1                  | 0/1                  |
| Hodgkin lymphoma                    | 0/3                  | 0/3                  |

Variable patterns of nuclear, membrane and/or cytoplasmic labeling were observed (Figure 1C-H). Antibody PASD1-1 stained the membrane and cytoplasm of the tumor cells strongly. Some moderate nuclear labeling was observed in some cases of DLBCL with PASD1-2. PASD1-1 staining was more frequent compared to PASD1-2 in most of the samples. Positive PASD1 staining was observed in patients with age range between 36-71 years old, higher in male cases than female by 54% and higher in Malay patients compared to Chinese by 77%. (Table 2).

**Table 2.** Demographic data of positive PASD1 expression among DLBCL and T-cell patients. Diffuse large B-cell lymphoma = DLBCL, TLL- T-cell lymphoblastic lymphoma, M = Male, F = Female, ML = Malay, CN = Chinese, + = positive staining.

| No | PASD1-1 | PASD1-2 | Diagnosis | Stage | Sex | Age | Race |
|----|---------|---------|-----------|-------|-----|-----|------|
| 1  | +       |         | DLBCL     | 4     | M   | 56  | CN   |
| 2  | +       |         | DLBCL     | 2     | M   | 71  | CN   |
| 3  | +       |         | DLBCL     | 4     | M   | 67  | ML   |
| 4  | +       |         | DLBCL     | 1     | F   | 71  | ML   |
| 5  | +       |         | DLBCL     | 2     | F   | 64  | ML   |
| 6  | +       |         | DLBCL     | 1     | F   | 61  | ML   |
| 7  | +       |         | DLBCL     | 1     | F   | 57  | ML   |
| 8  |         | +       | DLBCL     | 3     | M   | 61  | ML   |
| 9  |         | +       | DLBCL     | 2     | M   | 36  | ML   |
| 10 | +       | +       | DLBCL     | 3     | F   | 54  | ML   |
| 11 | +       | +       | DLBCL     | 1     | M   | 66  | CN   |
| 12 | +       | +       | DLBCL     | 4     | M   | 36  | ML   |
| 13 | +       |         | TLL       | 1     | F   | 40  | ML   |

**DISCUSSION**

In comparison to other CTA proteins, the PASD1 expression is very restricted due to its limited expression at the earliest stages of spermatogenesis in normal cells. This specificity makes it an ideal candidate for immunotherapy approach.

Our results also showed that PASD1-1 stained more samples compared to PASD1-2 suggesting that the presence of more PASD1\_v1 in these tissues compared to PASD\_v2. This may indicate an important role of PASD1\_v1 protein in the DLBCL initiation and progression. We must also highlight the sample limitation in this study and the data should be further validated with a higher number of samples to obtain the statistical significance of the data. Nevertheless, the expression of the PASD1 protein in 57.1% (12/21 cases) of DLBCL and 50% (1/2 cases) of TLL in Malaysian population were consistent with the frequency of previously reported data in the Caucasian population (8, 12). A higher number of staining in DLBCL samples compared to other lymphoma subtypes may suggest that PASD1 may represent a potential for DLBCL subtyping marker. We observed variable in signal intensity, strong signal in some tumors but weak in others. Such is common as a result of the stage and/or proliferative state of the tumor and also heterogeneity in CTA protein expression within a particular tumor category. Our results further support PASD1 protein to be the CTA that is widely expressed in DLBCL patients. Due to sample limitation, it was not possible to calculate the statistical significance of the correlation between PASD1 expression with age, sex and race.

**CONCLUSION**

Future studies relating PASD1 protein expression to clinicopathological data will determine whether the PASD1 expression has prognostic significance and/or a possible role in lymphoma progression. An important factor concerning PASD1 expression in these tumors is whether they are immunogenic and produce an immune response in these patients. An ongoing *in vitro* study of immunity in our local population will help to further validate this antigen as an immunotherapeutic target.

**LIST OF ABBREVIATIONS**

- Per ARNT SIM domain containing 1 - PASD1
- Cancer Testis Antigen - CTA
- Formalin-fixed Paraffin-Embedded - FFPE
- T-cell Lymphoblastic Lymphoma - TLL
- Tumor-associated antigens - TAA
- Diffuse large B-cell lymphoma - DLBCL
- Acute myeloid leukemia - AML
- Diaminobenzidine - DAB
- Immunohistochemistry - IHC

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