การแสดงออกของยีน PRAME ในผู้ป่วยไทยที่เป็นมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์และความสัมพันธ์กับโรค

ธิชยาภา พจนเสนี[1, 3] และ สว่าง เพชรวิเศษ[1, 2*]

[1]บัณฑิตศึกษา สาขาเทคนิคการแพทย์ คณะสหเวชศาตร์ มหาวิทยาลัยธรรมศาสตร์ ศูนย์รังสิต จังหวัดปทุมธานี
[2]ภาควิชาเทคนิคการแพทย์ คณะสหเวชศาสตร์ มหาวิทยาลัยธรรมศาสตร์ ศูนย์รังสิต จังหวัดปทุมธานี
[3]ภาควิชาอายุรศาสตร์ คณะแพทยศาสตร์โรงพยาบาลรามาธิบดี มหาวิทยาลัยมหิดล กรุงเทพมหานคร

บทคัดย่อ

ยีน preferentially expressed antigen of melanoma (PRAME) เป็นยีนที่สัมพันธ์กับการเกิดมะเร็งหลายชนิดในมนุษย์ ทั้งมะเร็งชนิดก้อนและมะเร็งเม็ดเลือด และมีรายงานว่า การแสดงออกของยีน PRAME มีความสัมพันธ์กับผู้ป่วยที่เป็นโรคมะเร็งชนิดรุนแรง จึงสามารถนำมาประยุกต์ใช้เป็นตัวชี้วัดการดำเนินของโรค ติดตามการรักษา และพัฒนาการรักษาโรคมะเร็งแบบมุ่งเป้า อย่างไรก็ตาม ยังไม่มีข้อมูลการศึกษาเกี่ยวกับยีน PRAME ในกลุ่มผู้ป่วยไทยที่เป็นโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์ งานวิจัยนี้จึงมีวัตถุประสงค์เพื่อศึกษาการแสดงออกของยีน PRAME และความสัมพันธ์กับโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์ในผู้ป่วยไทยจำนวน 50 ราย และคนปกติที่มีสุขภาพดี จำนวน 30 ราย โดยใช้วิธี real time quantitative reverse transcription PCR (real time RT-qPCR) ผลการศึกษาพบว่า ผู้ป่วยไทยที่เป็นโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์มีความถี่ของการแสดงออกของยีน PRAME ร้อยละ 58 และไม่พบการแสดงออกของยีน PRAME ในคนปกติ โดยการแสดงออกของยีน PRAME ในกลุ่มผู้ป่วยไม่สัมพันธ์กับปัจจัยเพศ จำนวนเม็ดเลือดขาวในกระแสเลือด ปริมาณฮีโมโกลบิน ปริมาณเม็ดเลือดแดงอัดแน่น จำนวนเกล็ดเลือด และจำนวนร้อยละของ blast ในไขกระดูกของผู้ป่วย (*p* = 0.704, 0.094, 0.764, 0.438 และ 0.598 ตามลำดับ) โดยสรุป การศึกษานี้เป็นครั้งแรกที่มีการตรวจพบและรายงานความถี่การแสดงออกของยีน PRAME ในผู้ป่วยไทยที่เป็นโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์ ซึ่งจะส่งผลดีต่อการพัฒนาวิธีการรักษาแบบมุ่งเป้า โดยใช้ยีน PRAME เป็นเป้าหมายหลักของการรักษาด้วยวิธีเซลล์และภูมิคุ้มกันบำบัด (cellular immunotherapy) รวมทั้งอาจเป็นประโยชน์ในการใช้เป็นตัวบ่งชี้ร่วมกับตัวบ่งชี้อื่นเพื่อติดตามการรักษาหรือการจัดแนวทางในการรักษาผู้ป่วยโรคมะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์

คำสำคัญ: PRAME, RT-qPCR, มะเร็งเม็ดเลือดขาวชนิดเฉียบพลันแบบมัยอิลอยด์

***ผู้รับผิดชอบบทความ E-mail address:** sawang.petvises@gmail.com
รับบทความ: 5 กรกฎาคม 2562 แก้ไขบทความ: 25 กรกฎาคม 2562 รับตีพิมพ์บทความ: 18 มีนาคม 2563

PRAME Gene Expression in Thai Acute Myeloid Leukemia and Disease Correlations

Tichayapa Phojanasenee[1, 3] and Sawang Petvises[1, 2*]

[1]*Graduate Program in Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani Province, Thailand*
[2]*Department of Medical Technology, Faculty of Allied Health Sciences, Thammasat University, Pathum Thani Province, Thailand*
[3]*Department of Medicine, Faculty of Medicine Ramathibodhi Hospital, Mahidol University, Bangkok, Thailand*

Abstract

Preferentially expressed antigen of melanoma (PRAME) is a tumor-associated antigen overexpressed in a wide variety of solid tumors and hematologic malignancies. Previous studies have shown high expression of PRAME in solid tumors with high potential as a prognostic marker and candidate target for immunotherapy. The PRAME expression in acute myeloid leukemia (AML) patients in Thailand has not yet been assessed. This study aimed to investigate the expression of PRAME gene in newly diagnosed Thai AML and its correlations with the disease. The real time quantitative reverse transcription PCR (real time RT-qPCR) was used to measure PRAME gene expression in 50 newly diagnosed Thai AML and 30 healthy control samples. The results revealed that 58% of AML samples expressed PRAME gene, while healthy control individuals showed no expression of the gene. PRAME expression levels in this study were not correlated with gender, white blood cell count, hemoglobin, hematocrit, platelet and number of blasts in the bone marrow of AML samples (p = 0.704, 0.094, 0.764, 0.438 and 0.598, respectively). This study is the first to reveal PRAME gene expression in Thai AML. The result showing the presence of PRAME at high proportion in AML could be advantageous for developing targeted cellular immunotherapy against this type of hematologic malignancy.

Keywords: PRAME, RT-qPCR, Acute myeloid leukemia

***Corresponding author E-mail address:** sawang.petvises@gmail.com
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Introduction

Preferentially expressed antigen of melanoma (PRAME) is a 509 amino acid protein encoded by PRAME gene located on chromosome 22q11, firstly characterized in 1977[1]. The PRAME gene expression is high in primary lesion and metastatic melanoma. The human PRAME protein is absent in normal adult tissues and cells of hematologic origin, except testis, adrenal gland, ovary and endometrium[2]. The profound overexpression of PRAME gene in cancers has led PRAME protein antigen to be classified as a member of cancer testis antigen (CTA) which is highly expressed in several types of solid tumors and hematologic malignancies. PRAME is highly expressed in small cell lung carcinoma, breast carcinoma, renal cell carcinoma, head and neck cancers, Hodgkin lymphoma, Wilm's tumor, medulloblastoma and neuroblastoma[4-9]. The high expression of PRAME is associated with disease progression and severity in some cancer types[10-14].

The mechanism underlying PRAME gene expression in cancers remains unknown. Several studies have proposed that PRAME expression could be induced by prior oncogenic protein such as BCR-ABL1 and RUNX1-RUNX1T1. The activation of BCR-ABL1 gene following the chromosome translocation t(9;22)(q43;q11.2) in chronic myeloid leukemia and the activation of RUNX1-RUNX1T1 following the chromosome translocation t(8;21)(q22;q22) in acute myeloid leukemia (AML) can lead to the induction of PRAME gene overexpression[6, 10, 11]. Another possible mechanism of PRAME gene expression is the hypomethylation status in cancer cells. The hypomethylation present in whole genomic site of cancers enable the activation of PRAME gene and finally lead to the presence of abundant protein antigen in cancer tissues[15, 16].

PRAME antigen was also found in myeloid and lymphoid malignancies. The expression of PRAME was reported in acute lymphoblastic leukemia (ALL) which shows relation to disease progression and severity[18, 19]. This might lead to the use of PRAME as biomarker for disease management and intervention. The PRAME gene expression in myeloid leukemia was less mentioned in terms of its disease correlations, particularly in Thai AML patients. The understanding of PRAME gene expression in AML would be more beneficial to the therapeutic approaches and intervention.

This study aimed to investigate the expression of PRAME gene in newly diagnosed Thai AML patients by using real time quantitative reverse transcription PCR techniques. The expression of PRAME in AML samples was compared to that in the normal healthy individuals as controls. The prevalence of expression and possible correlations of PRAME and disease parameters was assessed to gain more insight information and further application of new findings into

the development of novel therapy to combat the AML and other type of deadly cancers.

Material and methods

AML samples

A total of 50 samples (peripheral blood or bone marrow samples) were obtained from Thai AML patients (32 males and 18 females). The age of AML patients ranged from 16 to 89 years (median age; 56 years). The AML diagnosis was made in accordance with standard leukemic blasts morphology, cytochemistry, immunophenotype of blasts and cytogenetic analysis. EDTA blood samples collected from 30 healthy individuals with no obvious illness and diseases were used as controls along with the study of PRAME gene expression. The study has been approved by the Institute Biosafety Committee and Human Ethics of Thammasat University (064/2561).

RNA extraction and cDNA synthesis

RNA extraction was achieved by using QIAmp RNA blood mini kit (Qiagen, Germany). Reverse transcription was performed by iScript cDNA synthesis kit (Bio-Rad, USA). The primers specific for PRAME were obtained from the Primer-Blast program. The forward sequence of PRAME gene primer was 5'-ACATCCATGCATCTTCCTAC-3' and the reverse primer sequence was 5'-GGTTTC-CAAGGGGTTCATCA-3'. The primers specific for ACTIN gene were obtained from the study of Baraka and colleagues[22] (Table 1). The final concentration of primers was 10 μM. The RNA was stored prior to the quantitative analysis by real time PCR.

Table 1 PRAME and ACTIN primer sequences used in the study. The 20 bps of forward and reverse sequences of PRAME and ACTIN genes are listed. The product size of PRAME and ACTIN genes were 173 and 285 bps, respectively

Primer	Sequence	Nucleotide (bp)	Tm (°C)	Product size (bp)
***PRAME* forward**	5'-ACATCCATGCATCTTCCTAC-3'	20	56	173
***PRAME* reverse**	5'-GGTTTCCAAGGGGTTCATCA-3'	20	58	
***Actin* forward**	5'-AGCGAGCATCCCCCAAAGTT-3'	20	60	285
***Actin* reverse**	5'-GGGCACGAGGGCTCATCATT-3'	20	61	

Real time quantitative reverse transcription PCR (real time RT-qPCR)

The qPCR reaction mix was prepared by addition of 10 μL Luna Universal qPCR Mix (New England Biolabs Inc., USA), 0.5 μL forward primer, 0.5 μL reverse primer, 5 μL cDNA template and nuclease-free water to a final volume of 20 μL. The qPCR reactions for PRAME and ACTIN were incubated at 95°C for 1 minute as initial denaturation, followed by 40 cycles of 15 seconds at 95°C and 30 seconds at 57°C as annealing and extension steps. For result interpretation, the transcription levels of PRAME as target gene were normalized with ACTIN as reference gene to calculate the variable cDNA concentration in each sample. The threshold cycle value (Cq) of PRAME gene in each reaction less than 40 cycles was considered as positive for PRAME gene expression. The delta Cq was calculated by subtraction of Cq values for the reference gene from Cq values of the target gene to demonstrate the level of PRAME gene expression. To test for positivity of the test detection system, the cDNA of SH-Sy-5y neuroblastoma cell line was used as positive control for PRAME gene expression in this study.

Statistical analysis

Data were analyzed by using SPSS version 18 (SPSS Inc., Chicago, IL, USA). Numerical data were shown as mean, median and standard deviation. Qualitative data were shown as frequency and percentage. Chi-square test was used to analyze characteristic variables of studied groups. Spearman-rho method was used to test correlation between numerical variables. The p-value < 0.05 was considered as statistically significance.

Results

General characteristics of AML patients

AML has been classified as hematologic malignancy with high severity. The main causes of AML involve cytogenetic abnormality and gene mutations occurring simultaneously. This study investigated the presence of PRAME gene, a subgroup of cancer testis antigen (CTA) found in several types of cancers. The expression of PRAME gene by quantitative real time PCR have been tested in 50 newly diagnosed AML blood and bone marrow samples. These consisted of 32 males and 18 females, the age ranged between 16 to 89 years (median age; 56 years). The WBC counts ranged from 1.19 to 556.2 × 10^9 cells/L (median range; 46.3 × 10^9 cells/L) and blast count ranged from 30 to 95% (median range; 80%). The majority of AML patients were anemic as shown by the low hematocrit (median range; 23.8%) and hemoglobin level (median range; 7.95 g/dL). Thrombocytopenia was very common in AML patients as highlighted by the low platelet counts (median range; 39.5 ×10^9/L). (Table 2)

Table 2 General characteristics of Thai AML patients. The gender, age, white blood cell count (WBC), hemoglobin (Hb), hematocrit (Hct), platelet count and the number of blasts of AML patients are listed with the range, mean and median

Characteristics	Patient (N)
Gender	
Male	32
Female	18

Characteristics	Patient		
	Range	Mean ± SD	Median
Age (year)	16 - 89	55.04 ± 16.7	56
Male	16 - 89	56.31 ± 18.96	57
Female	28 - 73	52.78 ± 11.84	55
WBC ($x10^9$/L)	1.19 - 556.2	94.28 ± 118.73	46.3
Hb (g/dL)	4.2 - 11.8	7.85 ± 1.78	7.95
Hct (%)	11.3 - 37.9	23.71 ± 5.29	23.8
Platelet ($x10^9$/L)	3.0 - 370	76.66 ± 802.42	39.5
BM Blast (%)	30 - 95	71 ± 0.22	80

PRAME gene expression in Thai AML samples

The detection of PRAME gene expression in 50 Thai AML samples by real time RT-qPCR was performed simultaneously with 30 healthy controls. The results showed that 29 out of 50 AML samples (58%) were positive for PRAME gene expression, the threshold cycles (Cq) ranged from 25.26 to 36.00 (mean Cq; 31.27, median Cq; 31.90). The melting temperature (Tm) for PRAME gene detection was 83.00. The Cq for ACTIN reference gene ranged from 13.08 to 21.68 (mean Cq; 18.00, median Cq; 18.52) (Table 3). The amplification curve of positive PRAME gene samples and positive control (SH-Sy-5y) are shown in Fig. 1. There was no PRAME gene expression in peripheral blood collected from healthy control individuals indicating the cancer specific characteristic of PRAME gene. Randomly selected 5 PRAME gene positive samples were confirmed by DNA sequencing. All 5 PRAME positive samples were shown 100% identity to Homo sapiens PRAME mRNA covered all of 11 variants (data not shown).

Table 3 The threshold cycle (Cq) of PRAME gene expression in 50 Thai AML samples

Sample	Threshold cycle, Cq (n)		
	PRAME (Cq target)	***Actin*** (Cq reference)	Cq (Cq target – Cq reference)
P01	30.24	19.20	11.04
P02	27.71	16.69	11.02
P03	31.65	16.53	15.12
P04	27.70	16.69	15.12
P05	27.91	20.43	7.48
P06	29.43	15.69	13.74
P07	N/A	18.17	-
P08	29.88	19.22	10.66
P09	32.23	17.13	15.10
P10	25.26	20.06	5.2
P11	N/A	21.66	-
P12	N/A	19.65	-
P13	29.46	15.26	14.2
P14	30.13	18.91	11.22
P15	N/A	18.70	-
P16	N/A	19.93	-
P17	N/A	20.22	-
P18	34.31	15.53	18.78
P19	33.76	18.28	15.48
P20	N/A	21.00	-
P21	N/A	19.61	-
P22	N/A	16.92	-
P23	N/A	21.41	-
P24	N/A	21.68	-
P25	28.94	21.36	7.58
P26	32.43	17.24	15.19

Table 3 The threshold cycle (Cq) of PRAME gene expression in 50 Thai AML samples (Continued)

Sample	Threshold cycle, Cq (n)		
	PRAME (Cq target)	***Actin*** (Cq reference)	**Cq** (Cq target – Cq reference)
P27	26.29	18.72	7.57
P28	N/A	19.52	-
P29	N/A	17.64	-
P30	N/A	18.62	-
P31	33.60	21.00	12.60
P32	32.36	19.46	12.90
P33	36.00	16.11	19.89
P34	33.94	15.30	18.64
P35	32.90	16.30	16.60
P36	N/A	18.87	-
P37	31.90	14.30	17.60
P38	N/A	18.19	-
P39	N/A	20.35	-
P40	33.40	15.39	18.01
P41	N/A	19.67	-
P42	34.04	13.08	20.96
P43	34.75	13.89	20.86
P44	N/A	18.42	-
P45	33.15	13.22	19.93
P46	N/A	20.14	-
P47	34.13	15.35	18.78
P48	N/A	15.97	-
P49	29.95	19.42	10.53
P50	29.37	13.77	15.60
Range	**25.26 – 36.00**	**13.08 – 21.68**	**5.20 – 20.96**
Mean ± SD	**31.27 ± 2.75**	**18.00 ± 2.36**	**14.25 ± 4.39**
Median	**31.90**	**18.52**	**15.10**

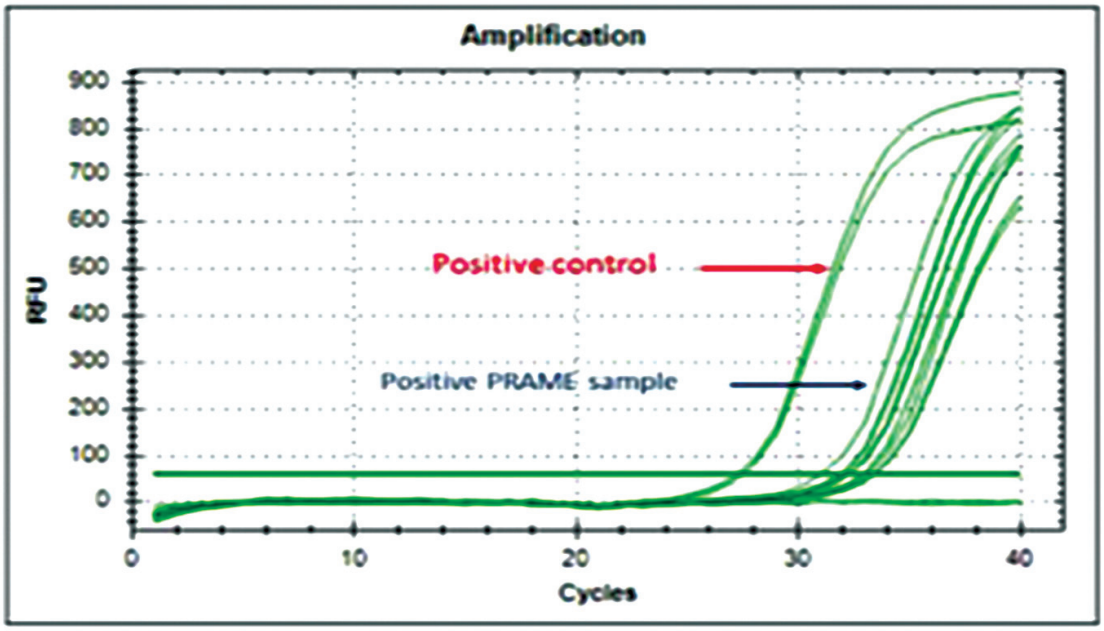

Fig. 1 The amplification curve of PRAME gene. The Cq of positive PRAME samples and positive control are depicted in amplification curves

PRAME gene expression and disease correlations

PRAME gene expression was revealed in 29 AML samples (58%) in this study. The correlation with general characteristic and hematologic parameters was conducted by using statistic tools to assess the significant level. There were 16 males and 13 females who were positive for PRAME gene expression. The *p*-values for gender and PRAME in male and female AML samples were 0.242 and 0.668, respectively. The expression of PRAME gene was also tested for its correlation with WBC counts, hemoglobin, hematocrit, platelet and number of blasts in bone marrow. Comparative statistical *p*-values between PRAME and WBC counts, hemoglobin, hematocrit, platelet and bone marrow blasts were 0.704, 0.094, 0.764, 0.438 and 0.598, respectively (Table 4). There were no significant correlations between PRAME gene expression and WBC counts, hemoglobin, hematocrit, platelet and number of blasts in bone marrow.

Discussion

PRAME gene is a member of cancer testis antigen (CTA) expressed in a wide variety of cancers including melanoma, breast cancers, head and neck cancers, Wilm's tumors, medulloblastoma, Hodgkin lymphoma, neuroblastoma, myeloid and lymphoid leukemia. The expression of PRAME has been related to disease progression, prognostic marker and severity in several types of

Table 4 The PRAME gene expression and its correlations with gender, WBC counts, hemoglobin, hematocrit, bone marrow blasts and platelet status

Parameter	*PRAME* (+) N (%)	*PRAME* (-) N (%)	*p*-value
Patient (N = 50)	29 (58)	21 (42)	
Gender			
Male (N = 32)	16 (50)	16 (50)	0.242
Female (N = 18)	13 (72.22)	5 (27.78)	
WBC (x10^9/L)			
≤ 50 (N = 26)	14 (53.85)	12 (46.15)	0.704
> 50 (N = 24)	15 (62.50)	9 (37.5)	
Hb (g/dL)			
≤ 8 (N = 26)	18 (69.23)	8 (30.77)	0.094
> 8 (N = 24)	11 (45.83)	13 (54.17)	
Hct (%)			
≤ 20 (N = 13)	8 (61.54)	5 (38.46)	0.764
> 20 (N = 37)	21 (56.76)	16 (43.24)	
BM Blast (%)			
20 – 40 (N = 7)	5 (71.43)	2 (28.57)	
41 – 60 (N = 12)	7 (58.33)	5 (41.67)	0.598
61 – 80 (N = 10)	5 (50)	5 (50)	
81 – 100 (N = 21)	12 (57.14)	9 (42.86)	
Platelet status			
Thrombocytopenia (N = 43)	24 (55.81)	19 (44.19)	0.438
Adequate (N = 7)	5 (71.43)	2 (28.57)	

malignancies. These highlight the beneficial of PRAME in terms of therapeutic intervention and management to get the best treatment of choice for deadly cancers. This study showed, for the first time, the expression of PRAME gene in newly diagnosed Thai AML patients. Amongst the 50 AML samples, PRAME gene expression was detected in 29 samples,

representing 58% of total AML patients. The expression of PRAME in our study is consistent with the finding of Epping MT *et al.* in which the role of PRAME in cancers was described[7]. The expression of PRAME in larger proportion of Thai AML would be advantageous for the development of targeted therapy in AML. Since the expression of PRAME is restricted to the cancerous tissues[15, 17, 19 - 21] the use of PRAME as candidate antigen for adoptive immunotherapy is very prominent and possible, for instance, PRAME-specific cytotoxic T lymphocyte (CTL), engineered chimeric antigen receptor T cells (CAR T cells), and the development of specific antibodies target the PRAME antigen.

The correlation between PRAME and general characteristics of Thai AML patients has also been investigated to gain more understanding of gene expression and disease status. The 50 Thai AML patients in the study were mostly anemic as shown by the low level of hematocrit and hemoglobin. The majority of AML patients were thrombocytopenic with a variety of leukocytosis. These demonstrated that the Thai AML patients were at high risk of severe anemia, bleeding tendency and more prone to infection due to loss of normal functional blood cells. The correlations between PRAME and gender, peripheral blood hematological parameters and bone marrow blasts were determined by statistical tools[2, 3, 10]. There was no correlation between PRAME and patient gender, WBC counts, hemoglobin, hematocrit, platelet number and bone marrow blasts. These might be due to the preferentially low sample size of the study. Since the annual occurrence of AML in Thailand is not high enough, the accumulation of patient samples is limited. Further studies need to be conducted for better outcome.

Overall, our study revealed, for the first time, the expression of PRAME in newly diagnosed AML in Thailand. Even though the proportion of PRAME gene expression in Thai AML patients did not differ when compared with the other ethnic and races, the expression of PRAME has shown a promising tumor target antigen that can be further explored[22]. The proportionally high expression of PRAME in AML patients might lead to the development of novel treatment to fight the course of hematologic malignancies. By virtue of PRAME being the most privilege CTA together with the capacity to be recognized by MHC II molecules, this would enable the recognition by cytotoxic T lymphocytes and other immune cells resulting in the functional immune response against PRAME positive cancers. These would ultimately lead to the adoptive use of PRAME as target antigen for cellular immunotherapy via different strategies. Furthermore, to gain more information on PRAME expression in AML, prospective studies equipped with more advanced scientific tools would be necessary to pursue for better beneficiary outcome in the field of cancer research.

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