Correlation between Preferentially Expressed Antigen of Melanoma and Tumour Necrosis Factor-Related Apoptosis-Inducing Ligand Gene Expression in Different Types of Leukaemia Patients

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Key Words

Preferentially expressed antigen of melanoma · Tumour necrosis factor-related apoptosis-inducing ligand · Leukaemia

Abstract

Introduction: Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) down-regulation by preferentially expressed antigen of melanoma (PRAME) is a general phenomenon in different types of solid tumours, but research on the correlation between PRAME and TRAIL gene expression in leukaemia patients is rare. **Method:** PRAME and TRAIL expression was detected in bone marrow samples from 80 newly diagnosed acute leukaemia (AL) patients and 40 chronic myeloid leukaemia (CML) patients using TaqMan-based real-time quantitative PCR methods, and a linear correlation analysis was performed on their levels of expression. A total of 15 normal bone marrow samples from individuals with non-malignant haematological diseases served as normal controls. **Results:** PRAME expression was higher in both AL and CML patients compared to controls (both $p < 0.001$). CML patients in both blast crisis (BC) and the accelerated phase (AP) had significantly higher PRAME levels than CML patients in the chronic phase (CP) ($p = 0.006$ and 0.0461 , re-

spectively). TRAIL expression was higher in both the acute myeloid leukaemia (AML) group and the acute lymphoblastic leukaemia (ALL) group than in the controls ($p = 0.039$ and 0.047 , respectively). In contrast, CML patients had lower TRAIL levels than controls ($p = 0.043$), and TRAIL expression in CML patients in the advanced phases (BC and AP) was significantly lower than in CML-CP patients ($p = 0.006$). In CML patients, there was a significant inverse correlation (Spearman's $R = -0.6669$, $p < 0.0001$) between PRAME and TRAIL gene expression, while a greater significant inverse correlation was found in patients in the advanced phases (BC and AP) ($R = -0.6764$). In addition, no correlation was observed in AML and ALL patients. **Conclusion:** The simultaneous detection of PRAME and TRAIL gene expression may be helpful to monitor condition changes in leukaemia patients and evaluate therapeutic effects in clinical practice, particularly in CML patients.

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Introduction

Preferentially expressed antigen of melanoma (PRAME) is a tumour antigen recognised by cytotoxic T cells (CTLs) and was originally identified in a melanoma

patient [1]. It is expressed in various malignant tumours, including solid tumours and haemopoietic neoplasia; however, it is not expressed or only weakly expressed in normal tissues [2]. Tumour necrosis factor-related apoptosis-inducing ligand (TRAIL), a new antitumour molecule, is a type II membrane protein belonging to the TNF superfamily [3], and it is abnormally expressed in a variety of cancers (higher or lower expression than normal tissues). It has been confirmed that TRAIL down-regulation by PRAME is a general phenomenon in different types of solid tumours; De Carvalho et al. [4] also found this phenomenon in chronic myeloid leukaemia (CML) patients. However, research on the correlation between PRAME and TRAIL gene expression in different types of leukaemia patients is relatively rare. In this study, we determined the expression of these two genes in bone marrow mononuclear cells from different types of leukaemia patients and performed a linear correlation analysis on this expression.

Materials and Methods

Patients and Controls

Bone marrow samples were collected from 80 de novo patients with acute leukaemia [AL: 61 acute myeloid leukaemia (AML) and 19 acute lymphoblastic leukaemia (ALL)] and 40 patients with CML from June 2011 to July 2012. AL was classified according to the French-American-British (FAB) classification. The CML diagnosis of patients enrolled in this study was confirmed by Philadelphia chromosome and BCR-ABL rearrangement detection. CML was classified into the chronic phase (CP), accelerated phase (AP) and blast crisis (BC) according to the criteria of the International Bone Marrow Transplant Registry. All bone marrow samples were tested for PRAME and TRAIL expression with quantitative RT-PCR. In addition, a control group of 15 normal bone marrow samples from individuals with non-malignant haematological diseases was tested, and the K562 cell line was used as a positive control and for sensitivity tests. The study was approved by the research ethics committee of our hospital, and informed consent was obtained from all participants.

Primer and Probe Design

Primers and probes were designed using Primer-Express software (PE Biosystems). Each probe was labelled with a fluorescent 5'-reporter dye (FAM: 6-carboxy-fluorescein) and a 3'-quencher (TAMRA: 6-carboxy-tetramethyl-rhodamine). The PRAME-specific primers and probe were as follows:

forward primer, 5'-CTCTATGTGGACTCTTTATTTTCC-TTAGA-3';

reverse primer, 5'-CGAAAGCCGGCAGTTAGTTATT-3';

probe, 5'-CTGGATCAGTTGCTCAGGCACGGTGA-3'.

The TRAIL-specific primers and probe were as follows:

forward primer, 5'-CTCTGTGGCTGTAACCTACGTG-3';

reverse primer, 5'-CAAGCAATGCCACTTTTGGA-3';

probe, 5'-CGAGCTGAAGCAGATGCAGGACAAGTAC-3'.

The GAPDH housekeeping gene was used to normalise PRAME and TRAIL expression.

Real-Time Amplification Protocol

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The RNA concentration and purity were determined by spectrophotometry. Reverse transcription was carried out with a Prime Script RT-PCR kit (TaKaRa, Japan). The standard reverse transcription reaction volume was 10 µl and contained 2 µl of 5 × RT buffer, 300 ng of template mRNA, 0.5 µl of oligo dT primer (50 µM), 0.5 µl of RT enzyme mix, 2 µl of random 6-mers (100 µM) and 2 µl of RNase-free dH₂O. This mixture was incubated at room temperature for 15 min and then maintained in a heat block at 85°C for 5 s. For PRAME, TRAIL and GAPDH transcripts, 2 µl of target cDNA was added to 20 µl of PCR mix containing 10 µl of Universal PCR Master Mix, 400 nM of each primer and 800 nM of the probe according to the manufacturer's protocol. The GAPDH housekeeping gene and the genes of interest were amplified in parallel. The reaction was initiated at 95°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5 s and annealing/extension at 60°C for 33 s. The data were recorded as threshold cycle (Ct) values with an ABI StepOne plus System (Applied Biosystems, Calif., USA) using analytical software from the same manufacturer.

Quantification with Real-Time RT-PCR

The average Ct value of GAPDH was subtracted from the average Ct value of each gene of interest. The calibrator was the mean Ct value obtained from 15 control marrow samples, which were assessed in parallel. The Δ Ct value obtained in leukaemia samples was then subtracted from the Δ Ct value obtained from the calibrator, giving a $\Delta\Delta$ Ct value. Because the amplification efficiencies for PRAME, TRAIL and GAPDH were comparable, the amount of mRNA of each gene normalised to GAPDH was given by the relation $2^{-\Delta\Delta Ct}$. A bone marrow sample was considered positive for PRAME expression if the expression level was 10-fold higher than the mean expression in control samples [5]. A bone marrow sample was considered positive for TRAIL expression if the expression level was 1-fold higher than the mean expression in control samples.

Sensitivity and CV Value

K562 cDNA was serially diluted 10-fold in water, and the limit of PRAME detection with real-time RT-PCR was 10^{-5} (fig. 1). The cDNA of mononuclear cells from a normal bone marrow sample was serially diluted 10-fold in water, and the limit of TRAIL detection was 10^{-5} (fig. 1). Intra- and inter-assay reproducibility was determined using 10-fold serial dilutions of K562 cDNA (1:1 to $1:10^{-5}$). The intra- and inter-assay CVs for real-time RT-PCR were 2.4 and 3.1%, respectively.

Statistical Analysis

Statistical analyses were carried out with GraphPad Prism, version 5.00 for Windows (GraphPad Software, San Diego, Calif., USA). Experimental values were compared using the Mann-Whitney U test, the Kruskal-Wallis test or the non-parametric Spearman's rank correlation test. Differences between experimental groups were considered significant at $p < 0.05$.

Fig. 1. a Sensitivity of real-time RT-PCR in detecting PRAME expression in K562 cDNA serially diluted 10-fold in water. **b** Sensitivity of real-time RT-PCR in detecting TRAIL expression in the cDNA of mononuclear cells from a normal bone marrow sample serially diluted 10-fold in water.

Fig. 2. a Correlation between relative PRAME and TRAIL expression in all CML patients (Spearman's $R = -0.6669$, $p < 0.0001$). **b** Correlation between relative PRAME and TRAIL expression in CML patients in the advanced phase (BC and AP) (Spearman's $R = -0.6764$, $p = 0.0002$). **c** Correlation between relative PRAME and TRAIL expression in CML patients in the CP (Spearman's $R = 0.1968$, $p = 0.4821$).

Results

Correlation

A correlation analysis was first performed on PRAME and TRAIL gene expression in CML patients, and the result showed that there was a significant negative correlation (Spearman's $R = -0.6669$, $p < 0.0001$) between the expression of these two genes (fig. 2), in agreement with

De Carvalho et al. [4]. In addition, a more significant correlation ($R = -0.6764$, $p = 0.0002$) was observed in patients who were in an advanced phase (BC or AP), but there was no correlation ($p = 0.4821$) in CML-CP patients (fig. 2). Unexpectedly, in AML ($p = 0.7322$) and ALL ($p = 0.6235$) patients, no correlation was observed between the levels of these two genes (fig. 3).

Fig. 3. a Correlation between relative PRAME and TRAIL expression in AML patients (Spearman's $R = 0.04676$, $p = 0.7322$). **b** Correlation between relative PRAME and TRAIL expression in ALL patients (Spearman's $R = 0.1238$, $p = 0.6235$).

Table 1. Expression of PRAME in bone marrow cells from leukaemia patients by real-time RT-PCR

Sample	n	n of positive	Ratio of positive, %	Relative expression ^a
AML M1	3	1	33.33	95.92±49.2
AML M2	27	15	55.55	868.66±233.98
AML M3	10	6	60	629.95±146.91
AML M4	13	6	46.15	101.22±26.7
AML M5	8	2	25	34.27±14.49
Total	61	30	48.39	523.46±152.29
CML-BC	15	12	80	3,844.86±739.89
CML-AP	10	4	40	985.6±278.17
CML-CP	15	2	13.33	6.92±4.38
Total	40	18	45	1,690.82±994.13
ALL	19	7	36.84	261.78±88.84
Control	15	1	6.67	5.49±3.82

^a Results are expressed as mean ± SD for relative expression compared to normal bone marrow, using the relation $2^{-\Delta\Delta C_t}$.

Table 2. PRAME and TRAIL expression of AML-M2 samples with AML1-ETO at diagnosis and at CR using real-time RT-PCR

Sample	Expression of PRAME at diagnosis	Expression of PRAME at CR ^a	Expression of TRAIL at diagnosis	Expression of TRAIL at CR ^a
P1	2,272.40	43.11	20.48	6.71
P2	12,865.30	4.08	12.21	17.29
P3	340.14	0.59	1.23	8.23
P4	770.69	30.70	3.56	0.64

P = AML-M2 patient with AML1-ETO.

^a CR is defined as <5% leukaemia cells in the bone marrow.

Relative Expression of PRAME in Patients with Different Types of Leukaemia

The PRAME levels were either extremely low or undetectable in control samples. PRAME expression in patients with AML and ALL was higher than in controls ($p < 0.001$ for both), and it was detected at high levels in AML M3 (60%), AML M2 (55.55%) and AML M4 patients (46.15%) (table 1). We also found that the PRAME expression in 4 patients with t(8;21) was much higher than other AML subtypes. Likewise, PRAME expression was also higher in the CML group compared to controls ($p < 0.001$) (table 1). The difference in PRAME expression between the CML-BC and CML-CP groups, and between the CML-AP and CML-CP groups was statistically significant ($p = 0.006$ and 0.0461 , respectively). Furthermore, the difference between CML-BC and CML-AP patients was statistically significant ($p = 0.045$). These results confirmed those of De Carvalho et al. [4] and Radich et al. [6], who demonstrated that PRAME was up-regulated during CML progression. Interestingly, we also found that there was an inverse correlation ($r = -0.35$, $p = 0.019$) between PRAME levels and the quantity of bone marrow blast cells in AML patients. Figure 4 shows PRAME expression in different types of leukaemia patients.

PRAME and TRAIL Levels in t(8;21) AML Patients Assessed for Minimal Residual Disease

The gene expression of PRAME and TRAIL in four t(8;21) AML patients was monitored at presentation and at complete remission (CR) (table 2). Three of the patients reached CR after 1 cycle of standard 3 + 7 induction chemotherapy (doxorubicin 30 mg/m²/day or idarubicin 12 mg/m²/day for 3 days plus cytarabine 100 mg/m²/day for

Fig. 4. Relative PRAME levels in control and patient groups (expressed in ΔCt). Horizontal lines represent the mean relative PRAME expression of each study group. A high ΔCt value indicates a low PRAME expression level.

7 days), and 1 patient reached CR after 2 cycles of induction chemotherapy. Interestingly, a marked decrease in PRAME expression was observed in all four samples tested between diagnosis and CR. Moreover, TRAIL expression increased in two samples and decreased in two samples.

Relative TRAIL Expression in Patients with Different Types of Leukaemia

TRAIL expression was significantly higher in AML and ALL patients compared to controls ($p = 0.039$ and 0.047 , respectively) (table 3), and it was expressed at similar levels in different subtypes of AML ($p = 0.376$). The difference in TRAIL expression between AML and ALL patients was not statistically significant ($p = 0.157$). TRAIL expression was lower in CML patients than in controls ($p = 0.043$), and its expression in CML cases at an advanced phase was significantly lower than in CML-CP patients ($p = 0.006$). The difference in the TRAIL level between CML-BC and CML-AP patients was not statistically significant ($p = 0.23$). Figure 5 shows TRAIL expression in patients with different types of leukaemia.

Discussion

PRAME is over-expressed in a variety of neoplasia, such as melanoma, Hodgkin lymphoma and myelocytic and lymphocytic leukaemia, but its expression is low or

Table 3. Expression of TRAIL in bone marrow cells from leukaemia patients by real-time RT-PCR

Sample	n	n of positive	Ratio of positive, %	Relative expression ^a
CML-BC	15	1	6.67	0.54 ± 0.43
CML-AP	10	1	10	0.47 ± 0.42
CML-CP	15	3	20	1.14 ± 0.86
Total	40	5	12.5	0.75 ± 0.59
AML	61	35	57.38	10.15 ± 8.16
ALL	19	10	52.63	8.96 ± 5.65
Control	15	3	20	1.65 ± 0.91

^a Results are expressed as mean $\pm$ SD for relative expression compared to normal bone marrow, using the relation $2^{-\Delta\Delta Ct}$.

absent in normal tissues [2]. In this study, we used real-time RT-PCR to detect PRAME gene expression, and relative gene expression data were analysed with the $2^{-\Delta\Delta Ct}$ method. Only one of fifteen normal bone marrow samples from individuals with non-malignant haematological diseases showed weak PRAME gene expression. In contrast, 30 of 61 patients (48.39%) with AML and 7 of 19 patients (36.84%) with ALL showed PRAME expression, and the difference in PRAME expression between AML and ALL cases was not statistically significant, which was in agreement with previous reports [7]. The highest prev-

Fig. 5. Relative TRAIL levels in control and patient groups (expressed in ΔCt). Horizontal lines represent the mean relative TRAIL expression of each study group. A high ΔCt value indicates a low TRAIL expression level.

alence has been found in M2 and M3 phenotypic subgroups and in cases with t(8;21) [8]. Tajeddine et al. [5] demonstrated that the expression of PRAME and AML1/ETO transcripts was highly correlated. In our study, PRAME was found in 100% of patients with the t(8;21) translocation in M2 and 60% of patients with the t(15;17) translocation in M3. We monitored PRAME expression in 4 PRAME-positive patients with the AML1/ETO fusion gene, and PRAME expression decreased significantly after induction therapy in all 4. This finding indicates that PRAME could serve as a marker for monitoring minimal residual disease (MRD) in AL. We also found an inverse correlation between PRAME expression and blast cells in the marrow of AML patients, which was in line with the findings of Steinbach et al. [9]. PRAME expression has been studied in a relatively limited number of cases with CML, and the data on PRAME expression are highly variable. PRAME expression was not detected in a study of 13 cases [8]. In another study by Matsushita et al. [7], 32 CML cases were evaluated, and PRAME expression was identified in 31%; there was a trend towards increased PRAME expression in advanced-stage disease. Watari et al. [10] proposed that PRAME was a BCR-ABL inducible gene, and this hypothesis was proven by De Carvalho et al. [4]. In our study, 15 cases had CP disease and 2 of these had PRAME expression (13.33%), 10 cases had AP disease and 4 of these had PRAME expression (40%) and 12 of the 15 cases with BC had PRAME expres-

sion (80%). We found an obvious trend towards increased PRAME expression in CML patients with advanced-stage disease, which was in line with previous research [4, 6, 7]. Van 't Veer et al. [11] suggested PRAME as an independent prognostic marker of poor clinical outcome in different solid tumours, while another group viewed PRAME as a marker of favourable prognosis in childhood AML patients [12].

TRAIL can only induce apoptosis in cancer cells, and increasing evidence has shown that TRAIL is capable of acting as a tumour suppressor. On the one hand, experiments using neutralising antibodies against TRAIL proved that it could significantly increase liver metastases in mice [13]. On the other hand, TRAIL-knockout mice were more susceptible to tumourigenesis induced by metastasis and chemical carcinogens [13]. In fact, according to some expression-profiling surveys in cancer, upon cancer progression, TRAIL expression is lost [14]. These data indicate that TRAIL down-regulation plays an important role in tumour initiation and progression. TRAIL expression has been studied in a relatively limited number of leukaemia cases. In our study, TRAIL expression in the CML group was significantly lower than in normal controls. Patients with lower TRAIL expression were in the advanced phases of the disease, while patients with higher expression were in the CP, which indicates that decreased TRAIL expression may play an important role in the pathogenesis of CML. In contrast to CML, TRAIL

expression in the AML and ALL groups was significantly higher than in controls. A previous study used RT-PCR to detect the TRAIL gene in leukaemia cells, and the results showed that malignant cells from most cases of primary AML and ALL had positive TRAIL expression [15], which is in line with our results. TRAIL is involved in the tumour cell evasion of immunosurveillance through killing activated human T cells, and TRAIL could be related to spontaneous tumour cell death and necrosis [16]. Therefore, we hypothesise that TRAIL up-regulation in AL might play a role in tumour initiation. In our study, TRAIL expression at CR showed high variability compared with its expression at presentation. This finding may indicate that TRAIL itself is not a candidate for monitoring MRD, and the simultaneous detection of TRAIL and PRAME is necessary to monitor MRD.

De Carvalho et al. [4] detected PRAME and TRAIL gene levels in peripheral blood mononuclear cells from CML patients using RQ-PCR. He found a significant inverse correlation between the expression of these two genes, and knocking down the PRAME gene with RNA interference restored TRAIL gene expression in a BCR-ABL-positive cell line. The BCR-ABL-mediated up-regulation of PRAME directly resulted in TRAIL down-regulation in BCR-ABL-positive cells [4]. TRAIL down-regulation by PRAME may be a more general phenomenon in a variety of tumours, as indicated by *in silico* analyses in gene-expression-profiling studies. De Carvalho et al. [4] hypothesised that TRAIL expression may be regulated by PRAME because PRAME is a dominant repressor of RA receptor signalling [17] and TRAIL is inducible by RA treatment. However, the mechanism via which PRAME suppresses the RA signalling pathway has only been well established in melanoma, and whether PRAME inhibits RA signalling in other tumours is not clear. In fact, in AL,

PRAME expression was not associated with down-regulation of RA signalling [18]. We detected PRAME and TRAIL gene expression in bone marrow mononuclear cells from leukaemia patients with RQ-PCR, and our result was in line with the conclusion of De Carvalho et al. [4] that there was a significant inverse correlation between the expression of these two genes in CML cases. More importantly, we found a more significant correlation between this expression in an advanced phase, but there was no correlation in CML-CP cases. In addition, no correlation was observed between the levels of these two genes in AL patients. To date, these observations have not been presented in the literature. The combined treatment of TRAIL production with imatinib enhances the TRAIL-induced apoptosis of BCR-ABL-positive cell lines, even K562, a TRAIL-resistant cell line [19], and it has been proven that K562 cells with an artificial reduction in PRAME expression are more sensitive to imatinib because of increased TRAIL expression [4]. Therefore, we hypothesise that interfering with PRAME expression combined with methods that stimulate TRAIL up-regulation may be a good alternative or co-adjuvant strategy for CML patients. In contrast, there was no inverse correlation between the levels of these two genes in AL cases, which may be because PRAME is not associated with down-regulation of RA signalling in AL cases; therefore, PRAME could not down-regulate TRAIL by suppressing the RA signalling pathway.

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