Cadherin-13 in primary and blast crisis chronic myeloid leukaemia: declining expression and negative correlation with the *BCR/ABL* fusion gene

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ABSTRACT

Expression of the *CDH*13 gene in chronic myeloid leukaemia (CML) patients at different clinical stages, and its relationship with the *BCR/ABL* fusion gene, is investigated. Expression of the *CDH*13 gene and the *BCR/ABL* fuse gene is investigated in peripheral blood from 30 healthy adults, 25 primary CML patients and 25 CML patients in blast crisis using the EvaGreen real-time reverse transcriptase polymerase chain reaction (RT-PCR). Results showed that *BCR/ABL* fusion gene expression in the blast crisis CML patients was 4.72-fold higher than that in patients with primary CML. Expression of CDH13 mRNA in primary and blast crisis CML patients was lower than in the healthy adults, reduced 64% and 75%, respectively. Expression of CDH13 showed a negative correlation with the *BCR/ABL* fusion gene. The data indicate that the decline of CDH13 expression accompanied the different clinical stages of CML and probably was involved in over-expression of the *BCR/ABL* fusion gene.

KEY WORDS: Genes ABL.
H-cadherin.
Leukemia, myelogenous, chronic, BCR-ABL positive.

Introduction

Cadherins are a superfamily of adhesion molecules that mediate Ca^{2+}-dependent cell–cell adhesion. The cadherin-13 (CDH13, also called H-cadherin and T-cadherin) gene, a new member of the cadherin superfamily, recently was isolated and has been mapped to 16q24.[1] CDH13 is a unique member of the cadherin family linked to the membrane through a glycophosphatidyl inositol (GPI) anchor.[2,3] However, unlike most of the other proteins in the cadherin family, it lacks a cytoplasmic domain.

The exact cellular function of this protein has yet to be completely elucidated. Loss of expression and aberrant methylation of the *CDH*13 gene have been identified in human cancer cell lines and human primary tumours such as lung, breast, gastric, colorectal and ovarian cancers.[4–7]

It is hypothesised that this gene functions as a tumour suppressor gene. Although it has been reported that some chronic myeloid leukaemia (CML) patients present with the silencing of CDH13 expression by aberrant promoter methylation,[8] the expression of CDH13 in the different clinical phases of CML and its relation with the *BCR/ABL* fusion gene has not been evaluated.

Materials and methods

Patients and healthy adults

Twenty-five primary CML patients and 25 CML patients in blast crisis were diagnosed according to standard criteria[9] between August 2004 and June 2007. Thirty health adults comprised the control group.

Total RNA isolation

Peripheral blood from patients and healthy men were anticoagulated with EDTA-K_2 and mononuclear cells were isolated using a Ficoll-Paque density gradient by centrifugation (Beckman GS-15R). Total cellular RNA was extracted with Trizol (Invitrogen Gibco) following the manufacturer's protocol, and the quantity and purity of RNA were determined by ultraviolet spectrophotometry (Beckman DU-640).

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Reverse transcription reaction

Total RNA (1 μg) and 200 ng random primer (Songon Biological Engineering, China) were added in 15 μL diethylpyrocarbonate (DEPC)-treated H_2O, incubated at 65°C for 5 min and cooled quickly on ice. Then, DEPC H_2O (2.0 μL) 5*RT buffer (containing 375 mmol/L KCl, 250 mmol/L Tris-HCl, 3 mmol/L $MgCL_2$ and 50 mmol/L DTT) (5.0 μL), 10 mmol/L dNTP (1.25 μL), 50 units/μL RNAsin (0.75 μL) and MMLV reverse transcriptase (200 units/μL , Promega) (1.0 μL) were added, centrifuged and incubated at 37°C for 60 min, and then stored at –20°C.

Primers

The primers were designed using Primer Primers 5.0 software. CDH13 primers were: forward, 5′-GAT GGC GGC TTA GTT GCT-3′; reverse, 5′- CGA AAG ATC GGT CGG TTG -3′. BCR/ABL primers were: forward, 5′-CTC CAG ACT GTC

CAC AGC ATT CCG -3′; reverse, 5′-CAG ACC CTG AGG CTC AAA GTC AGA -3′. GAPDH primers were: forward, 5′-CAA CTT TGG TAT CGT GGA AGG ACT C-3′; reverse, 5′- AGG GAT GAT GTT CTG GAG AGC C -3′. All primers were synthesised by Songon Biological Engineering.

Real-time RT-PCR

A real-time reverse transcriptase polymerase chain reaction (RT-PCR) method was performed using a Lightcycler (Roche). The PCR method was carried out in a 20 µL final volume containing a) 25 mmol/L $MgCl_2$ (2 µL), b) 4 pmol/µL sense and antisense primers (1.0 µL each), c) 10 mmol/L dNTP (0.4 µL), d) 2 µg/µL BSA (1.0 µL), e) EvaGreen (1.0 µL, Boitium), f) PCR buffer (2.0 µL, Qiagen), g) complementary DNA (cDNA, 2.0µL), h) hot start *Thermus aquaticus* (*Taq*) DNA polymerase (0.5 unit, Qiagen) and i) H_2O up to 20 µL.

After initial activition of the hot start *Taq* DNA polymerase at 95°C for 15 min, temperature cycling was initiated. Each cycle consisted of denaturation at 95°C for 5 sec, hybridisation at 58°C for 10 sec, elongation at 72°C for 20 sec and 83°C for 5 sec, followed by a single fluorescence measurement. A total of 40 cycles were performed.

Cycling conditions for BCR/ABL and GAPDH were: initial activition at 95°C for 15 min followed by 40 cycles at 95°C for 5 sec, 58°C for 20 sec and 72°C for 20 sec. The fluorescence signal was acquired at the end of the elongation step. For mathematical modelling, it is necessary to determine the cycle threshold (CT) for each transcript.[10] The cycle threshold is defined as the point at which the fluorescence rises appreciably above the background fluorescence.

Gel electrophoresis

The PCR products (10 µL), mixed with 2 µL loading buffer, were added to 2% (w/v) agarose gel (Bio Basic, Canada) containing ethidium bromide (0.5 µg/mL) and electrophoresed with 1× TAE buffer at a constant 100 V for 25 min. The gels were visualised and photographed under ultraviolet (UV) light using an FR200A image system (Furi Technology, China).

Standard curve

Complementary DNA from healthy adults and K562 cells were diluted to 20, 2, 0.2, and 0.02 ng (corresponding to total RNA) with TE as the standard template. Real-time PCR was performed for GAPDH, BCR/ABL and CDH13.

Table 1. Mean CT value of the *CDH*13 gene from healthy adults and CML patients in CP and BC.

Group (cases)	*CDH*13 $CT_{AVERAGE}$	*GAPDH* $CT_{AVERAGE}$	$2^{-\Delta\Delta CT}$
CML, primary (*n*=25)	34.11	20.28	0.36
CML in BC (*n*=25)	35.00	20.65	0.25
Healthy adults (*n*=30)	32.55	20.19	

Table 2. Mean CT value of the *BCR/ABL* fusion gene from CML patients in CP and BC.

Group (cases)	*BCR/ABL* $CT_{AVERAGE}$	*GAPDH* $CT_{AVERAGE}$	$2^{-\Delta\Delta CT}$
CML in BC (*n*=25)	30.48	20.65	4.72
CML, primary (*n*=25)	32.36	20.28	

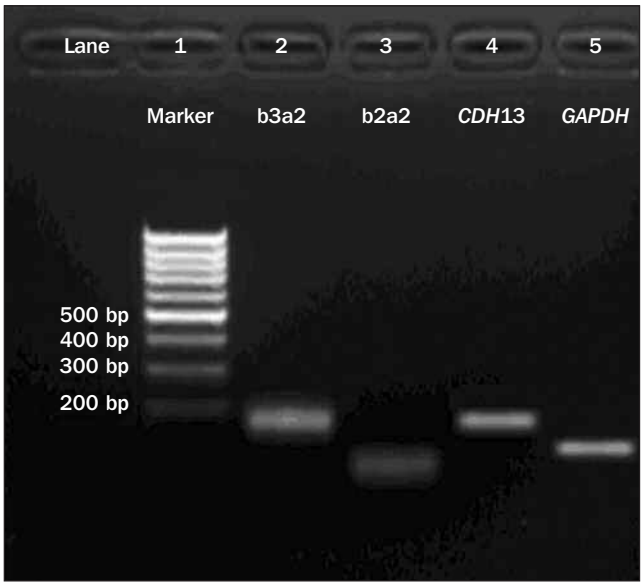

Fig. 1. PCR results showing the amplified products after agarose gel electrophoresis. Lane 1: molecular weight marker; lanes 2 and 3: *BCR/ABL* fusion gene (b3a2 and b2a2, 165 bp and 90 bp); lane 4: *CDH*13 gene (183 bp); lane 5: *GAPDH* gene (129 bp).

Statistical analysis

The mean CT value of CDH13 from healthy adults and the CML patients in the chronic phase and in blast crisis were compared using the CT value of *GAPDH* as an internal control. ΔCt was the difference in Ct values derived from the target gene (in each sample assayed) and the *GAPDH* gene, while ΔΔCt represented the difference between the paired samples. The n-fold differential ratio was expressed as $2^{-\Delta\Delta Ct}$. Statistical analysis of the association between *CDH*13 and *BCR/ABL* gene expression was performed using simple correlation analysis, while statistical inference of correlation was performed using *t*-test.

Results

Gel electrophoresis and real-time RT-PCR

Gel electrophoresis showed that the PCR products of CDH13, BCR/ABL and GAPDH showed specific bands, and the size of the CDH13, BCR/ABL and GAPDH amplicons matched the designed primers according to the molecular weight marker (Fig. 1).

The standard curve coefficient correlation of CDH13 and GAPDH was 1.0. Negative control (distilled water) showed no significant primer amplification (Fig. 2). Efficiency of CDH13 and GAPDH amplification was similar. The melting curve showed that the *CDH*13, *BCR/ABL* and *GAPDH* genes each had a single peak (Fig 3).

CDH13 expression in CML patients

The mean CT values of CDH13 and GAPDH from healthy adults and the CML patients are shown in Table 1, The expression of CDH13 mRNA from the primary and blast crisis CML patients (64% and 75%, respectively) were

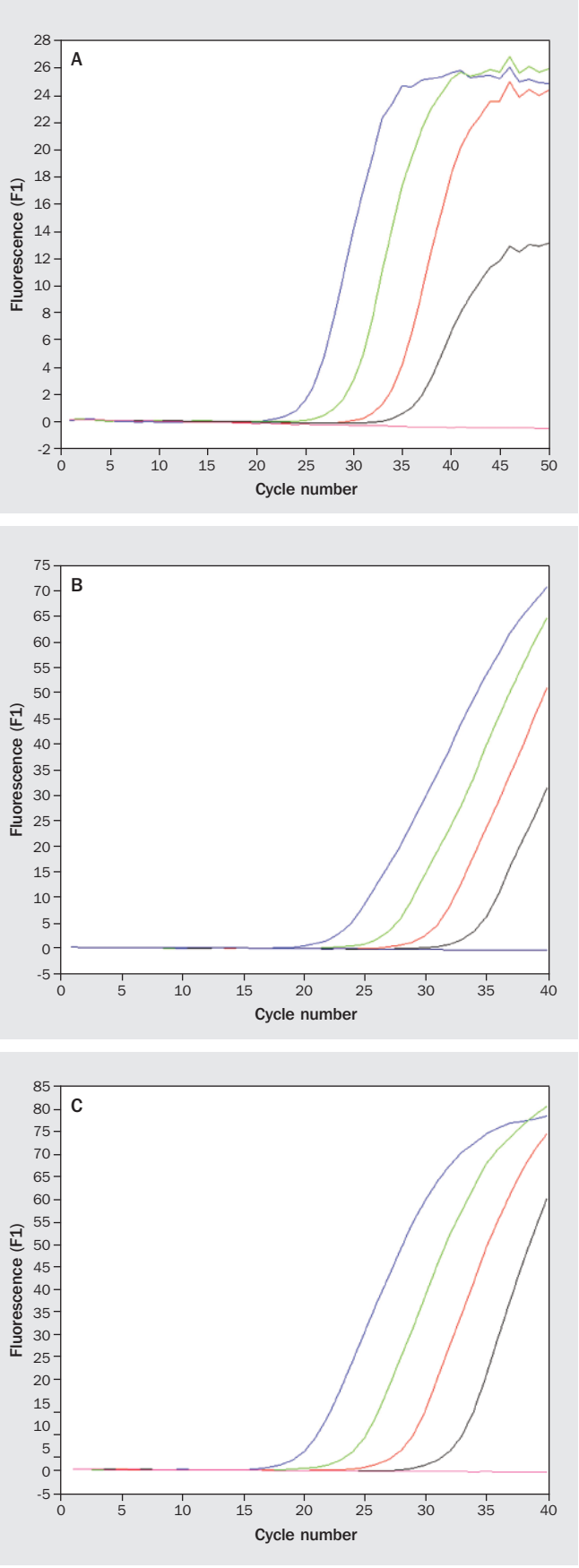

Fig. 2. Amplication curves of *CDH*13 (A), *BCR/ABL* (B) and *GAPDH* (C) genes (corresponding to 20, 2, 0.2 and 0.02 ng total RNA). See these images in colour at www.bjbs-online.org

reduced compared to the healthy adults. Expression of CDH13 mRNA from the blast crisis CML patients was lower than that in the primary CML patients. The mean CT values for the *BCR/ABL* fusion gene and GAPDH from the primary and blast crisis CML patients are shown in Table 2. Expression of the *BCR/ABL* fusion gene in the blast crisis CML patients was 4.72-fold higher than in the patients with primary CML.

Correlation analysis of the CDH13 and BCR/ABL expression

Association analysis of CDH13 and BCR/ABL expression level was performed using simple correlation analysis. Statistical inference of correlation coefficient was performed using *t*-test. *CDH*13 gene expression showed a negative correlation ($r=-0.36$, $P<0.05$, $t=2.252$) with *BCR/ABL* fusion gene expression.

Discussion

Chronic myeloid leukaemia is a myeloproliferative disorder arising from the clonal expansion of an altered pluripotent haematopoietic stem cell. It evolves in three clinical stages: the chronic phase, the accelerated phase and blast crisis.

Chronic myeloid leukaemia is characterised by a chimaeric *BCR/ABL* gene on the Philadelphia (Ph) chromosome, which encodes a 210-kDa fusion protein, This fusion protein has altered kinase activity, which is the source of its oncogenicity by affecting RAS, PI3K/AKT and JAK/STAT signal transduction pathways.[11–13]

The present results show that the amount of BCR/ABL messenger RNA (mRNA) in cells from patients in blast crisis are higher than in those in the chronic phase, indicating that their expression rises with disease progression.

The epigenetic change originated by over-expression of BCR/ABL may be involved in the blast crisis process. Investigation indicates that the expression of BCR/ABL damages DNA repair gene function,[14,15] blocks cell apoptosis,[16,17] and differentiation of myeloprecursor cells.[18] Therefore, BCR/ABL level may be essential in determining the phenotype of the leukaemic clone at different stages of the disease.[19]

Furthermore, subsequent mechanisms that alter adhesion to stromal cells and the extracellular matrix[20] are also implicated in the malignant transformation by BCR/ABL. Reduced cell–cell adhesiveness is considered to be important for both the early and late carcinogenic steps.

Chronic myeloid leukaemia is characterised by premature release of haematopoietic progenitor cells to the peripheral blood. This may be a result of an aberrant adhesion of leukaemic progenitors to bone marrow stromal cells. Cell adhesion molecules (CAM) on the surface of CML cells may be partially responsible for these changes in adhesion characteristics. The integrin, selectin and immunoglobulin families have been reported to be involved in defective cell adhesion in CML.[21–23]

In recent years, a large family of transmembrane glycoproteins, the cadherins, have attracted more interest. These are involved in the contact inhibition of cell growth by inducing cell-cycle arrest,[24] and act as an invasion suppressor in the initiation and progression of human cancers.[25]

CDH13 (H-cadherin) is a new member of the cadherin superfamily, which plays an important role in selective cell recognition and adhesion. Expression of CDH13 is

decreased by methylation in a variety of human cancers, indicating that the *CDH*13 gene functions as a tumour suppressor gene.

Although the mechanism by which CDH13 results in cell growth suppression has yet to be elucidated, some results demonstrate that CDH13 growth suppression is associated with G2 phase arrest and requires p21CIP1/WAF1 expression.[26] There is evidence that loss of CDH13 is associated with enhanced tumourigenicity of human non–small-cell lung cancer and prostate cancer.[27,28]

In the present study, we show that the *CDH*13 gene expression is decreased in cells from primary and blast crisis CML patients. Despite the crucial role of the *CDH*13 gene in cell–cell adhesion and in the contact inhibition of proliferation, the expression status of this gene suggests that low expression might partly be responsible for increased circulation of immature haematopoietic cells in the peripheral blood.

Although over-expression of DNA methyltransferases (DNMTs) has been proposed as a mechanism for aberrant genome methylation, it does not explain the specific regional hypermethylation in cancer cells. It has been reported that some CML patients present with low CDH13 expression due to aberrant promoter methylation.[8] However, in the chronic phase of CMl, levels of DNMTs are not significantly different from those in normal bone marrow cells,[29] indicating that over-expression of DNMTs is not involved in the methylation of the *CDH*13 gene.

Our results show that the expression of CDH13 in blast crisis CML patients was lower than in primary CML patients, and showed a negative correlation with *BCR/ABL* fusion gene expression. This supports the hypothesis that BCR/ABL fusion protein may act as a docking protein to recruit downstream molecules to actin, and involves the epigenetic changes of CDH13 methylation during transformation.

It has been reported that the acute promyelocytic leukaemia (PML)-retinoic acid receptor alpha (RARalpha) fusion product recruits histone deacetylase (HDAC) and DNA methyltransferase (DNMT) activities on retinoic acid (RA)-target promoters, thus silencing them and blocking differentiation.[30–32] Whether the *CDH*13 gene is methylated by this mechanism requires further investigation.

Abnormal circulation and unregulated proliferation of CML progenitors is related to BCR/ABL-induced abnormalities in β1 integrin-mediated adhesion and signalling,[33–35] which functions as an extracellular matrix (ECM) receptor. Study indicates that expression of CDH13 regulates the level of surface β1 integrin and enhances cell matrix adhesiveness.[36] Thus, low expression of CDH13 may be implicated in the BCR/ABL-induced abnormal association of integrin receptors and provide new insights into mechanisms underlying abnormal integrin function in CML progenitors.

In conclusion, our results suggest that the low CDH13 expression occurs as a multistage process in CML and may participate in defective cell adhesion in CML. High expression of BCR/ABL may be implicated in the modulation of CDH13 and may contribute to the development of CML. □

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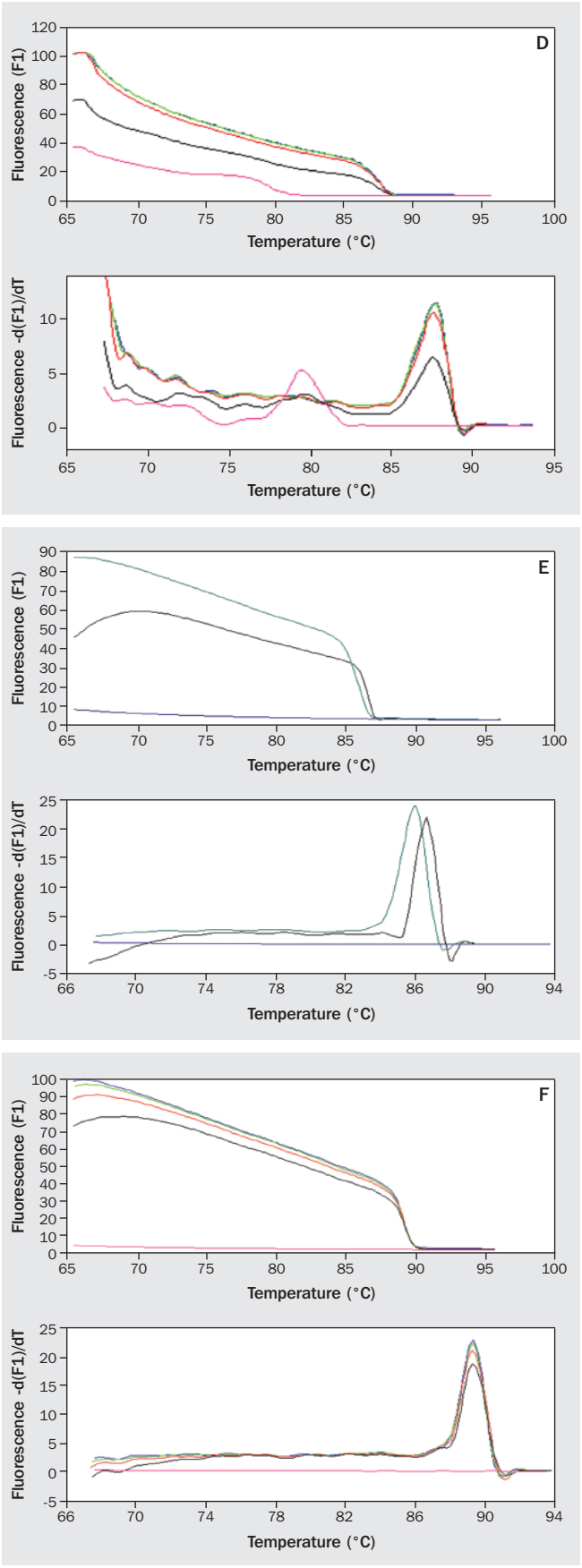

Fig. 3. Melting curve of *CDH*13 (D, Tm 87.3˚C), *BCR/ABL* (E, b2a2: Tm 85.8˚C; b3a2: Tm 86.5˚C) and *GAPDH* (F, Tm 88.2˚C)
See these images in colour at www.bjbs-online.org

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