Differential display analysis of mRNAs in chronic myelogenous leukaemia

M.K. El-Awady,[1] N.M. Abdalla,[2] A.M. Salem,[2] S. Samir[1] and A.M. Nour[1]

التحليل التظهيري التفريقي للرنا المرسال في ابيضاض الدم النقيي المزمن

مصطفى العوضي، نادية محمد عبد الله، أحمد محمد سالم، سَمَر سمير وعادل محمد نور

الخلاصة: يمثل تحليل الرنا المرسال المتعبِّر باستخدام تفاعل سلسلة البوليمراز الخاص بالتظهير التفريقي، أداةً قوية لتحديد خصائص الجينات الضالعة في المسارات الخبيثة، وقد تؤدي إلى تحديد بعض الواصمات المتعلقة بمختلف أطوار ابيضاض الدم النقيي المزمن. وقد قمنا بدراسة وجود المُنتسَخات *BCR-ABL* في 25 من مرضى ابيضاض الدم النقيي المزمن في الطور المزمن أو الهجمة الأرومية. ثم قمنا بتحليل تعبير منتسخات رنا الكريات البيض في أطوار هذا المرض. وقد استخدمت طريقة تفاعل سلسلة البوليمراز DD لفحص حالات ابيضاض الدم المزمن النقوي المنشأ المصحوب بـمُنتَسَخات *BCR-ABL*، بالمقارنة بحالات ابيضاض الدم النقيي المزمن غير المصحوب بـمُنتَسَخات *BCR-ABL* يمكن اكتشافها. وقد جاءت النتائج التي توصلنا إليها مؤيدة لاستخدام طريقة التظهير التفريقي، ليس فقط لتحديد خصائص الجينات المتعبِّرة والمتعلقة بابيضاض الدم النقيي المزمن تظهيراً تفريقياً، وإنما أيضاً لتحديد أماكن النماذج التي يمكن تنفيذها بوصفها بصمات لها قيمتها بالنسبة لكل طور من أطوار هذا المرض.

ABSTRACT Analysis of expressed mRNAs with differential display-polymerase chain reaction (DD-PCR) is a powerful tool for the characterization of genes involved in malignant pathways and might identify markers for different phases of chronic myelogenous leukaemia (CML). We examined the presence of *BCR-ABL* transcripts in 25 CML patients in either the chronic phase or blast crisis. We then analysed the expression of leukocytic RNA transcripts in CML phases. DD-PCR technique was used to examine CML cases with *BCR-ABL* in comparison with CML cases lacking detectable *BCR-ABL* transcripts. Our results support the use of differential display not only for characterization of the CML differentially expressed genes but also to locate patterns that can be implemented as valuable fingerprints for each phase of CML.

Analyse de la représentation différentielle des ARNm dans la leucémie myéloïde chronique

RESUME L'analyse des ARNm exprimés par représentation différentielle-amplification génique est un instrument puissant pour la caractérisation des gènes impliqués dans le développement de la malignité et pourrait identifier les marqueurs des différentes phases de leucémie myéloïde chronique. Nous avons examiné la présence de transcrits *BCR-ABL* chez 25 patients atteints de leucémie myéloïde chronique se trouvant en phase chronique ou crise blastique. Nous avons ensuite analysé l'expression des transcrits ARN leucocytaires en phases de leucémie myéloïde chronique. La technique de représentation différentielle-amplification génique a été utilisée pour examiner les cas de leucémie myéloïde chronique avec *BCR-ABL* par rapport aux cas de leucémie myéloïde chronique n'ayant pas de transcrits *BCR-ABL* détectables. Nos résultats vont dans le sens de l'utilisation de la représentation différentielle non seulement pour la caractérisation des gènes des leucémies myéloïdes chroniques exprimés de façon différentielle mais aussi pour la localisation des modèles qui peuvent être appliqués comme empreintes digitales pour chaque phase de la leucémie myéloïde chronique.

[1]*Departments of Human Genetics and Natural and Microbial Products; National Research Centre, Cairo, Egypt.*
[2]*Department of Biochemistry, Faculty of Science, Ain Shams University, Cairo, Egypt.*
Received: 25/06/00; accepted: 09/11/00

Introduction

Differential gene expression results in the programming of several biological processes including development, disease, exposure to hazards, injury and death of an organism. Differential display polymerase chain reaction (DD-PCR) was first described by Liang and Pardee [*1*] and was shown to be a reliable method for examining gene regulation and expression cloning of unknown sequences. Since the introduction of DD-PCR, over 100 reports have been published that document either successful applications or method improvements [*2*]. With differential display, each RNA sample is first reverse transcribed with a degenerate anchored oligo-dT primer set that anneals to the 5' end of the poly A tails of mRNAs. Each primer set, e.g. T12MN, will in theory reverse transcribe one-fourth of the total mRNA population. A decamer oligonucleotide of arbitrary sequence that can hybridize to the 3' termini of any first strand cDNA is used to amplify fragments representing most mRNA species in a given cell. Thus, the procedure allows for the amplification of an mRNA sub-population without specific knowledge of sequence information.

Although the 9 t(9;22) chromosomal translocation resulting in the Philadelphia (Ph) chromosome formation and expression of different forms of *BCR-ABL* transcripts seems to play a pivotal role in the pathogenesis of chronic myelogenous leukaemia (CML), the underlying molecular mechanisms of the disease are poorly understood. The disease is characterized mainly by two distinct phases: chronic and blast crisis. The former is the initial phase of CML, a long-lasting disorder with a median duration of 3.5–5 years before evolving to a more aggressive phase, blast crisis [*3*].

Upon translation of *BCR-ABL*, the 8.5 kb mRNA, a new protein with a molecular mass of 219 kDa (P210 BCR-ABL) is synthesized. It differs from C-ABL, a 145 kDa protein (P145 C-ABL). In some cases, a different oncoprotein product of the translation of *BCR-ABL*, a 185 kDa protein (P185 BCR-ABL) has been found [*4*]. The normal tyrosine kinase activity of C-ABL is deregulated because it binds to BCR which in turn is characterized by markedly increased autophosphorylation activity [*5*].

Several mouse models involving expression of the BCR-ABL fused protein have demonstrated a causal relationship with the development of myeloid and lymphoid malignanacies [*6*]. In 5%–10% of patients with typically clinical features of CML, Ph-chromosome was not detected in cytogenetic studies [3]. One-third of these patients displayed *BCR-ABL* translocation when analysed with a reverse transcriptase-PCR method. These patients have similar clinical features and the same response to interferon therapy [*7*]. The remaining patients with the classic features of chronic phase CML showed neither cytogenetic nor molecular changes detectable on chromosomes 9 and 22.

We examined gene expression patterns in CML patients with or without *BCR-ABL* translocation in either chronic or blast crisis phases.

Methods

The study comprised 25 CML patients (9 males and 16 females). Chronic CML was found in 22 cases and blast crisis in 3 cases. Patient ages ranged from 16 years to 56 years. Four patients (2 males and 2 females), with normal blood count and age range of 27–45 years acted as controls.

Total cellular RNAs were extracted from blood samples using a single-step acid-guanidinium thiocyanate-phenol method [*8*].

For the detection of *BCR-ABL* transcripts, 1 µg of leukocytic cellular RNA or RNA from K560 cells (ATCC, CCL243) was subjected to reverse transcription (RT reaction) in the presence of 200 µmol dNTPs, 10 pmol reverse primer (R1) 5' TTCCACTGGCCACAAAAT 3' [*9*], 200 units of superscript reverse transcriptase (GIBCO-BRL, United States of America) and 20 units of RNasin (Clontech, United States of America) in a 50 µL reaction mixture at 37 °C for 1 hour, followed by boiling for 10 minutes. Then 10 µL of cDNA was amplified in the presence of 25 pmol of forward (F10) 5' TTCAGAAGCTTCTCCCTG 3' [*9*] and of reverse primer (R1), 250 µmol dNTPs, 1 unit Taq DNA polymerase (Promega, United States of America). PCR conditions were 94 °C for 45 seconds, 54 °C for 30 seconds and 72 °C for 2 minutes for 34 cycles, followed by an extension cycle at 72 °C for 10 minutes. *BCR-ABL* products were identified as either 227 or 252 bp bands on 3% agarose gel stained with ethidium bromide.

For the differential display of CML RNA, 200 ng of leukocytic RNA from chronic phase CML patients, blast crisis CML patients and control subjects were reverse transcribed in the presence of 20 µmol dNTPs, 10 µmol downstream primer T12AC and 200 units of superscript reverse transcriptase (GIBCO-BRL) for 1 hour at 37 °C, followed by boiling for 10 minutes. One-tenth of the RT reaction volume was amplified in the presence of 2.5 µmol T12AC as a downstream primer, 0.5 µmol OPA 16 (5' AGCCAGCGAA 3'), OPA18 (5' AGGTGACCGT 3'), or LTK3 (5' CTTGATTGCC 3') as an upstream primer [*1*], 2 µmol dNTPs, 2 units Taq DNA polymerase, 1 µL ^{35}S-dATP (1000 Ci/mmol). Amplification was carried out at 94 °C for 30 seconds, 40 °C for 2 minutes, 72 °C for 30 seconds for 35 cycles followed by one extension cycle at 72 °C for 10 minutes. Radiolabelled products were resolved on polyacrylamide sequencing gel containing 8 mol urea, then the gels were subjected to autoradiography.

Results

Characterization of CML patients

We examined 25 CML patients by RT-PCR for the presence of *BCR-ABL* chimeric RNAs. Karyotype analyses were performed for blast crisis patients. The results are shown in Table 1 and Figure 1.

To examine genetic heterogeneity among control patients, peripheral blood leukocytic RNAs were analysed with DD-PCR using 3 separate pairs of primers T12AC/OP16 (A), T12AC/OPA18 (B) and T12AC/ltk3 (C). Figure 2 shows the abundance of ^{35}S-labelled cDNAs in the four control subjects (N1–N4). All three primer combinations showed equal amounts of cDNA species across the four control subjects, indicating that variations in relative abundance of RNA expression among normal individuals were minimal.

Two pairs of primers (A and B) were used in DD-PCR to examine RNAs from CML patients with detectable *BCR-ABL* specific RNA or without, as well as from a control subject. Figure 3 shows several cDNAs that demonstrated differential expression in CML patients. Two cDNAs (a and d) were overexpressed in Ph-negative compared with Ph-positive and control cDNA. Four cDNAs (e, g, j and n) were exclusively expressed in Ph-negative CML but not detected in either Ph-positive CML

Table 1 **Characterization of the patients**

Phase description	No.	Detection of *BCR-ABL* Negative	Positive	Type of *BCR-ABL* 3/2[a]	2/2[a]	Cytogenetics
Chronic	22[b]	1	21	11	10	–
Blast crisis	3[c]	–	3	2	1	46, XX,t(9;22)(q34q11) (2 patients)
						46, XX,t(9;22)(q34q11) t22 double Ph+ve (1 patient)

[a] *3/2 for fusion of exon 3 of* BCR *with exon 2 of* ABL *and 2/2 for fusion of exon 2 of* BCR *with exon 2 of* ABL.
[b] *13 females and 9 males.*
[c] *3 females. Subjects were aged 16.5–56 years.*

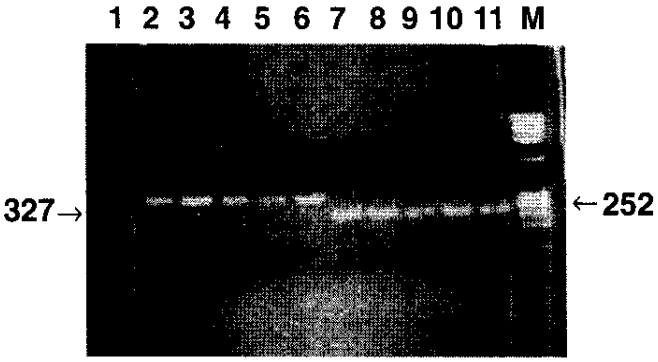

Figure 1 **Reverse transcriptase polymerase chain reaction (RT-PCR) detection of different forms of *BCR-ABL* transcripts in patients with chronic myelogenous leukaemia. Total RNAs were extracted from the patients, RT-PCR was performed using forward and reverse procedures. Amplified products, 327 and 252 bps, are shown in lanes 3–11. Lanes 1 and 2 are negative and positive controls respectively. M is fX174 Hae III digest as a molecular weight marker.**

or control cDNAs. Two cDNAs (h and o) were expressed in CML patients whether Ph-positive or Ph-negative but were not detected in normal individuals. The last group of cDNAs (c, i, l and m) were expressed in all 3 categories of subjects with minor differences.

Expression of chronic and blast crisis specific cDNAs was examined using T12GA as a downstream primer and OPA16 (A) or OPA18 (B) as upstream primer on RNA extracted from CML subjects during chronic phase and blast crisis

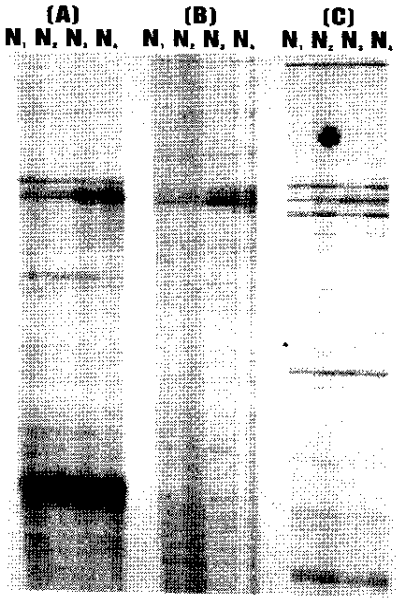

Figure 2 **Interindividual variation among normal subjects. Total RNAs were extracted from normal subjects (N1, N2, N3 and N4)and reverse transcribed in the presence of downstream primer (T12GA). Polymerase chain reaction was performed using downstream primer (T12GA) and upstream primers (OPA18, OPA16 and Ltk3). Radiolabelled products were resolved on 6% denaturing polyacrylamide gel electrophoresis and subjected to autoradiography.**

Figure 3 **Differential display analysis of Philadelphia (Ph) positive and negative patients with chronic myelogenous leukaemia. Total RNAs were extracted from Ph-positive (S), Ph-negative (V) and normal (N) participants and reverse transcribed in the presence of downstream primer (T12AC). Polymerase chain reaction was performed using downstream primer (T12AC) and upstream primers (OPA18 and OPA16). Radiolabelled products were resolved as described for Figure 2.**

and from normal subjects. Figure 4 shows that several genes expressed candidate cDNAs as specific markers for different stages of CML. cDNAs (a, b, c, e, f, g, h and j) displayed hyperexpression in chronic phase CML compared with controls, while all were expressed at very low rates in the crisis phase. Furthermore, some cDNAs (d, i, k, and l) were expressed almost exclusively in blast crisis, indicating that they may serve as specific markers for crisis phase of CML.

Discussion

Differential display analysis allows the study of semi-quantitative differences in

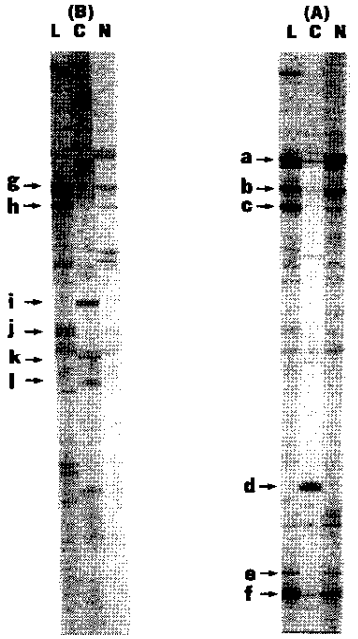

Figure 4 **Differential display analysis of chronic and blast crisis phases of chronic myelogenous leukaemia. Total RNAs from a patient with chronic myelogenous leukaemia with chronic phase (L) followed by blast crisis (C) phase and a normal subject (N) were extracted and reverse transcribed in the presence of downstream primer (T12GA). PCR was performed using downstream primer (T12GA) and upstream primers (OPA18 and OPA16). Radiolabelled products were resolved as described for Figure 2.**

gene expression associated with pathological states and genetic stress, especially during malignancy. It provides a means for identification and isolation of expressed genes where knowledge of sequences is not available. The technique involves reverse transcription of mRNAs using anchored oligo-dT primers, PCR amplification with both the anchored and arbitrary decamers, labelled deoxynucleotide triphosphates and high resolution denaturing polyacrylamide gel electrophoresis for product analysis [*10*]. The differential display technique was developed with the goal of identifying differentially expressed genes, i.e. detecting individual mRNA species that are subject to changes in different sets of mammalian cells [*1,11*]. The method provides a pattern of mRNA composition of cells. This mRNA fingerprint is useful in the same way as the two-dimensional protein gels for observing alterations in gene expression. The cDNAs can be reamplified, cloned, sequenced and compared with sequences in gene banks. Furthermore, reamplified cDNAs can be used as probes for Northern or Southern blot hybridization and to clone genes from genomic or cDNA libraries for further molecular characterization [*10*].

The majority of DD-PCR studies have involved applications of *in vitro* treatments of cultured cells where cell populations were rather homogeneous. The use of this technique for *in vivo* studies is rare. Due to possible interindividual variations in leukocytic gene expression, the choice of control subjects must be validated prior to *in vivo* application of the method. Most of the published data about these types of applications select controls according to the clinical status of the subjects as well as the tissue used [*12,13*]. Arbitrary RNA fingerprinting has been the method of choice to study interindividual variations. It has revealed that normal mammals show no major variations among individual mRNAs with only slight differences, which are attributed to polymorphism of a few species of RNA [*14*].

We focused on the clinical and routine laboratory findings for each volunteer. Controls were of the same age category as CML patients to avoid genetic variations in

gene expression. They also had similar haematological profiles. Furthermore, the volunteers had a healthy physical history and no evidence of bacterial, fungal or viral infections. With the selected PCR primers, no obvious variations were observed. Although different primer sets express distinct cDNA patterns, none displayed any significant variations from one individual to another.

Although CML was the first malignant disorder in which a consistent chromosome abnormality was identified when Nowell and Hungerford [*15*] identified the Ph-chromosome, the actual mechanism of the genesis of CML has not yet been elucidated. Many haematological disorders have been described and associated with the presence of the Ph-chromsome [*16,17*]. Furthermore, CML without the molecular translocation of *BCR-ABL* has been described with clinical symptoms and response to therapeutic agents similar to those with *BCR-ABL* [*3,18*].

Transgenic mice have been reported to develop CML when interferon consensus sequence binding protein is disrupted. Moreover, these mice developed blast crisis phase after a certain period. Interestingly, biphasic CML developed without any cytogenetic or molecular evidence of aberrant expression of *ABL* oncogene or translocation of this oncogene to the *BCR* region [*19*]. These results imply that there may be more than a molecular mechanism for progression and aggressive termination of this disease [*6,20–22*].

Our results showed that CML patients, whether Ph-negative or Ph-positive, had their own specific pattern of RNA expression. Some of these transcripts were expressed in both categories while others were exclusively expressed in one type of the disease or the other.

Patients in the blast crisis phase of CML do not have a long survival time. They have a medial survival of 3–6 months because of the late prognosis of this critical phase and poor response to the chemotherapy [*23,24*]. The lack of precise diagnostic and prognostic tools for determining the initiating events of this phase represents the principal obstacle in the management of the fatal phase. Cytogenetic studies and detection of allelic loss such as P^{53} and β-interferon with molecular methods have not show high reproducibility [*25,28*].

We found that the blast phase has a distinct pattern from the chronic phase of the same patients. The blast crisis has less banding pattern in comparison with the chronic phase and control subjects. A similar observation has been reported by Tatematsu et al., who attributed this phenomenon to chromosomal instability resulting from insufficient telomerase at the start of the blast crisis phase [29]. We found novel transcripts that appeared prominently during this phase.

The patterns obtained in our study are of great value for CML patients. They can be employed for the diagnosis of each phase, chronic or blast crisis, for type identification (Ph-positive or Ph-negative) and also for monitoring the efficacy of drugs during each phase of CML. Furthermore, cloning and identification of consistently expressible markers for prognosis of CML are underway.

References

1. Liang P, Pardee AB. Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction. *Science*, 1992, 257:967–71.

2. Galindo JE et al. Differential display of RNA. In: Caetano-Anolles G, Gresshoff PM, eds. *DNA markers: protocols, applications, and overview*. New York, Wiley-Liss Incorporated, 1997:225–36.

3. Cortes J et al. Philadelphia chromosome negative chronic myelogenous leukemia with rearrangement of the breakpoint cluster region: long-term follow-up results. *Cancer*, 1995, 75:464–70

4. Guo JQ, Wang JYJ, Arlinghaus RB. Detection of BCR-ABL proteins in blood cells of benign phase chronic myelogenous leukemia patients. *Cancer research*, 1991, 51:3048–57.

5. Muller AJ, Young JC, Pendergast AM. *Bcr* first exon sequences specifically activate the *bcr*-tyrosine kinase oncogene of Philadelphia chromosome-positive human leukemias. *Molecular and cellular biology*, 1991, 11:1785–92.

6. Sawyers CL, Denny CT, Witte ON. Leukemia and the disruption of normal hematopoiesis. *Cell*, 1991, 64:337–50.

7. Dobrovic A. et al. Molecular diagnostic of Philadelphia negative CML using the polymerase chain reaction and DNA analysis: clinical features and course of M-*bcr* negative and M-*bcr* positivo CML. *Leukemia*, 1991, 5:187–90.

8. Chomeznski P, Sacchi N. Single-step method of RNA Isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical biochemistry*, 1987, 162:156–60.

9. Lion T et al. Use of quantitative polymerase chain reaction to monitor residual disease in chronic myelogenous leukemia during treatment with interferon. *Leukemia*, 1995, 9:1353–60.

10. Polymerase chain reaction. In: Ausubel FM et al., eds. *Current protocols in molecular biology*. New York, John Wiley and Sons, 1993.

11. Liang P, Averboukh L, Pardee AB. Distribution and cloning of eukaryotic mRNAs by means of differential display: refinements and optimization. *Nucleic acids research*, 1993, 21:3269–75.

12. Thigpen AE et al. Increase expression of early growth response-1 messenger ribonucleic acid in prostatic adenocarcinoma. *Journal of urology*, 1996, 155:975–81.

13. Gonsky R et al. Identification of rapid turnover transcripts overexpressed in thyroid tumors and thyroid cancer cell lines: use of a targeted differential RNA display method to select for mRNA subsets. *Nucleic acids research*, 1997, 25:3823–31.

14. Welsh J et al. Arbitrarily primed PCR fingerprinting of RNA. *Nucleic acids research*, 1992, 20:4955–70.

15. Nowell PC, Hungerford DA. Chromosome studies on normal and leukemic human leucocytes. *Journal of the National Cancer Institute*, 1960, 25:85–109.

16. Bloomfield CD et al. The Philadelphia chromosome in acute leukemia. *Virchows archive B: cell pathology*, 1978, 29:81–91.

17. Hagemeijer A. Grosveld G. Molecular cytogenetics of leukemia. In: Henderson ES, Lister TA, Greaves MF, eds. *Leukemia*. Philadelphia, WB Saunders Company, 1996:138–84.

18. Kurzrock R et al. Philadelphia chromosome-negative chronic myelogenous leukemia with outbreak point cluster region rearrangement: a chronic myelogenous leukemia with a distinct clinical course. *Blood*, 1990, 75:445–52.

19. Holtschke T et al. Immunodeficiency and chronic myelogenous leukemia-like syndrome in mice with a targeted mutation of the ICSBP gene. *Cell*, 1996, 87:307–17.

20. Holt JT, Render RL, Nienhuis AW. An oligomer complementary to c-myc messenger RNA inhibitors proliferation of HL-60 promyelocytic cells and induces differentiation. *Molecular and cellular biology*, 1988, 8:963–71.

21. Ratajczak MZ et al. Acute and chronic-phase myelogenous leukemia colony-forming units are highly sensitive to the growth inhibitory effects of c-myb antisense oligodeoxynucleotides. *Blood*, 1992, 79:1956–61.

22. Afar DE et al. Differential complementation of *bcr-abl* point mutations with c-myc. *Science*, 1994, 264:424–6.

23. Kantarjian HM, Keating M, Talpaz M. Chronic myelogenous leukemia in blast crisis: analysis of 242 patients. *American journal of medicine*, 1987, 83:445–54.

24. Derderian PM, Kantarjian HM, Talpaz M. Chronic myelogenous leukemia in the lymphoid blastic phase: characteristics, treatment response, and prognosis. *American journal of medicine*, 1993, 94:69–74.

25. MacBride OW, Merry D, Givol D. The gene for human p53 cellular tumor antigene is located on chromosome 17 short arm (17q13). *Proceedings of the National Academy of Sciences of the USA*, 1986, 83:130–8.

26. Neubauer A, Neubauer B, Liu E. Polymerase chain reaction based assay to detect allelic loss in human DNA: loss of beta-interferon gene in chronic myelogenous leukemia. *Nucleic acids research*, 1990, 18:993–8.

27. Feinstein E, Cimino G, Gale RP. p53 in chronic myelogenous leukemia in acute phase. *Proceedings of the National Academy of Sciences, USA*, 1991, 88:6293–7.

28. Nakai H et al. Frequent p53 gene mutations in blast crisis of chronic myelogenous leukemia, especially in myeloid crisis harboring loss of a chromosome 17q. *Cancer research*, 1992, 52:6588–93.

29. Tatematsu K et al. A novel quantitative 'stretch PCR assay', that detects a dramatic increase in telomerase activity during the progression of myeloid leukemias. *Oncogene*, 1996, 13:2265–74.