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CONSTRUCTION OF REFERENCE PLASMIDS FOR THE QUANTIFICATION OF BCR-ABL FUSION TRANSCRIPTS IN CHRONIC MYELOID LEUKEMIA BY QUANTITATIVE REAL-TIME PCR

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ABSTRACT

Chronic myeloid leukemia (CML), is a myeloproliferative disorder characterized by rapid increased proliferation of the granulocytes. The hallmark of CML is the presence of the Philadephia chromosome, which is a product of translocation between the chromosomes 9 and 22 and as a result, a fusion gene is formed between the *BCR* gene (chr. 22) and *ABL* (chr.9). Several variants of the *BCR-ABL* fusion transcript exist which is based on the different breakpoint locations between the *BCR* and *ABL* genes. The most known variants are designated p190, p210 and p230 fusion transcript. Since the amount of *BCR-ABL* fusion transcript is correlated with the tumor load, therefore is important to quantify the expression of *BCR-ABL* in the patient samples. For p210 fusion transcript, a reference plasmid was created and the quantification was facilitated by having a standard curve made by serial dilution of the reference plasmid. Unfortunately, reference plasmid for p190 and p230 remain absent. Here in this study, we designed and construct two reference plasmids for quantification of the p190 and p230 fusion transcripts. Unfortunately, the construction of the reference plasmid for p190 was unsuccessful, after ligation, none of the colonies analyzed contained the 1000-bp insert of the p190. In contrast, the p230 reference plasmid, named pBL-230, was constructed in which contain a 620-bp fragment of the p230 fusion transcript. We showed that serial dilutions of pBL-230 could be accurately quantified by two different UPL probes and can be used for quantification of p230 fusion transcript in CML patients.

ÖZET

Kronik Myeloid Lösemi (KML), granülositlerin hızla artan proliferasyonuyla seyreden bir myeloproliferatif hastalıktır. KML, 9 ve 22 kromozomları arasındaki bir translokasyonla oluşan Philadelphia kromozomunun varlığı ile karakterizedir ve bu translokasyon sonucunda BCR (22. kr) ve ABL (9. kr) arasında bir füzyon gen meydana gelir. BCR ve ABL genlerindeki kırık bölgelerine bağlı olarak farklı BCR-ABL füzyon transkriptleri oluşmaktadır. En bilinenleri p190, p210 ve p230 füzyon transkriptleridir. BCR-ABL füzyon transkript miktarı tümör yükü ile doğrudan ilişkili olduğu için hasta örneklerinde BCR-ABL anlatımını sayısal miktar olarak belirlemek önemlidir. Daha önce p210 transkripti için bir referans plazmid oluşturulmuştur ve bu plazmidin seri dilüsyonları ile elde edilen standart eğrisi ile sayısal miktar tayini yapılmaktadır. Ancak maalesef benzer bir plazmid p190 ve p230 kırıkları için mevcut değildir. Bu çalışmada hem p190 hem de p230 transkriptlerinin miktar tayinini yapabilecek bir referans plazmid oluşturmayı amaçladık. Ancak p190 plazmidi bütün uğraşlarımıza rağmen başarıya ulaşamadı, elde ettiğimiz kolonilerin hiç biri p190'ın 1000bç'lik fragmanını içermiyordu. Bunun aksine p230 referans plazmidi (pBL-230) 620bç'lik p230 fragmanı ile klonlanabildi. pBL-230'un seri dilüsyonları ile iki farklı UPL prob kullanarak KML hastalarında p230 transkript miktarını tayin edebildik.

Introduction

Chronic Myeloid Leukemia (CML) is a clonal, myeloproliferative disease derived from a single, myeloid progenitor cell with the Philadelphia (Ph) chromosome [1]. The Philadelphia chromosome is formed by the translocation between the chromosome (chr.) 9 and 22. This has resulted in the *ABL* gene, located on chr. 9 is fused with the *BCR* gene located in the chr. 22 [2]. During the early stages, CML usually progresses slowly over weeks or months, and it develops gradually with three stages: the chronic stage, the accelerated stage and the blast stage. These stages are distinguished by the number of myeloid blast cells (immature white cells) in the blood and bone marrow and by the severity of symptoms. The Ph chromosome can be found in over 90% of CML patients [3].

In CML, different variants of fusion gene transcripts are detected which depend on the different breakpoint locations between the *BCR* and *ABL* genes [4-5]. All these variant fusion gene transcripts are designated as p190, p210 and p230. Each of the fusion genes produces oncoproteins at different length (190kD, 210 kD and 230kD respectively).

The incidence of CML is approximately 1-2 per 100,000 people per year (1). Among the CML types, the most frequent variant is p210, then p230 and less frequent one is p190. The p190 Philadelphia chromosome is mostly found in precursor B cell acute lymphoblastic leukemia (pre-B ALL) while both the p210 and p230 types are CML related. The p190 variant do not carry on *BCR* amino acid residues, which increase tyrosine kinase expression compared to the p210 and p230. This might suggest that p190 has less tyrosine kinase activity between the variants. Although the function of the *BCR* oncogene is not yet certain, the protein has serine/threonine ki-nase activity and is a GTPase-activating protein [6]. Moreover, *ABL* gene on chromosome 9 (is a proto-oncogene that encodes a protein tyrosine kinase involved in a variety of cellular processes, including cell division, adhesion, differentiation, and response to stress [7].

As mentioned earlier, CML develops in different phases in which 85% of patients are diagnosed at chronic phase (1). At this phase, it is crucial that the patients are treated accurately. To determine the treatment but also for clinical approaches, it is important to determine which variants of the fusion transcript is expressed in the tumor cells. Since the amount of *BCR-ABL* fusion transcript is correlated with the tumor load, therefore is important to quantify the expression of *BCR-ABL* in the patient samples [8-9]. Moreover, the quantitative and qualitation assessment of the *BCR-ABL* fusion transcript during and after is not only needed to evaluate the effect of the treatment but it is also crucial to detect possible early relapse of CML.

Quantification of fusion transcripts by real-time PCR (RQ-PCR) is very convenient for monitoring minimal residual disease because it can detect the low level of expression [10]. Traditionally the quantification of the BCR-ABL expression is based on the comparison with a clinical sample with known copy number of BCR-ABL fusion transcript. For p210 fusion transcript, a reference plasmid was created and the quantification was facilitated by a standard curve created by serial dilution of the reference plasmid. Unfortunately, reference plasmid for p190 and p230 remain absent.

Here in this study, we designed and construct two reference plasmids, which can be used to quantify the p190 and p230 fusion in patients' samples. For this purpose, two short fragments of p190 and p230 fusion transcript respectivlely were amplified and cloned the pSK(+) Bluescript vector plasmid. The reference plasmid will be validated and used to quantify the fusion transcript in patient samples.

Materials and Methods

Patient samples and CML cell line

Six CML patient samples were from Aziz Sancar Institute of Experimental Medicine (DETAE), Istanbul University and used with informed consent. The CML AR-230 cell line was cultured in DMEM medium supplemented with 1% penicillin and streptomycin, 10% heat0inactivated fetal calf serum at 5% CO_2 and 37^{0}C.

Primer Design

Three primers were designed to amplify the fragments of the variant p190 and p230 fusion transcripts for cloning. For the variant p190 fusion transcript, the forward primer was located at the exon 1, while in variant p230 fusion transcript the forward primer is located at exon 17/18. The reverse primer used for both variant p190 and p230 is located at exon 4. For quantitative RQ-PCR, specific primers were used and described by Van Dongen *et. al.* [12] The sequences all primers are listed in Table 1.

RNA isolation and cDNA synthesis

To clone the variant p190 fusion transcript, a pre-B ALL patient sample (16818) who is known to be positive for p190 was used as template. For the p230 fusion transcript, CML AR-230 cell line was used as template. Total RNAs were isolated using the GeneElute RNA isolation it. (Sigma). Total RNA was quantified by Nanodrop.

For cDNA synthesis, 1 ug/10ul RNA were pre-incubated at 65°C for 10 minutes and 10 ul cDNA reaction mix containing 10x Ca buffer, $MgCl_2$ (0.2M), DTT(100mM), Random Primer (dN6), Oligo-dT (100ug/ul), MilliQ, RNAout, Superscript II was added, The cDNA synthesis done at 42°C for 50 min. The cDNA synthesis is then stopped by incubation of 3 min at 95°C and 80ul of water was added to each sample to adjust to the concentration of 10 ng/ul. The cDNA synthesis was validated by housekeeping gene B-actin amplification.

Cloning of the variant p190 and p210 BCR-ABL fusion transcripts

To construct the reference plasmids, a 1000-bp EcoRI-BamHI p190 and a 620-bp EcoRI-BamHI p230 fragments respectively, were amplified with specific primers (Table 1) from cDNA derived from AR230 cell line and a P190 positive patient sample, digested with restriction enzymes and purified from the agarose gels. The fragments were then cloned into EcoRI/BamHI-digested pSK+Bluescript plasmid. The reference plasmids were validated by restriction enzymes digestion to confirm the insertion of the p190 and p230 fragments respectively.

Quantification of CML patient samples for p230 fusion transcripts

Total RNAs of the CML patients were isolated from PBMC. One ug total RNA was used for cDNA synthesis according to the procedure described earlier. For the RQ-PCR, 50 ng of cDNA of each sample were used in the reaction. The house-keeping gene cyclophilin was used to quantify the amount of fusion transcript a serial dilution (106-101 copies) of the reference plasmid containing the p230 fusion was prepared. All samples were done in duplicate. All RQ-PCR were performed on the LightCycler 96 (Roche) and the Ct-values were analyzed by the lightcycler 96 software (Roche).

RESULT

Amplification of the p190 and p230 inserts from CML patient 16818 and AR-230 cells

To construct the reference plasmids containing the fragments of p190 and p230 fusion transcripts, specific primers were designed to amplify a 1000bp fragment of the p190 fusion transcript and a 620 bp fragment of p230 fusion transcript. The amplifications were performed with the PFU taq polymerase at various annealing temperatures. The size of the inserts was verified by agarose gel electrophoresis (Figure 1).

Construction of the reference plasmids

The amplified fragments were cut out from the gel, purified and digested with restriction enzyme EcoRI and BamHI. Afterwards, both digested fragments were ligated into EcoRI/BamHI-digested pSK(+)bluescript vector plasmid. A insert:vector ratio of 3,5:1 was used for the p190 and for p230 the ratio was done in 4:1. All ligations were done overnight at 16°C. After the ligation, 5 ul of the ligation mixture used to transform 100 ul DH5a competent cells, plated on LB-agar plates supplemented with X-gal and IPTG and incubated overnight at 37 ^{0}C. A difference in blue/white colonies on the agar plates was observed between the ligations of p190 and p230. In p190 ligation, not only more colonies were seen on the plate compared to the p230 ligation, but also the ratio blue/white is higher in p190 (5/1) than in p230 (1/1) (Figure 2).

Sixteen white colonies from the p190 plates and 15 white colonies from the p230 plates were picked and overnight mini cultures were prepared. Plasmid DNAs were isolated and digested with EcoRI and BamHI to confirm the insertion of the fragments into the vector plasmid. Surprisingly none of the colonies derived from the p190 plates contains the 1000-bp insert (Figure 3a). We have repeated the procedure twice with newly amplified inserts gave the same results.

In contrast, 5 out of 15 white colonies from the p230 plates showed the correct insert ligated into the vector plasmid (Figure 3b). One of these 5 colonies, designated as pBL-230, was used for large scale plasmid production and is used for the quantification of p230 fusion transcript in CML patients.

Real - Time Quantitative-PCR

To quantify the copy number of p230 fusion transcript in CML patient samples, a serial dilution of the pBL-230 plasmid were prepared to create a standard curve. Two different Universal Library Probes (UPL) probes (10 and 25) were chosen and used for quantification. There is no substantial different with the probes. They both gave similar Ct values with the standard curves (Figure 4a).

Six CML patient samples were used in this study. These patients were screened initially for the presence of p190 and p210 fusion transcripts and the results were negative. Fifty nanogram cDNA were used for the RQ-PCR and both UPL probes were used to detect the fusion transcript. After the analysis, none of the six samples showed expression of the p230 fusion transcript (Figure 4b and Table 2).

DISCUSSION

Chronic myeloid leukemia (CML), is a myeloproliferative disorder characterized by rapid increased proliferation of the granulocytes. The hallmark of CML is the presence of the Philadephia chromosome, which is a product of translocation between the chromosomes 9 and 22 and as a result a fusion gene is formed between the *BCR* gene (chr. 22) and *ABL* (chr. 9). Several variants of the *BCR-ABL* fusion transcript exist which is based on the different breakpoint locations between the *BCR* and *ABL* genes. The most known variants are designated p190, p210 and p230 fusion transcript. P190 is mostly found in pre-B-ALL while both p210 and p230 are observed in CML.

Since the amount of *BCR-ABL* fusion transcript is correlated with the tumor load, therefore is important to quantify the expression of BCR-ABL in the patient samples. Traditionally the quantification of the *BCR-ABL* expression is based on the comparison with a sample with known copy number of *BCR-ABL* fusion transcript. For p210 fusion transcript, a reference plasmid was created and the quantification was facilitated by a standard curve made by serial dilution of the reference plasmid. Unfortunately, reference plasmid for p190 and p230 remain absent.

Here in this study, we designed and constructed two reference plasmids for the quantification of the p190 and p230 fusion transcripts. First, we designed specific primers, which amplify a 1000-bp fragment and a 620-bp fragment of p190 and p230 fusion transcript respectively. The frag-ments were cloned into the pSK(+)Bluescript vector plasmid. Blue/white colonies were observed in both ligations, however due to unexplainable condition, the number of the colonies was always higher in p190 ligation compared to p230 ligation. Surprisingly when the colonies were evaluated for the presence of the inserts, none of the 16 white colonies contain the correct 1000-bp insert in the p190 agar plate. This indicates that the amplified p190 insert was non-specific at start. Several modifications to the PCR condition such as Mg concentration, annealing temperature, addition of DMSO and different DNA polymerases remained unsuccessful. Currently new primes were designed and being evaluated. In contrast, 5 out of the 15 white colonies from the p230 agar contain the correct 620-bp insert. One of these 5 colonies was used to produce large quantity of the p230 reference plasmid, which is designated as pBL-230.

A standard curve was prepared by serial dilution (10^6-10^1 copy numbers) of the reference pBL-230 plasmid. The standard curves quantified with two UPL probes were almost identical and were used as reference to quantify six CML patient samples. These samples were previously analyzed as both

p190 and p210 negative. After the RQ-PCR analysis, the quality of the cDNA in all samples were sufficient as seen with the high Ct values of the housekeeping gene cyclophilin. However, p230 fusion transcript expression was not detected in all these patients. These results suggest that these patients might expression the rare BCR-ABL variants p195, p200 and p225 (6).

Table 1: Sequences and additional information of primers that is used for cloning and quantitative RQ-PCR.

Oligo Name	Sequence	Temperature
BCR-e1-A	GACTGCAGCTCCAATGAGAAC	59.8°C
ABL-a3-E3	TGACTGGCGTGATGTAGTTGCTT	60.6°C
BCR-e19	TTCGGAGTCAAGATTGCTGTG	57.9°C
BCR-e1_BAMHI	TCGGATCCCCTGGTGAAGGTCAACGACA	69.5°C
BCR-e17_18_BamHI	TCGGATCCTCGCCATGAATGGGATCGAA	68.1°C
ABL-e4_EcoRI	ACGAATTCCTCTCACTCTCACGCACCAA	66.6°C

Table 2: The threshold cycle values of CML patients. All values are average of duplicates.

Patient	Ct-value (cyclophilin)	Ct value (p230)
26	21.00	40
742	21.09	40
223	20.81	40
1510	21.66	40
119	22.31	40
CTF	22.00	40

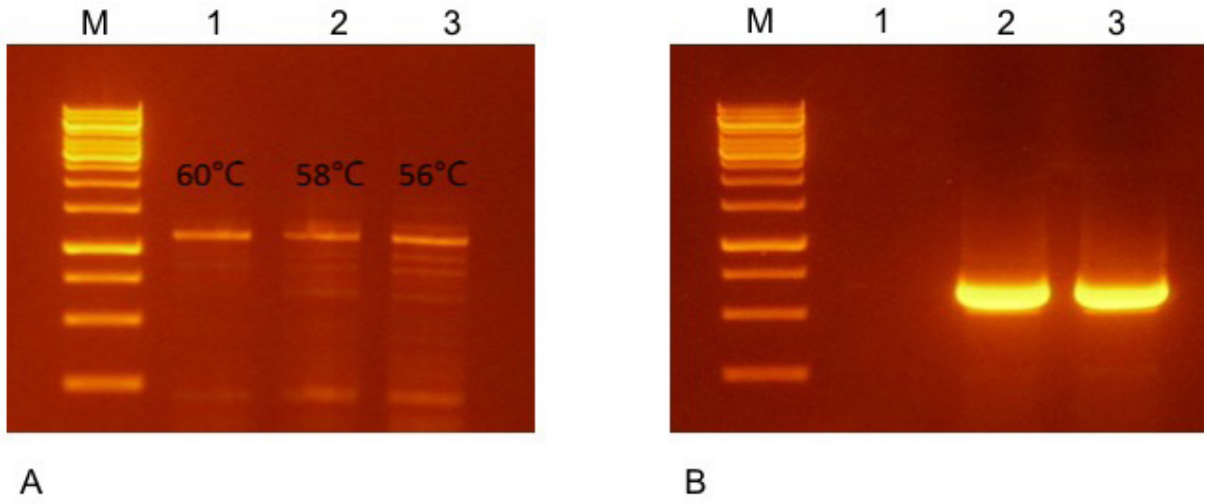

Figure 1. Amplification of the p190 (A) and p230 (B) inserts from CML patient 16818 and AR-230 cells. A. Amplification of p190 insert under different annealing temperatures. M= 1kb ladder. B. Amplification of p230 insert from AR-230 cells. Lane 2 and 3 are duplicates.

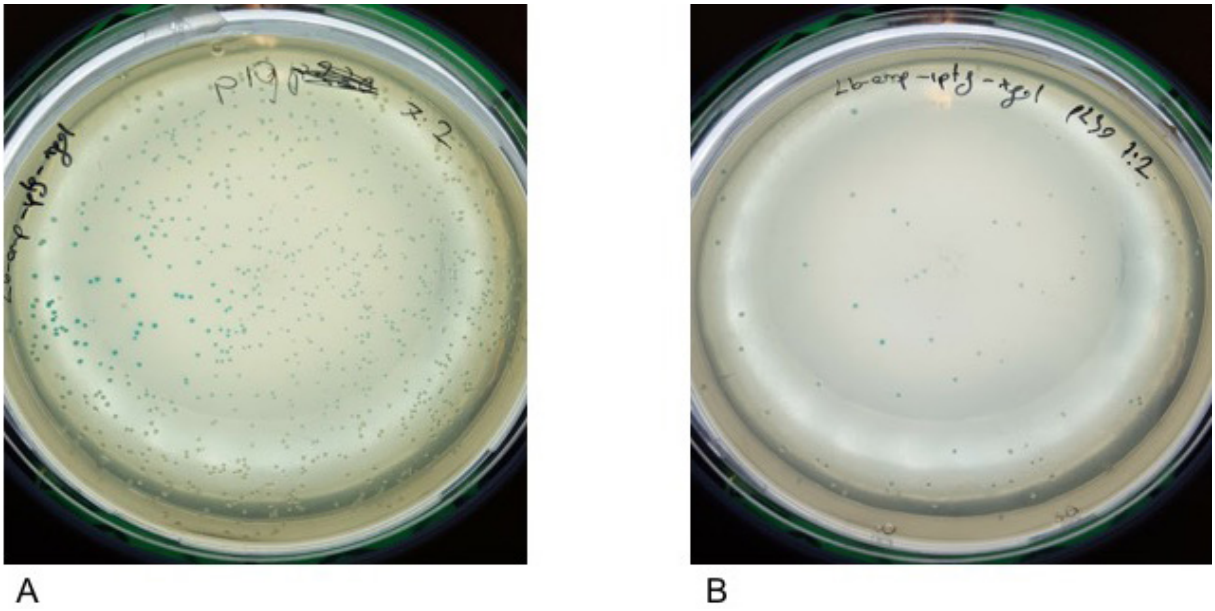

Figure 2. Blue-White colonie screening. Ligation mixtures were transformed into DH5a cells and plated on LB-agar plates supplemented with X-gal and IPTG.

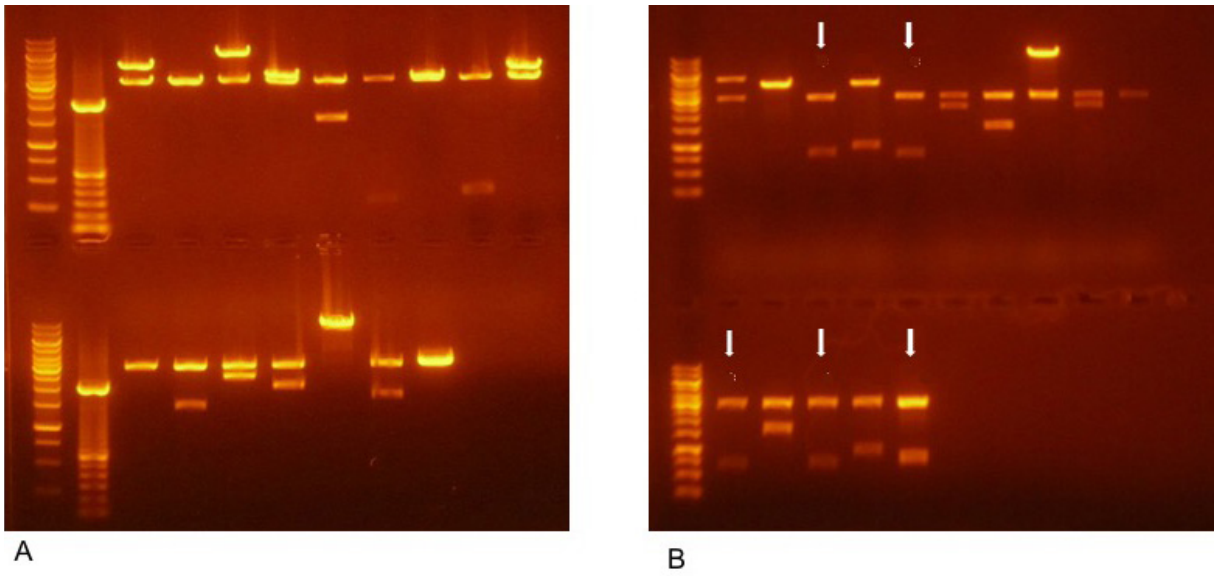

Figure 3. White colonies from the both agar plates were grown, plasmid DNA was isolated and digested with restriction enzyme to confirm the correct inserts for p190 (A) and p230 (B). In the p230 agar plate, five colonies were found to have the correct insert ligated into the pSK(+)Bluescript vector (Fig. 3B, arrow marked).

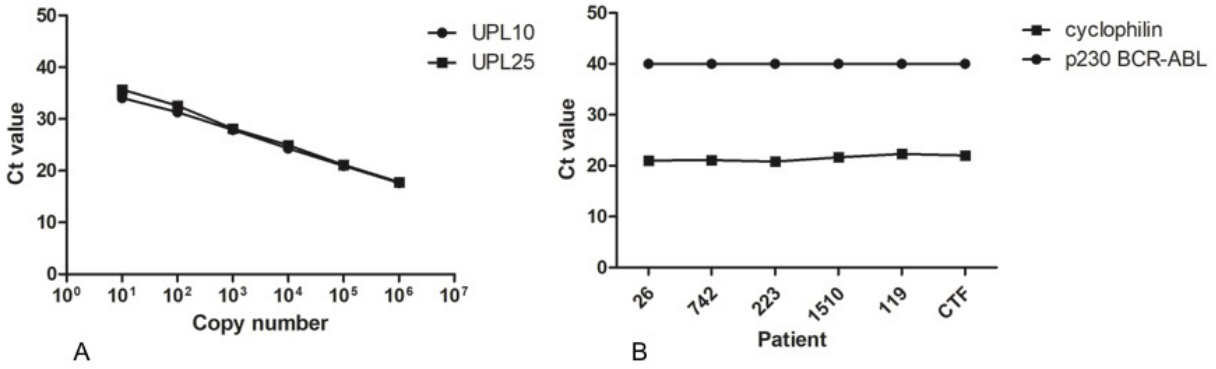

Figure 4. Standard curves of the reference plasmid pBL-230. Serial dilution of the newly made pBL-230 plasmid were prepared and RQ-PCR were performed with two UPL probes to determine the corresponding Ct values (4A). The Ct values of cyclophilin and p230 BCR-ABL assessed by quantitative RQ-PCR (4B).

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