

Which has greater effects on leukomogenesis in Philadelphia chromosome positive leukemias: fusion or cell type?

[Philadelphia kromozomu pozitif olan lösemilerde lökomogenez üzerinde hangisi daha etkilidir: Füzyon veya hücre tipi?]

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ABSTRACT

Aim: The occurrence of different breakpoint cluster region/c-abl oncogene 1 (*BCR-ABL*) fusion forms in several types of Philadelphia chromosome positive leukemia suggests these proteins have alternative leukomogenic activity. Distinct types of leukemia diverge from each other with respect to levels of telomerase and telomere lengths. It is known that telomeres and telomerase are essential to the regulation of cell life-span and division. It has been hypothesized that the difference in leukomogenic activities of distinct forms of *BCR-ABL* fusion could be related to the telomere length and telomerase activity of the hematopoietic cells transformed by these proteins.

Material and methods: We have compared the effects of p190*BCR-ABL* and p210*BCR-ABL* on telomerase activity, telomere length and proliferative rate of myeloid (32D cl3) and lymphoid (Ba/F3) cell lines transformed by both fusion forms. Telomerase activity, telomere length and cell proliferation capacity were measured by Telomerase Repeat Amplification Protocol (TRAP), Terminal Restriction Fragments (TRF) and mitotic index respectively.

Results: In myeloid cells, a difference was found between the p190*BCR-ABL* and p210 *BCR-ABL* with regard to telomerase related proliferative behaviour. p210*BCR-ABL*, unlike p190 *BCR-ABL*, is associated with increased mitotic index in both cells' origin.

Conclusion: Our results suggest that the leukomogenic activities of *BCR-ABL* fusion proteins in myeloid cell might be related to the telomerase activity and telomere length of the cells. To realize intracellular dynamics associated with cell proliferation, other targets for *BCR-ABL* fusion protein and telomerase should be investigated.

Key Words: p190 (bcr-abl) Fusion Proteins, p210 (bcr-abl) Fusion Proteins, Telomerase Reverse Transcriptase, bcr-abl Proto-Oncogenes, cell line transformed

Conflict of interest: Authors have no conflict of interest.

ÖZET

Amaç: *BCR-ABL* (Breakpoint cluster region/c-abl oncogene 1) füzyonlarının, farklı Philadelphia kromozomu pozitif lösemi tiplerinde birbirinden değişik olması, bu füzyonların ürünü olan proteinlerin farklı lökomojenik aktiviteye sahip olabileceklerini düşündürür. Bu lösemi tipleri, telomeraz aktivitesi düzeyi ve telomer boyuna göre de birbirlerinden ayrılmaktadır. Telomerlerin ve telomerazın, bölünme ve hücre yaşam süresinin düzenlenmesinin temel gereklerinden olduğu bilinmektedir. Çeşitli *BCR-ABL* füzyon proteinlerinin lökomojenik aktivitelerinin farklı olmasının, bu proteinlerle transforme olan hematopoetik hücrelerin telomeraz aktivitesi ve telomer boyu ile ilişkili olabileceği hipotezi ileri sürülebilir.

Gereç ve yöntemler: p190*BCR-ABL* ve p210*BCR-ABL* füzyon proteinleri ile transforme edilen myelositer (32D cl3) ve lenfositler (Ba/F3) hücre hatlarında, bu proteinlerin telomeraz aktivitesi, telomer boyu ve proliferasyon oranı üzerine olan etkilerini karşılaştırdık. Telomeraz aktivitesi, telomer boyu ve hücre proliferasyon kapasitesi, sırasıyla, Telomerase Repeat Amplification Protocol (TRAP), Terminal Restriction Fragments (TRF) ve mitotik indeks yöntemleri kullanılarak incelenmiştir.

Bulgular: Myelositer hücrelerde, telomerazla ilişkili proliferasyon davranışının p190 *BCR-ABL* ve p210*BCR-ABL* füzyon proteinlerinde farklı olduğu bulundu. p210*BCR-ABL* füzyon proteini, p190*BCR-ABL* füzyon proteininden farklı olarak her iki hücre tipinde de artmış mitotik indeksle birliktelik gösterdi.

Sonuç: Bulgular, myelositer seri hücrelerinde *BCR-ABL* füzyon proteinlerinin lökomojenik aktivitelerinin, bu hücrelerin telomeraz aktivitesi ve telomer boyu ile ilişkili olabileceğini düşündürmektedir. Hücre proliferasyonu ile ilişkili hücre içi dinamiklerin açıklığına kavuşturulabilmesi için, *BCR-ABL* füzyon proteini ve telomerazın başka hedeflerinin araştırılması gereklidir.

Anahtar Kelimeler: p190 (bcr-abl) füzyon proteini, p210 (bcr-abl) füzyon proteini, telomeraz revers transkriptaz, *Bcr-Abl* protoonkogenleri, transforme hücre eşsöyleri

Çıkar çatışması: Yazarların çıkar çatışması yoktur.

Introduction

The Philadelphia (Ph) chromosome, a characteristic cytogenetic marker of chronic myeloid leukemia (CML), is caused by reciprocal translocation of the 3' region of *ABL* gene onto the 5' region of the *BCR* gene [1]. The resulting chimeric fusion protein, BCR-ABL, demonstrates increased tyrosine kinase activity, localized mostly in cytoplasm, and has the ability to transform hematopoietic cells *in vitro*, and induces leukemia in mice. There are three distinct forms of BCR-ABL as a consequence of different translocation breakpoints on chromosome 22 [2].

It is known that the three different forms of *BCR-ABL* are associated with distinct forms of leukemia. In virtually all CML patients, the BCR-ABL protein has a molecular mass of 210 kDa, whereas in 50% of adults and 80% of children with Ph (+) acute B lymphoid leukemia the BCR-ABL protein is smaller, with a molecular mass of 185 or 190 kDa [3].

The association of different forms of the *BCR-ABL* oncogenes with distinct types of leukemia raises the question of whether different forms of BCR-ABL proteins have intrinsically different leukemogenic activity in hematopoietic cells.

Li et al. [4] proposed two models to explain this phenotype/genotype correlation. They suggested that, it is possible that the three forms of *BCR-ABL* have different intrinsic leukemogenic activities or the three fusion genes might have identical leukemogenic properties, but their expression might be largely restricted to different hematopoietic lineages [4].

BCR-ABL tyrosine kinase provides a specific target for CML therapy as the tyrosine kinase activity of this oncogene is essential for transforming ability. A tyrosine kinase inhibitor (Gleevec, imatinib mesylate) is currently used in CML as a gold standard therapy. Gleevec competes with ATP for binding to the ATP-binding site of BCR-ABL and prevents a conformational change to the active form of fusion protein and inhibits BCR-ABL. About 96% of CML patients exhibited complete hematologic responses (CHR) and major cytogenetic responses (MCR) to Gleevec treatment, whereas approximately 55% of acute lymphoblastic leukemia (ALL) patients showed positive responses to Gleevec treatment [5]. A poor prognosis is observed in Ph (+) adult acute lymphocytic leukemia patients [6]. It is emphasized that the allogeneic haematopoietic stem cell transplantation is the only known curative treatment for Ph (+) ALL patients [7]. After imatinib-based regimens, most Ph (+) ALL patients who do not undergo allogeneic stem cell transplantation relapse [8]. It is reported that the morphology is more atypic and the prognosis is worse in Ph (-) CML patients compared with Ph (+) CML cases [9-11]. The different relationship between Ph positivity and the prognosis and the response to the treatment in ALL and CML can be explained by the origin of malignant cell

types. Since the presence of Ph chromosome is also a sign of poor prognosis in acute myelogenous leukemia (AML) [12], it is reasonable to predict that different fusion proteins may be more effective than cell types. Besides of these observations, recent studies have suggested that treatment of Gleevec could inhibit telomerase activity through suppressing human telomerase reverse transcriptase (hTERT) mRNA and hTERT phosphorylation level [5, 13, 14].

Telomerase expression is one of the hallmarks of human cancer. In the majority of normal somatic human tissues, telomerase activity is absent, whereas at least 85% of human tumors have positive telomerase activity [15]. Telomerase is a ribonucleoprotein which adds telomeric repeats onto the ends of chromosomes during the replicative phase of the cell cycle [16]. The human telomerase enzyme consists of RNA component and reverse transcriptase. Telomerase utilizes its own RNA (hTR) as a template to add the hexanucleotides (TTAGGG) to the telomeric ends [17].

In this study, we aimed to clarify cell-dependent and/or fusion-dependent effects of BCR-ABL protein on prognosis of leukemia. Here we have examined differences of two distinct forms of BCR-ABL fusion protein on leukomogenic effects in myeloid and lymphoid cells. Additionally, the relation between their leukomogenic effects and telomerase activity was investigated.

Materials and methods

The study was carried out on 32D cl3 (myeloid), Ba/F3 (lymphoid), 190*Bcr-Abl* and 210 *Bcr-Abl* transduced 32D cl3 and Ba/F3 cell lines. P210 and P190 transformed cells were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin at 37°C and 5% CO₂. Interleukin-3 (IL3) was added to the medium (10%) to stimulate growing 32D cl3 and Ba/F3 cells which do not carry any genetic rearrangement [1]. The existence of the *Bcr-Abl* fusion gene was confirmed in 190*Bcr-Abl* and 210*Bcr-Abl* transduced 32D cl3 and Ba/F3 cell lines using FISH method with LSI BCR-ABL ES/DC (Vysis#39766) probe.

Telomerase assay

Telomerase activity was measured using TRAP-eze™ telomerase detection kit (Intergen#S7700) according to the provided protocol. Cells were washed twice with PBS and approximately 10⁵-10⁶ cells were resuspended in 200 µl lysis buffer (5% CHAPS {3-[(3-cholamidopropyl) dimethylammonio] -1- propanesulfonate}, 10 mM Tris-HCl, 1 mM EGTA, 1 mM MgCl₂, 5 mM mercaptoethanol, 0.1 mM benzamidine, 10% glycerol). The suspension was kept on ice for 30 min, centrifuged at 12,000g for 20 min at 4°C. The supernatants (150 µl) were collected and the protein concentration was measured using Bradford method, after which the supernatants were frozen and stored at -80°C.

TRAP-eze™ telomerase detection kit was used for the measurement of telomerase activity according to the manufacturer's protocol. This PCR-based telomerase assay is a modification of the method originally described by Kim et al. [15]. Briefly, approximately 100 ng of protein were added to the TRAP reaction mixture (5 µl 10 X TRAP reaction buffer, 1 µl 50 X dNTP mix, 1 µl TS primer, 1 µl TRAP primer mix), 2 U Taq DNA polymerase and dH₂O in a total volume of 50 µl. Reaction mixtures were incubated at 30°C for 30 minutes, and then subjected to 34 PCR cycles at 94°C/30 seconds, 59°C/30 seconds, and 72°C/60 seconds. As negative control, every sample extract was evaluated after heat inactivation of telomerase. Thus, analysis of each sample was performed as two assays: one with a test extract and one with a heat-treated test extract. Heat treat was carried out by incubating 10 µL of each sample at 85°C for 10 minutes prior to the TRAP assay to inactivate telomerase. Protein extract from telomerase positive cell pellet provided in the kit was used as positive control sample. In addition, TSR8 control template provided in the kit was used as internal control for the quantitation of the telomerase activity. 2 µL 1X CHAPS Lysis Buffer was used to test the presence of products in the primer-dimer/PCR contamination for each reaction set. The PCR products were analyzed by electrophoresis at 350 V for 2.5 hours on a 12%polyacrylamide gel. The gel was stained with SYBR Green I nucleic acid gel stain (Invitrogen) and visualized by Gel Logic 200 Image Analyzer. Images were analyzed with the Kodak 1D Software. Telomerase activity was calculated using the formula: TA (TPG, Total Product Generated) = $\{[X-X_0/C] / [r-r_0/C_R]\} \times 100$, where, X is the intensity of the telomerase ladder of the test sample, X₀ is the inactivated counterpart of it, C is the intensity of the internal standard in the test sample, r is the intensity of the TSR8 quantitation control, r₀ is the signal of the region of the gel lane corresponding to the 1X CHAPS Lysis Buffer control and C_R is the intensity of the internal standard in TSR8 quantitation control.

Telomere length assay

Total DNA was isolated by standard protocol with phenol/chloroform [18], then 10 µg genomic DNA was digested overnight with 10 U of HinfI (Q-BIOgene #ER-HIF 200) at 37°C. Following 0.8% agarose gel electrop-

horesis, gels were denatured and transferred to nylon membranes and then cross-linked with UV light [19]. We detected telomere fragments by Southern hybridization with 5'end [γ 32P] labeled telomeric probes, which consist of four TTAGGG telomeric repeats. When the membranes were dried, they were exposed to a storage phosphoric screen (BAS-IP MS 2325) for 30-40 minutes. The position and density of the telomeric hybridization signals were evaluated with Bas-1800 II™ Imaging System. Mode telomere length was determined using Aida 2.43™ software by comparing the position of the greatest signal in each lane of the Southern Blot to a standard curve derived from the migration of DNA standards with known molecular weight.

Mitotic Index (MI)

Mitotic Index score was used to evaluate cell proliferation capacity. Colcemid was added to the cultures 4 hours before harvesting. Cell suspension was fixed in methanol and glacial acetic acid (v/v 3:1) after hypotonic treatment (0.075 M KCl). Cytogenetic preparations were stained with Giemsa and analyzed by a light microscope at 400× magnification. The mitotic index was determined by counting the number of metaphase plaques in one thousand cells, scored in four different slides for each experiment.

Results

Differences in telomerase activity between myeloid and lymphoid cell lines according to fusion proteins

Telomerase activity of wild myeloid cells was dramatically lower than its transformed form by either 190*Bcr-Abl* (12-fold) or 210*Bcr-Abl* (5-fold). In lymphoid cell lines, telomerase activity of wild type cells was lower than that of fusion gene transfected ones. p190BCR-ABL positive cells have remarkably higher telomerase activity than p210BCR-ABL positive lymphoid cells (Table 1, Figure 1). Telomerase activity was higher in myeloid cell lines according to lymphoid cells when considering wild cell lines. In contrast, the telomerase activity of p190BCR-ABL positive lymphoid cells was higher than that of 190*Bcr-Abl* transformed myeloid cells. The highest telomerase activity was found in 190*Bcr-Abl* transformed cell lines independent of their origin.

Table 1. Cell proliferation capacity (MI), telomerase activation (TRAP) and telomere length (TRF) of all cell lines

Cell Line	MI (#metaphase plaques/ 1000 cells)	TRAP (TPG)	TRF (kb)
32D cl3	0.043	0.019138	0.812
32D cl3-190 ^{BCR/ABL}	0.002	0.245749	0.674
32D cl3-210 ^{BCR/ABL}	0.057	0.094921	1.068
Ba/F3	0.001	0.003653	1.115
Ba/F3-190 ^{BCR/ABL}	0.198	0.360805	0.976
Ba/F3-210 ^{BCR/ABL}	0.139	0.045031	1.068

Proliferation capacity according to fusion gene/cellular origin

MI of wild myeloid cell lines was dramatically different from that of wild lymphoid cell lines (0.043 and 0.001, respectively). Proliferation capacity showed difference in myeloid cell lines, according to fusion proteins type. The MI decreased in 190*Bcr-Abl* transformed myeloid cell line, but contrarily increased by 210*Bcr-Abl* transformation. Cell proliferation capacity clearly increased by both transformations in lymphoid cell (Table 1, Figure 2).

Relation between telomerase activity and cell proliferation capacity

Cell proliferation capacity increased in 210*Bcr-Abl* transformed myeloid cell line but decreased in 190*Bcr-Abl* transformed line. Interestingly, telomerase activity increased in both of these cell lines, especially in 190*Bcr-Abl* transformed line (Table 1, Figure 3).

In lymphoid cell lines, when compared to wild-type and transformed cell lines, there was a positive correlation between telomerase activity and mitotic index. (Table 1, Figure 4).

Telomerase activity and telomere length in myeloid and lymphoid cell lines

Despite dramatically increased telomerase activity in 190*Bcr-Abl* transformed myeloid cell line, shorter telomere length was observed in these cells. In addition, telomere length was longer in 210*Bcr-Abl* transformed myeloid cell line that has less increased telomerase activity than 190*Bcr-Abl* transformed cell. Finally, both forms of BCR-ABL fusion protein increased telomerase activity in myeloid cell lines but their effects on telomere length differ related to the type of fusion (Table 1, Figure 5, 6).

Increase in telomerase activity together with telomere shortening in 190*Bcr-Abl* transformed cell lines was unrelated to cell origin. In contrast, despite the increase in telomerase activity, changes in telomere length in 210*Bcr-Abl* transformed cell lines differed with regard to the origin of the cell.

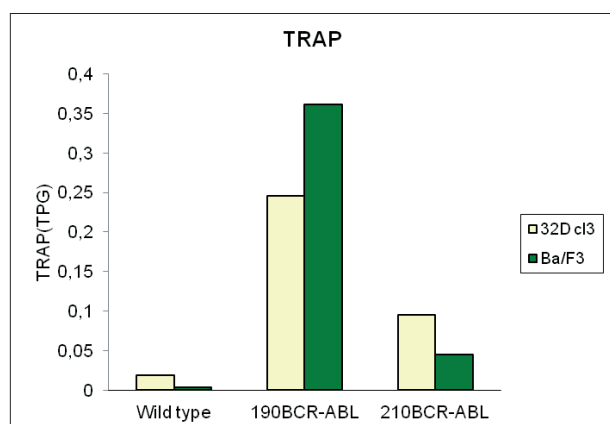


Figure 1. Telomerase activities (TRAP) of myeloid and lymphoid cell lines (wild, 190*Bcr-Abl* transformed, 210*Bcr-Abl* transformed)

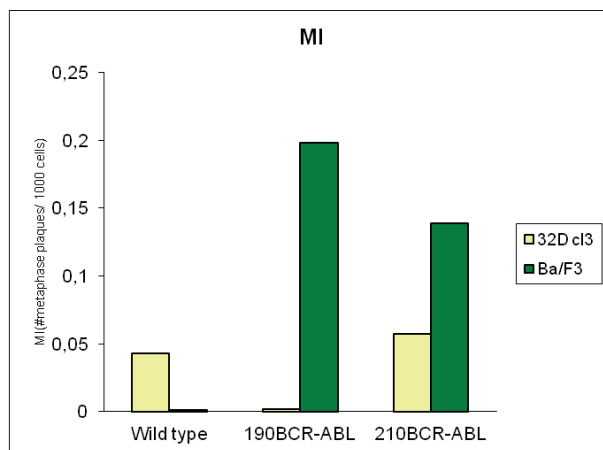


Figure 2. Mitotic indexes (MI) of myeloid and lymphoid cell lines (wild, 190*Bcr-Abl* transformed, 210*Bcr-Abl* transformed)

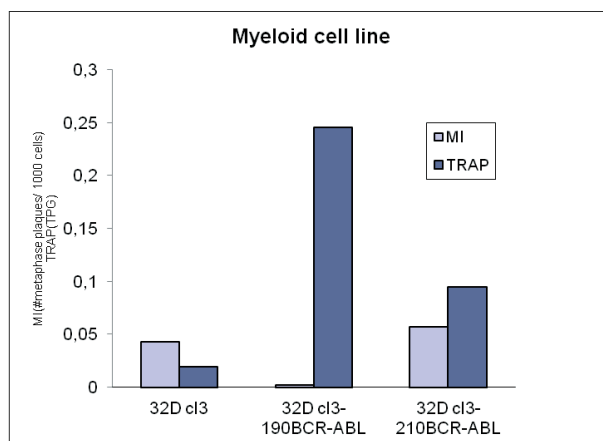


Figure 3. Relation of telomerase activity (TRAP) and mitotic index (MI) in the myeloid cell lines

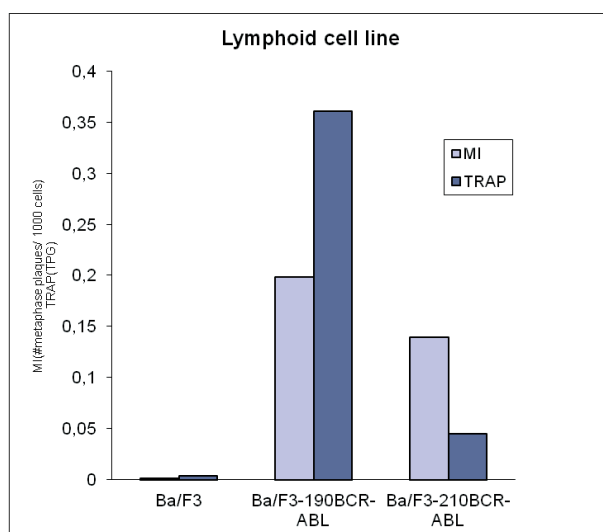


Figure 4. Relation of telomerase activity (TRAP) and mitotic index (MI) in the lymphoid cell lines

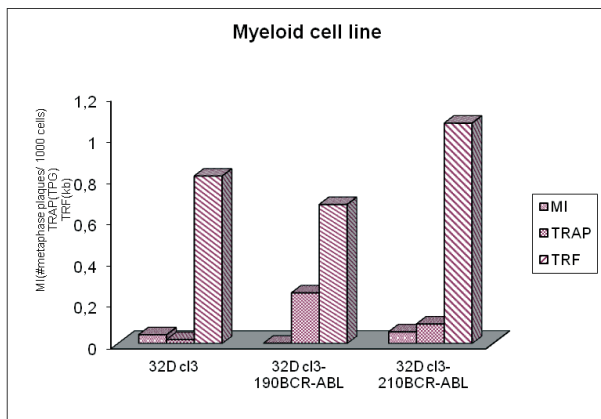


Figure 5. Comparative results of mitotic index (MI), telomerase activity (TRAP) and telomere length (TRF) in myeloid cell lines

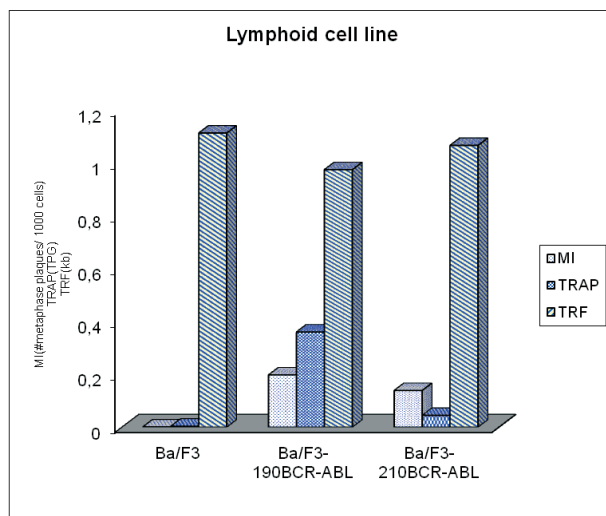


Figure 6. Comparative results of mitotic index (MI), telomerase activity (TRAP) and telomere length (TRF) in lymphoid cell lines

Comparison of telomerase activity, telomere length and cell proliferation capacity in myeloid and lymphoid cell lines

In lymphoid cell lines, regardless of fusion protein type, increase in telomerase activity was observed with telomere length shortening according to the wild type. Cell proliferation capacity also increased in these cell lines. Considering the myeloid series, the higher telomerase activity with shortest telomere length was found in 190*Bcr-Abl* transformed cell. Unexpectedly, this cell line decreased cell proliferation capacity. There were differences between 210*Bcr-Abl* and 190*Bcr-Abl* transformed myeloid cell lines.

Discussion

BCR-ABL fusion protein that is the product of the Philadelphia chromosome (Ph) has constitutional tyrosine kinase activity, which is caused by the absence of NH₂ terminal autoregulation region of cABL [20-23]. The various fusion genes (P190, P210 and P230) have different

molecular mass according to break point of BCR and show distinct tyrosine kinase activity. The enhanced leukemogenic activity of P210 without any effect on differentiation was shown. The higher tyrosine kinase activity of P190 than that of P210 were identified [4]. These fusion proteins are found in distinct forms of leukemia. Although P210 is always expressed in all cells of CML, P190 is found in *Chronic Lymphocytic Leukemia* (CLL), ALL and AML with considerably lower frequencies than in CML.

The relation between Ph chromosome and clinical prognosis is still unknown, but two models have been suggested to explain the effect of different fusion proteins on clinic prognosis. One of the models proposes that p210 and P190 have different leukemogenic activity. The other one claims that the cell type in which gene fusion occurs is more important than the type of fusion protein [4, 24-26].

Li et al. [4] reported that these IL-3 dependent myeloid and lymphoid cell lines become independent of IL-3 for survival and growth after transformation. Although, the proliferation rate of p190 transformation was higher than p210 transformation in lymphoid cells, this proliferation capacity did not show any difference related to fusion protein in myeloid cells [4]. In our study, it was observed that p190 or p210 transformed myeloid cells had the capability of growing and surviving without IL3. But p210 transformed myeloid cells had higher proliferation rate than p190 transformed type. In lymphoid cells, the proliferation capacity was increased with both type of fusion proteins, but p190 was more effective than p210 as similar to the former study [4].

Telomere length and telomerase activity are related to the proliferation capacity of the cell. Norback and Roos [27] reported that, telomere length progressively became shorter after each cell division despite telomerase activity in normal hematopoietic progenitor cells.

Telomerase reactivation is observed in almost 90% of human cancers and all immortal cancer cells but not present in normal tissues and somatic cells except stem cells and progenitor cells. Although telomere length becomes shorter after every division in normal cell, telomere length is relatively constant but shorter compared to normal cells in malign cells [28]. It has been shown that, telomerase activity regulates the cell proliferation capacity, but this regulation is achieved not only by controlling the telomere length but also via different interactions with several key regulators of cell cycle.

Short telomere length with increased telomerase activity was observed in the diagnosis of AML [29-32]. But in same phase of CML, low telomerase activity with short telomere length was reported [29-36]. Telomerase activity is increased in blastic crisis of CML. The telomerase reactivation has been triggered by not only shortened telomere length but also increased blastic cell ratio [29,31,32]. In our study we observed significantly incre-

ased telomerase activity and shortened telomere length in p190BCR-ABL transformed lymphoid cells as similar to the previous reports [29-33]. But, the telomere length was not significantly different from the wild lymphoid cell line although the telomerase activity increased in the p210 transformed cells. This discrepancy might be a result of the differences between the pathways which trigger telomerase reactivation in these two cell lines. Telomerase reactivation might occur before telomere shortening in p210 transformation, while in p190 positive cells short telomere length is the major factor to stimulate the telomerase reactivation. According to these results, it can be concluded that, p210 but not p190 has direct effect on telomerase activity. Tauchi et al. [37] have supported this observation. They reported that the efficiency of imatinib on apoptosis on K562 cell line, which derived from a CML patient in blast crisis, increased in the presence of a telomerase inhibitor. They suggested a relationship between BCR-ABL fusion proteins and telomerase. BCR-ABL fusion proteins, especially p210, might supportively play role on cell proliferation together with telomerase; they might have synergic effect on cell proliferation either in the same pathway or independently. Tauchi et al. [37] also showed that only imatinib treatment on K562 cells has dramatically decreased telomerase activity with shortened telomere length.

Although these results have supported our argument, Hartmann et al. [38] reported that imatinib treatment has no effect on either telomerase activity or telomere length on BaF3 and *Bcr-Abl* positive BaF3185 cell lines. In their previous study [39] they observed an increase in telomere length of CML patients receiving imatinib treatment. They explained the elongation of telomere length by two hypotheses. This difference could be due to a shift from Ph (+) toward Ph (-) hematopoiesis. Alternatively, imatinib could have a direct stimulatory effect on telomerase activity in Ph (+) (and/or Ph (-) cells.

The difference between the relations of two fusion proteins with telomere length could be explained alternatively by cell proliferation capacity. The mitotic index in p190 transformed lymphoid cells was higher than that in p210 transformed one in the present study. Because of this difference, shortening of telomere length might be achieved faster in p190 transformed cells despite the telomerase reactivation. In our study, we found 12-fold increase in telomerase activity following p190 transformation while p210-transformed cells exhibit 5-fold increased activity relative to the wild type in myeloid series. This observation is consistent with increased *in vitro* tyrosine kinase activities of p190 and p210 proteins. Li et al. reported [4] that p190 and p210 had respectively 7 and 5.4-fold increased *in vitro* phosphorylation activity. According to these two investigatory results, it is possible to conclude that an exponential correlation is present between phosphorylation and telomerase stimulation functions of BCR/ABL.

Compared with the wild type, prolonged telomere length

was found in 210*Bcr-Abl* transformed myeloid cell. Despite the increased activation of telomerase, shortened telomere length was found in 190*Bcr-Abl* transformed myeloid cell, as well as in 190*Bcr-Abl* transformed lymphoid cell. These results might be regarded as a clue for the existence of a possible direct effect of p210 on telomerase activation.

In this study, extremely high telomerase activity was found with short telomere length in p190-transformed myeloid cells as similar with the former studies about AML [30-33]. In addition to this, high telomerase activity of p210-transformed cells has been concordant with the data of previous studies which have reported that telomerase reactivation occurred in the blastic crisis of CML.

The differences between the effects of p210 and p190 on telomerase activity and telomere length might be one of the reasons causing different leukemogenic activity in hematopoietic cells. Although the relation between telomerase and BCR-ABL fusion proteins could not be explained exactly, a combination of tyrosine kinase inhibitors with telomerase inhibitors has been suggested as an attractive therapy for CML patients [40].

In conclusion, our results suggest that p210 has a direct effect on telomerase activity independent of telomere length. As a result of this, we concluded that other target/targets for BCR-ABL and telomerase might be responsible for these intracellular dynamics. In order to clarify these intracellular dynamics; genes of negative/positive regulators, their protein products and the association between telomerase and BCR-ABL should be analyzed.

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