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Characteristics of the TCR Vβ repertoire in imatinib-resistant chronic myeloid leukemia patients with *ABL* mutations

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Diversity in the T cell receptor (TCR) repertoire provides a miniature defense ability for the T cell immune system that may be related to tumor initiation and progression. Understanding the T cell immune status of leukemia patients is critical for establishing specific immunotherapies. Previous studies have reported abnormal *TCR* repertoires and clonally expanded *TCR Vβ* T cells in chronic myeloid leukemia in chronic phase (CP-CML). In this study, we investigated the distribution and clonality of the *TCR Vβ* repertoire in 4 cases with imatinib-resistant CML in blast crisis (BC-CML) with abelson murine leukemia viral oncogene homolog 1 (ABL1) kinase domain mutations (KDMs). Examination of *TCR Vβ* expression and clonality was performed by reverse transcription-polymerase chain reaction (RT-PCR) and GeneScan analysis. Significantly skewed *TCR Vβ* repertoires were observed in BC-CML patients with different KDMs, and 4 to 8 oligoclonally expanded *TCR Vβ* subfamilies could be identified in each sample. Intriguingly, a relatively highly expanded *Vβ9* clone with the same length as complementarity-determining region 3 (CDR3) (139 bp) was found in all three CML patients in lymphoid blast crisis (LBC-CML) who had different KDMs, but the clone was not detected in the only CML patient in myeloid blast crisis (MBC-CML). In conclusion, restricted *TCR Vβ* repertoire expression and decreased clone complexity was a general phenomenon observed in the BC-CML patients with different KDMs, indicating the T-cell immunodeficiency of these patients. In addition, clonally expanded *Vβ9* T cell clones may indicate a specific immune response to leukemia-associated antigens in LBC-CML patients.

T cell repertoire, chronic myeloid leukemia, blast crisis, imatinib resistance, BCR-ABL mutation

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Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell disorder characterized by the translocation t(9q34;22q11), which results in the creation of a novel *BCR-ABL* fusion gene with abnormal tyrosine kinase activity [1,2]. Imatinib mesylate (IM) is a targeted molecular drug that serves as frontline therapy for all phases of CML for newly diagnosed patients that works by binding to the tyrosine kinase domain of BCR-ABL and inhibiting its function [3–5]. Although high response rates are observed for CML patients who undergo IM treatment, a significant number of patients develop primary or acquired resistance to IM [1,2]. Clinical resistance to IM for CML patients has been attributed to several mechanisms, and the dominant mechanism appears to be the acquisition of point mutations in the *ABL* kinase domain (KD) (30%–90% of patients de-

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velop resistance) that lead to altered affinity for IM by the BCR-ABL1 protein [6,7]. However, due to IM treatment resistance, a proportion of CML patients easily progress to blast crisis phase (blastic transformation may be myeloid, lymphoid, or undifferentiated/mixed), which is an advanced stage of CML disease progression that contributes to the expansion of primitive cells and not mature granulocytes [8–10]. To date, greater than 90 *ABL* gene mutants have been described [6,11]. Different KD mutants confer different sensitivities to ABL kinase inhibitors, and the correlation between the imatinib resistance level and frequency of mutations has been analyzed in several studies; however, the functional importance of these mutants in terms of whether certain mutations affect disease progression or the immune response remains unclear.

T cell receptor (TCR) diversity is one of the defining characteristics of the adaptive immune system. This diversity is prerequisite for guaranteeing that an organism can fight against a universe of foreign antigens and maintain a balanced immune status. The human T cell system is estimated to contain approximately 3×10^{11} cells, which results in estimates for TCR β chain diversity for young adults in the range of 2×10^{6} to 2×10^{7} cells, and each TCR β chain can combine with 25 or more TCR α chains [12,13]. T cell clone diversity originates from T cell differentiation in the thymus, which is characterized by the formation of the complementarity-determining region (CDR) according to rearrangement of multiple variable (V), diversity (D), and joining (J) gene segments. In addition, the random insertion or deletion of nucleotides between gene segments (V-N-D-N-J) increase the clonal diversity [14,15]. Specific recognition of an antigen mediates the clonal expansion of T cells expressing a unique *TCR Vβ* gene of a characteristic CDR3 size. Examination of CDR3 may reveal the immune response characteristics of an individual and identify antigen-specific TCRs. In our previous study, we found that restricted use of the *TCR Vβ* subfamily is a common characteristic of CML patients, and the oligoclonally expanded *TCR Vβ21* subfamily was detected in 27.6% of CML patients and 30.8% of Ph+ B-ALL cases, which may be due to clonal expansion in response to leukemia-associated antigens, such as the BCR-ABL fusion protein [16,17]. In light of these findings from tyrosine kinase inhibitor (TKI) sensitive CML patients, we investigated whether clonal *TCR Vβ* restrictions are detectable and whether different characteristics of the *TCR Vβ* repertoire distribution pattern could be found in IM-resistant BC-CML patients who carry different KDMs.

1 Materials and methods

1.1 Samples

After obtaining patient consent, peripheral blood or bone marrow from four CML patients who were in blast crisis phase (assigned P1 to P4) that had hematologic or cytogenetic resistance to imatinib were collected (P3 had AML resembling myeloid blast crisis (MBC), and the other three patients had B-ALL resembling lymphoid blast crisis (LBC)). Different BCR-ABL KDMs were detected in the patients, including T315I in patient 1 (P1), E255K in patient 2 (P2), Y-253F in patient 3 (P3), and F317L combined with a S417Y mutation in patient 4 (P4). Mononuclear cells were isolated from fresh peripheral blood or bone marrow by Ficoll-Hypaque gradient centrifugation. RNA was extracted from the samples according to the manufacturer's recommendations (TRIzol, Gibco, USA). RNA quality was analyzed in a 1.0% agarose gel stained with ethidium bromide. Two micrograms of RNA was reverse transcribed into first-strand cDNA with random hexamer primers using the reverse transcriptase of the Superscript II Kit (Gibco). cDNA quality was confirmed by RT-PCR of the *β2* microglobulin gene.

1.2 RT-PCR for *TCR Vβ* subfamily member amplification

Twenty-four *TCR Vβ* sense primers and a single *TRBC* primer were used in unlabeled PCR for amplification of the *TCR Vβ* subfamily members. Subsequently, runoff PCR was performed with fluorescent primers labeled at the 5′ end with a FAM (5-Carboxyfluorescein) fluorophore (*Cβ*-FAM) (TIB MOLBIOL GmbH, Germany). PCR was performed as described in our previous study [18].

1.3 GeneScan analysis for *TCR Vβ* subfamily clonality

Unlabeled PCR products (2.0 μL) from subfamily members with positive expression were subjected to one run off reaction cycle with a fluorophore-labeled *Cβ*-fam primer. The labeled runoff PCR products (2.0 μL) were heat-denatured at 94°C for 4 min with 9.5 μL formamide (Hi-Di Formamide, ABI, USA) and 0.5 μL Size Standards (GENESCAN™-500-LIZ™, Perkin Elmer, ABI).The samples were then loaded in a 310 POP-4™ gel (Performance Optimized Polymer-4, ABI) and resolved by electrophoresis using a 310 DNA sequenator (Perkin Elmer, ABI) for size and fluorescence intensity determination using GeneScan software [18,19].

2 Results

2.1 Skewed expression patterns of the 24 *TCR Vβ* subfamily genes in CML patients with *ABL KD* mutations

All of the BC-CML patients with a KD mutation had a significantly skewed *TCR* repertoire with 11 to 16 of the 24 *TCR Vβ* subfamilies detected in each patient. The CML patient with a Y253F mutation in the *ABL* gene (P3) failed

to express most of the *TCR Vβ* subfamilies as only 11 of the 24 could be detected. Of all of the detected subfamilies, *Vβ9*, *Vβ16*, *Vβ19* and *Vβ21* were commonly expressed in all samples, while *Vβ1*, *Vβ2*, *Vβ4*, *Vβ5*, *Vβ6*, *Vβ8*, *Vβ15*, *Vβ18*, *Vβ22* and *Vβ23* were expressed in three of the four samples, demonstrating a relatively higher frequency. In addition, there were three subfamilies(*Vβ3*, *Vβ11* and *Vβ20)* that were not expressed in all samples, and *Vβ7*, *Vβ12* and *Vβ17* were detected in only one sample. These six subfamilies appear to have a relatively lower usage frequency than the other subfamilies (Figure 1).

2.2 Identification of Vβ T cell clonal expansion in different patients

Based on GeneScan analysis, we found that a small proportion of polyclonal peaks (multi-peaks) were distributed in the *TCR Vβ* subfamilies expressed in each sample, while a clonotypic expansion pattern, which includes monoclonality (a single-peak pattern), oligoclonality/oligoclonal tendency (a high peak together with one or a few lower peaks) and biclonality (bi-peaks), were more common patterns for each sample, ranging from four to eight clonally expanded subfamilies in each sample. P2 and P3 had a greater number of expanded *Vβ* subfamilies compared with P1 and P4 (Figure 1), and oligoclonal *TCR Vβ5*, *Vβ8*, *Vβ9*, *Vβ18*, *Vβ19* or *Vβ23* were identified in two or more CML samples (Figure 2). Thus, we further compared the CDR3 lengths (the size of peaks corresponding to the type of clone in the same TCR Vβ subfamily in the detected sample) in the different samples. We found that the *Vβ9* subfamily was the most frequently clonally expanded *TCR β* family, a clone peak similar to the size of CDR3 (139 bp) could be detected in three of the four samples (P1, P2 and P4), and the fluorescence intensity captured by the DNA sequenator had a relatively high signal compared with most of the other subfamilies in each sample (Figure 2C). In addition, the oligoclonally expanded *Vβ5*, *Vβ8*, *Vβ18*, *Vβ19* and *Vβ23* subfamilies could be detected in two samples (Figure 2 A, B, D–F), and the CDR3 size in their primary clones appeared to be different. However, a similar clone peak could be found when comparing the oligoclonal peaks with each peak in the multi-peak background of other samples. For example, the oligoclonally expanded *Vβ8* clone of 270 bp in P3 could also be detected in the multi-peak background of P1 and P2 (Figure 2B). Oligoclonally expanded *TCR Vβ4*, *Vβ6*, *Vβ10*, *Vβ16*, *Vβ21* and *Vβ22* were each identified in only one sample. Additionally, we found the same phenomenon in which a similar primary clone was found in some of the *Vβ* subfamilies (Figure 3 A–F) e.g., oligoclonally expanded TCR *Vβ4* was found in sample P4 with a CDR3 oligoclonal peak at 185 bp, while a clone with a similar size as that of CDR3 could be found in the multi-peak background of the P1 and P2 samples (Figure 3A).

3 Discussion

Several studies have shown that skewed *TCR Vβ* subfamily expression is a common characteristic of leukemia patients [20,21], and clonally expanded T cells might indicate a specific T cell immune response recognizing tumor-specific or associated antigens [19,22]. Skewed expression of *TCR Vβ* subfamily members is also a significant characteristic of IM-resistant BC-CML patients with KDMs compared with healthy donors who express nearly all of the 24 *TCR Vβ* subfamilies [19,23]. The number of completely detectable subfamilies (11 to 16) is similar to that found for CML (6.89±4.63 TCR Vβ subfamilies), AML (7.58±4.93) and B-ALL (7.67±4.66) patients in our previous studies [2,16]. This result indicates that BC-CML patients with IM-resistant KDMs also have skewed usage of the TCR Vβ repertoire. However, BC-CML patients in which 14 or 16 *TCR Vβ* subfamilies have been identified appear to have more diverse immune responses due to the clonal evolution induced by the extra chromosomal abnormalities observed in approximately 80% of BC patients e.g., Phchromosome duplication, trisomy 8 or 19 and 17p loss [8,24], and this requires further investigation.

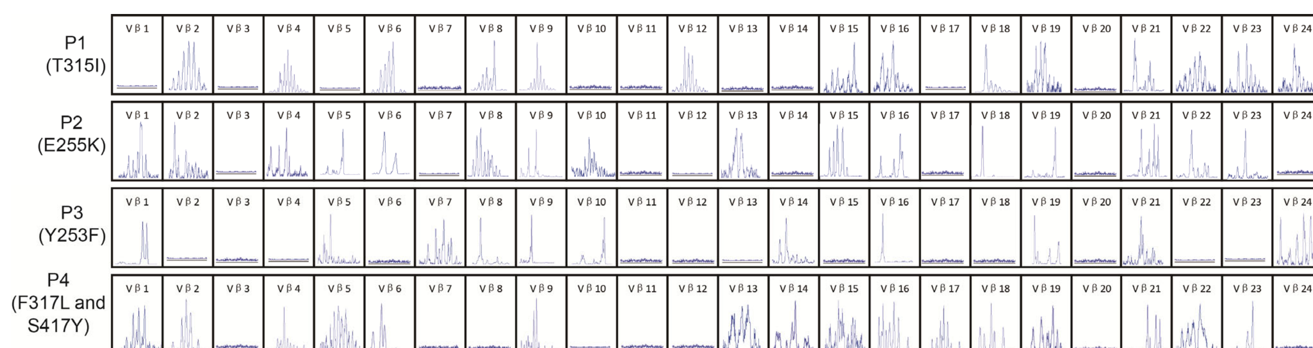

Figure 1 (color online) The distribution and clonality of the 24 TCR Vβ subfamilies in BC-CML patients with different ABL kinase domain mutations.

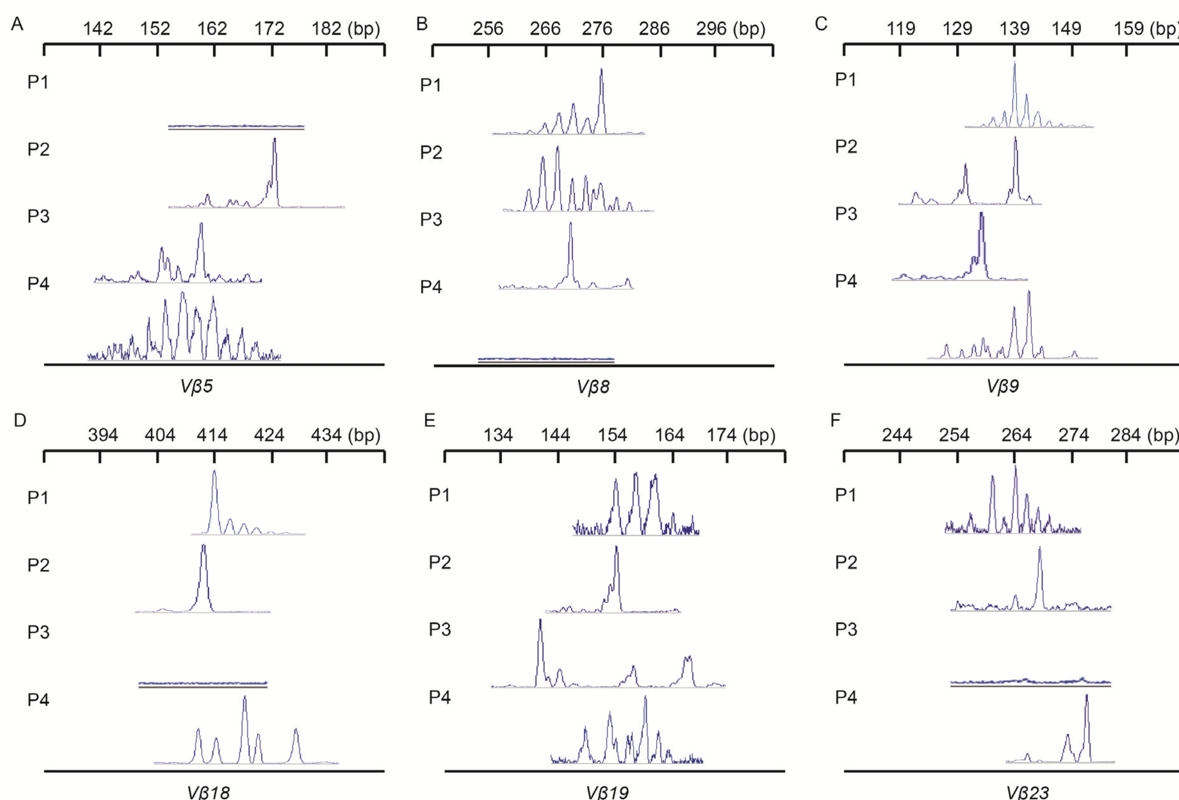

Figure 2 (color online) Comparison of the CDR3 clonality and length in the TCR Vβ subfamilies (*Vβ5*, *Vβ8*, *Vβ9*, *Vβ18*, *Vβ19* and *Vβ23*) in BC-CML samples. Oligoclonally expanded T cells could be identified in two or three samples. In some *Vβ* subfamilies, *Vβ* clones that are the same size as CDR3 could be found in either oligoclonally expanded *Vβ* clones or among polyclones, such as that for *Vβ8,* which had a 270 bp CDR in patient P3 that could also be detected in the multi-peak background of P1 and P2 (B). However, in some cases, the CDR3 size appeared to be relatively different in the same *Vβ* subfamily in different samples e.g., *Vβ23* (F).

Clonally expanded T cell repertoires were detected in all of the samples in this study, and P2, which has an E255K mutation, and P3, which has a Y253F mutation, had eight clonally expanded *TCR Vβ* subfamilies, accounting for 50% (8/16) and 73% (8/11) of the detectable subfamilies in each sample, respectively. Clonal T cells have previously been identified in normal individuals, but overall, these normally represent fewer than 5% of the CDR3 profiles in normal donors [20,23]. This abnormal TCR repertoire expression pattern revealed two patient characteristics. First, the disappearance of the polyclonal pattern found in several subfamilies represents a serious decrease in T cell diversity because T cell spectra are characterized by a Gaussian distribution containing six to eight peaks (polyclonality) for each subfamily in GeneScan analysis of healthy donors, and this pattern represents a repertoire that guarantees sufficiently diverse T cell clones that may be used against different antigens. This TCR expression pattern may explain why leukemia patients are more susceptible to virus infection. For example, patient No.3 died several days after sample collection, and her serious immune deficiency status may be a reason for her poor outcome. In addition, whether this abnormal T cell spectrum pattern was associated with different *ABL* kinase mutations is a question that needs to be answered in future research. Second, highly expressing oligoclonal *TCR* gene patterns have been reported to be associated with the highly expressed Ph chromosome in relapsed CML patients after bone marrow transplantation, and these patterns gradually decrease together with disease remission after donor lymphocyte infusion, indicating that a specific T cell response to antigens might derive from CML-specific *BCR-ABL* translocations or other leukemia-associated antigens [23].We were unable to precisely quantify which clonally expressed subfamily expanded the most; thus, it is difficult to determine which T cell clone may have expanded in response to tumor-associated antigens in different samples. Moreover, a relatively highly expanded *Vβ9* clone (semi-quantified by fluorescence intensity) that was the same size as CDR3 (139 bp) was found in patients P1, P2 and P4 who are CML patients with LBC. It appears that these *Vβ* clones may respond to a similar leukemia antigen in patients e.g., some leukemia-related antigens were displayed in patients with LBC-CML. Previously, we also detected the oligoclonally expanded *Vβ9* subfamily (the main clone demonstrating a 145 bp CDR3) in a CML patient that had Ph-negative ALL and was in the relapsed stage, and this

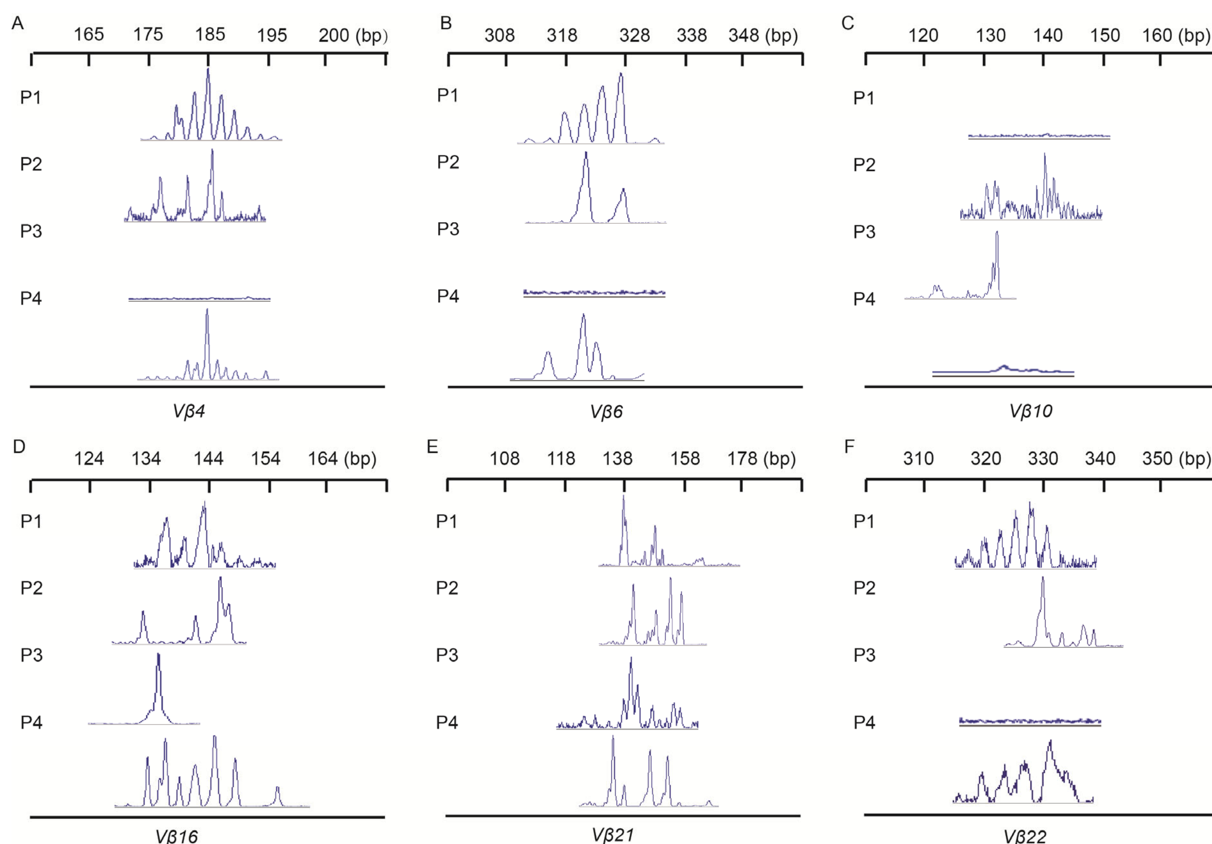

Figure 3 (color online) Comparison of the CDR3 clonality and size for*Vβ4*, *Vβ6*, *Vβ10*, *Vβ16*, *Vβ21* and *Vβ22* in the BC-CML samples. Oligoclonally expanded T cells could be identified in only one sample; however, a similar primary clone was found for the same *Vβ* subfamily members indifferent samples e.g.,*Vβ4*.

oligoclonally expanded subfamily was not observed in the CML-CP stage or post chemotherapy when the patient relapsed with ALL [25]; however, the nucleotide sequence of *Vβ9* clones should be analyzed to determine whether a conserved CDR3 structure exists with a size similar to CDR3 (139 or 145 bp) in different samples.

In conclusion, IM-resistant BC-CML patients containing different point mutations in the *ABL* gene demonstrated T cell immunodeficiency in the *TCR Vβ* repertoire not only in the number of absent *TCR Vβ* subfamilies but also in the decreased complexity of the TCR subfamilies expressed. The CML patient with MBC who had an E255K mutation (P3) had the most serious deficiency in the *TCR Vβ* repertoire, which appears to correspond to the worse clinical status of the patient. Whether specific ABL kinase domain mutations are associated with the severity of the deficiency in the *TCR Vβ* repertoire requires further studies focusing on analyzing the TCR repertoire with larger numbers of patients with specific types of KDMs. In addition, different numbers of clonotypic expansion patterns were found in all of the patients, and there was no identical oligoclonality discovered for the same *TCR Vβ* subfamily in the four patients; however, the same *Vβ9* clone expanded in all three of the LBC-CML patients, which may indicate that despite T-cell immunodeficiency, BC-CML patients may have the ability to specifically respond to leukemia-associated antigens. Furthermore, using high-throughput sequencing techniques to precisely determine the complete competency of the immune repertoire will help determine more accurate TCR subgroups for tumor antigen recognition and help in precisely and individually treating leukemia. On the other hand, epigenetic factors are increasingly recognized to play causative roles in cancer development as well. Whether aberrations of DNA methylation and histone modifications in CML cells have influence on the TCR repertoire is an interesting question awaiting further investigation [26].

The author(s) declare that they have no conflict of interest.

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