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Original article

Torque teno viruses implications on chronic myeloid leukemia

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Abstract

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by the presence of BCR-ABL+ and Ph+. According to recent studies, CML occurrence and evolution are influenced by a series of risk factors, including viruses. Multiple studies suggested that Torque teno viruses (TTVs) could modulate the treatment response and the evolution of hematological diseases. This study focuses on identifying the prevalence and clinical significance of TTVs in CML patients. The main aim was to determine if TTVs presence can be correlated with the onset of disease. We performed a retrospective study (2018-2022) that included 72 blood samples from patients diagnosed with CML. All the 3 anelloviruses were detected using hemi-nested PCR. The overall frequency of TTVs in blood samples was 93%. In our study group, most patients were carriers for the *Torque teno virus* (TTV), *Torque teno midi virus* (TTMDV) and *Torque teno mini virus* (TTMV) in 88%, 57% and 63% of samples. The largest group of carriers was represented by patients with all 3 anelloviruses (51,38%), followed by TTV (22,22%). In our study group represented by CML patients at diagnosis, the prevalence of TTVs is correlated with the molecular load of BCR-ABL. Further research and follow-up of patients with TTV are needed in the future, as well as the identification of new factors that can help to personalize treatment.

Keywords Chronic Myeloid Leukemia, Philadelphia chromosome, BCR-ABL fusion gene, Torque teno virus, hemi-nested PCR

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Introduction

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by the presence of the Philadelphia chromosome and BCR-ABL fusion gene. CML represents approximately 15% of newly diagnosed cases of leukemia in adults (Miranda-Filho et al. 2018; Jabbour and Kantarjian 2022). Philadelphia (Ph) chromosome that results from t(9;22)(q34;q11), is pathognomonic for CML (Guo et al. 1994; Washburn et al. 2021), (5-8%) of patients complex rearrangements can occur (Jones et al. 2009; Jabbour and Soverini 2009; Hakim et al. 2021). BCR-ABL1 fusion gene encodes 3 types of proteins (p210, p190, p230), known for their tyrosine kinase activity (Melo et al. 1993; Kim et al. 2018). The p210 protein is specific for CML through b2a2 and b3a2 transcripts. Most cases of CML have breakpoints in ABL intron 1 or intron 2 and in BCR exons b2 or b3. After translocation, fusion gene variants (such as b2a2 and b3a2) are formed. The p210 fusion protein will be translated from both variants(Pane et al. 2002).

CML treatment was revolutionized with the discovery of tyrosine kinase inhibitors (TKI). Validation of treatment efficacy, such as hematological, cytogenetic and molecular responses, improved treatment management for CML patients (Baccarani et al. 2013). Most chronic phase patients can achieve those responses during TKI therapy. Advances in molecular biology have presented the opportunity to monitor residual disease at the molecular level (Cortes et al. 2011; Tibes and Mesa 2012).

The introduction of TKIs (Imatinib, Nilotinib, Dasatinib, Bosutinib, Ponatinib, Asciminib) as standard treatment in chronic phase of CML has improved the patient‘s prognosis, overall survival and life expectancy (Hochhaus et al. 2017). In a few cases, TKIs can lead to mutations in the BCR-ABL kinase domain and resistance to therapy (Amarante-Mendes et al. 2022).

According to recent studies, CML occurrence and evolution are influenced by a series of risk factors: physical agents (ionizing radiation), chemical pollutants (chemotherapy, benzene, chemical compounds from cigarette smoke), certain biological agents (some viruses), other diseases and age (Lim et al. 2014; Bispo et al. 2020).

Torque teno virus (TTV), *Torque teno mini virus* (TTMV), and *Torque teno midi virus* (TTMDV) are common in the general population (1-12% of blood donors) and in 4% of patients without parenteral risk factors (Prescott et al. 1998; Okamoto et al. 1999; Pineau et al. 2000; Kodama et al. 2011). TT viruses were identified in patients with hemophilia (68%), and 17.8% of those with hematological malignancies (Maeda et al. 2000; Bagheri 2012). Multiple tissues have been found to carry these viruses, including bone hematopoietic cells, peripheral blood mononuclear cells (PBMCs), and polymorphonuclear cells (PMNs) (Okamoto et al. 1999; Gallian et al. 2000; Ishimura et al. 2010). Co-infections with multiple variants of *Torque teno* viruses (TTVs) or with other human viruses may contribute to the evolution of some diseases (Maeda et al. 2000; Ninomiya et al. 2007; Spandole et al. 2009; Rocchi et al. 2009) including acute and chronic hematologic malignancies (Hino and Miyata 2007; Bagheri 2012; Shaheli et al. 2015). Also, TTVs can modulate the treatment response (Pineau et al. 2000; Kincaid et al. 2013). Mixed infections of the TTVs may cause more clinical complications in patients with leukemia(Bagheri 2012; Shaheli et al. 2015).

Consequently, it is currently speculated that TTVs, if it does not contribute to the occurrence of pathological conditions, could influence their evolution. Although there are a few studies regarding the correlations of TTVs with different pathologies (Sarairah et al. 2020), molecular pathways and interactions of these viruses associated with leukemia, remain unknown.

This study focuses on identifying the prevalence and clinical significance of TTVs in CML patients. The main aim was to determine if TTVs presence can be correlated with the onset of disease.

Materials and methods

Sample selection

Our study included a group of 72 patients with CML, ranging in age from 17 to 97 years (median age of 56 years). Being a retrospective study (2018-2022), peripheral blood samples of CML patients at diagnosis were used and it was preferred since the presence of TTVs can change during the evolution of the disease. The group of interest was selected based on the cytogenetics and molecular results (Ph+ and BCR-ABL+). This study was conducted in compliance with the principles of the Helsinki Declaration and approved by the ethics committee of the Fundeni Clinical Institute. Prior to inclusion in the study, informed written consent was obtained from all patients for the scientific use of their data.

DNA extraction

DNA was extracted from peripheral blood, using Pure Link™ Genomic DNA Mini Kit (Invitrogen, USA), according to the manufacturer’s instructions. DNA concentrations of the obtained samples were measured using a NanoDrop spectrophotometer (Spectro-photometer ND-1000).

PCR amplification

A two-steps hemi-nested PCR protocol was optimized in order to determine the presence/absence of all three

anelloviruses (TTV, TTMDV, TTMV) according to the Ninomiya et.al. technique with an additional step for hot-start enzyme. Target regions were amplified using HotStarTaq® DNA Polymerase (Qiagen, Germany) with the mixed primers NG779/NG780 (sense) and NG781/NG782 (antisense) in a reaction volume of 10 µL using 0.04 µL of each primer (Ninomiya et al. 2008). The first PCR amplification program was represented by: the initial step at 95°C/15 min, 2 cycles (94°C/2min, 55°C/30 sec, 72°C/30sec), 35 cycles (94°C/30 sec, 55°C/30 sec, 72°C/30sec) and a final extension step at 72°C/ 1 min.

All three anelloviruses genomes were amplified individually using 1 µL of each of the viral amplicons from the first PCR. We performed a second round of PCR with DNA-specific primers for the detection of TTV- NG779/NG780 (sense) and NG785 (antisense), TTMDV – NG795 (sense) and NG796 (antisense), and TTMV - NG792/ NG793/ NG794 (sense) and NG791 (antisense).

The second PCR amplification program was the following: initial step at 95°C/ 15 minutes, 2 cycles (94°C/ 2 minutes, 55°C/ 30 seconds, 72°C/ 30 seconds), 30 cycles (94°C/ 30 seconds, 55°C/ 30 seconds, 72°C/ 30 seconds) and a final extension step at 72°C/ 1 minute. Information about the primer sequences can be found in Ninomiya et.al. research. (Ninomiya et al. 2008) The PCR final amplicons were migrated in 2% agarose gel to detect a band specific to each anellovirus species.

Statistical analysis

Statistical analysis was performed using the R studio (3.4.4 version). This software was used to determine statistical significance (p-value <0.05). Also, for obtaining the phylogram of TTVs, we used Clustal X software. The basic line plot was created using https://www.bioinformatics.com.cn/en, a free online platform for data analysis and visualization.

Results

Demographic and clinical profile of study subjects

According to Table 2, there were no significant differences regarding sex or age. In terms of molecular findings, the BCR-ABL transcripts were represented by b3a2 and b2a2. In 18 patients, the coexistence of both transcripts was observed and in one case, transcripts e1a2 and b2a2 were observed.

Table 1. The clinical and paraclinical characteristics of subjects selected for this study

Variable	No. of patients
Female	37
Male	35
Mean age (years)	56
Smokers	2
Patients with leukocytosis	55
Patients with hepatosplenomegaly	30
Transcript type	
b3a2	27
b2a2	25
b3a2 + b2a2	18
b2a2 + e1a2	1

Molecular evidence of the TTV genome in CML patients

After hemi-nested PCR was performed, the PCR final amplicons were migrated in 2% agarose gel to detect a band specific to each anellovirus species. Amplification products length measured 112 to 117 bp (TTV DNA), 88 bp (TTMDV DNA), and 70 to 72 bp (TTMV DNA). It can be observed from Figure 1, we identified co-infections of two or three anelloviruses in the same sample.

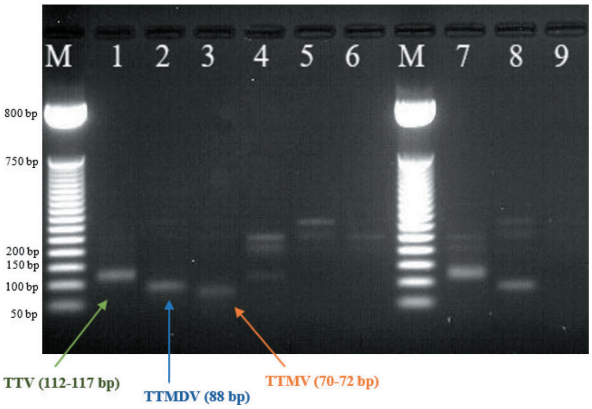

Figure 1: Electrophoresis (2% agarose) for detecting TTV, TTMDV and TTMV (M: Molecular weight marker 50bp DNA Step Ladder (Promega); Lines 1-3: patient presenting TTV-TTMDV-TTMV coinfection; Lines 4-6: patient with a positive result for TTV and negative results for TTMDV and TTMV; Lines 7-9: patient with TTV-TTMDV coinfection and negative for TTMV).

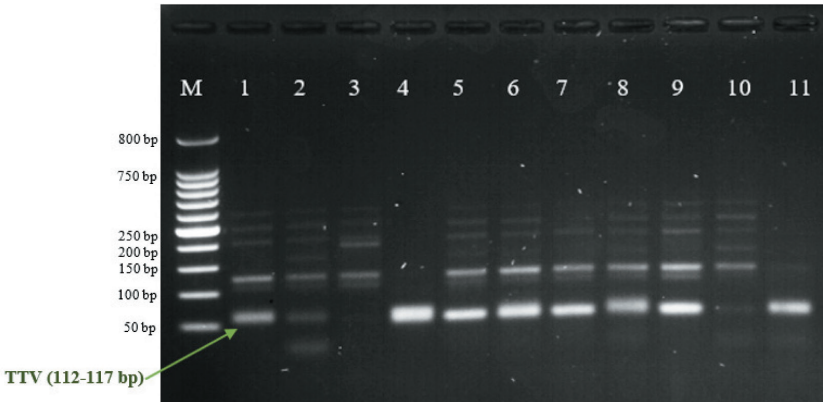

Figure 2. Electrophoresis (2% agarose) for detecting TTV (M: molecular weight marker 50bp DNA Step Ladder (Promega); 1-11: patients presenting TTV infection).

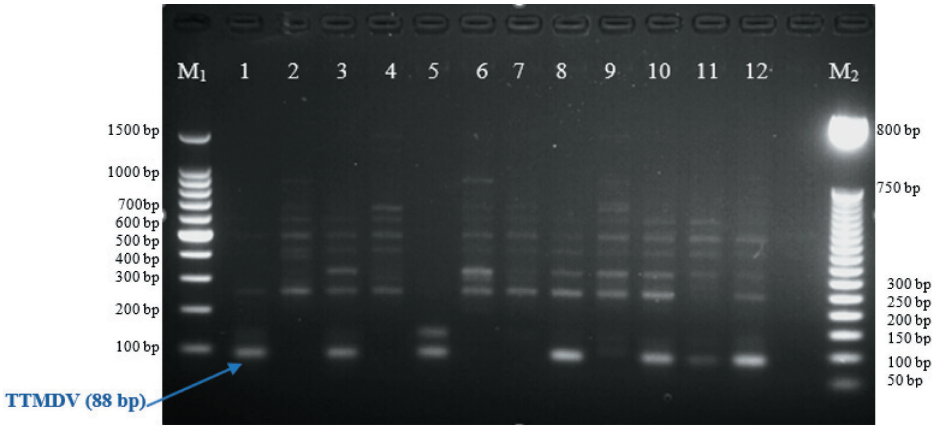

Figure 3. Electrophoresis (2% agarose) for detecting TTMDV (M_1: molecular weight marker BenchTop 100 bp DNA Ladder (Promega);1-12: patients presenting TTMDV infection; M_2: Molecular weight marker 50bp DNA Step Ladder - Promega).

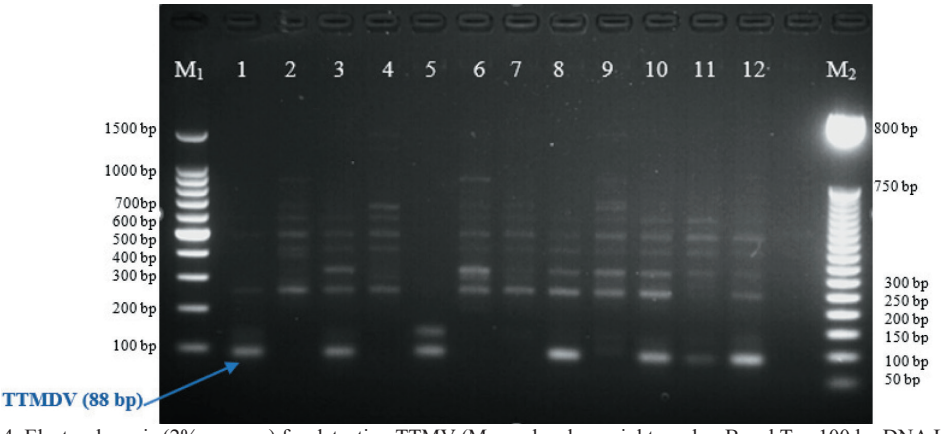

Figure 4. Electrophoresis (2% agarose) for detecting TTMV (M_1: molecular weight marker BenchTop 100 bp DNA Ladder (Promega);1-12: patients presenting TTMV infection; M_2: Molecular weight marker 50bp DNA Step Ladder - Promega).

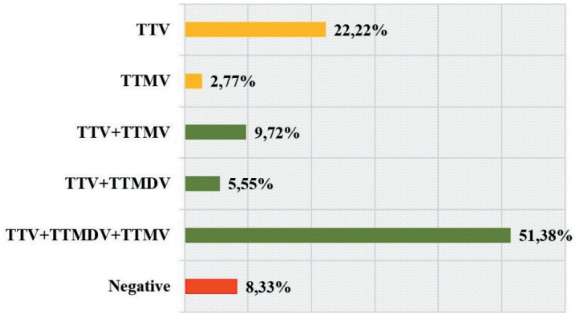

Figure 5. The variants of Torque Teno viruses identified in patients with CML (completely negative – red; single anelloviruses identified – yellow; coexistence of 2 or 3 anelloviruses – green)

Data analysis

After hemi-nested PCR was performed, we observed that the overall frequency of the 3 variants in blood samples was remarkably high (93%). Also, the majority of patients were carriers of TTV (88%), followed by TTMV (63%) and TTMDV (57%). Furthermore, an important aspect is given by the coexistence of 2 or 3 anelloviruses in the same sample. Regarding the distribution of carriers, the largest group was represented by patients for whom the presence of all 3 anelloviruses was identified (51,38%), followed by TTV (22,22%) and association of TTV and TTMV (9,72%). As can be seen from Figure 5, except for TTV, the singular presence of other viruses is rarely present in TTMV (2.77%). Also, in 8.33% of the subjects, the presence of none of the anelloviruses was identified.

As we can observe in Figure 6, the highest distribution of TTVs is registered in the case of b2a2 and b3a2, regarding the carriers of all the anelloviruses (n=13, respectively n=12). The next major category is represented by the association of both transcripts (b2a2 and b3a2) in the case of the coexistence of TTV and TTMDV (n=11). Surprisingly, although according to Figure 6, the coexistence of the 3 TTVs is found in a majority way (51.3%), we did not identify this situation in the case of any subject who was diagnosed with two BCR-ABL transcripts (b2a2+b3a2 and e1a2+b2a2).

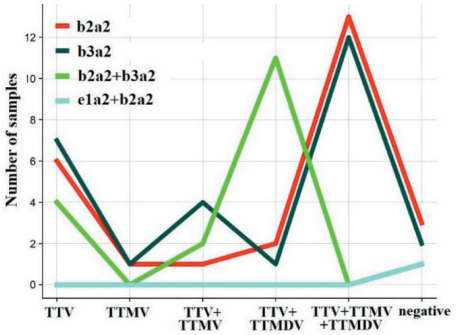

Figure 6: Distribution of TTVs based on BCR-ABL transcript type

Discussion

To our knowledge, these findings are among the few studies involving the association between TTV anelloviruses and CML. In the last decades, the study of TTVs has been full of controversies regarding their role and importance in various pathologies, especially hematological ones. Even though it has been observed that these viruses are frequently found in the general population, they seem to have a role in modulating the therapeutic response, as well as in the evolution of the disease(Spandole-Dinu et al. 2018).

In a recent study, it was observed that the presence of TTV is directly proportional to the molecular response of CML. Thus, the absence of TTV was correlated with disease-optimal molecular response (Galimberti et al. 2020). Most patients at the onset of CML were carriers of at least one of the TTVs variants. These data are consistent with those presented above, according to which the TTV load is proportional to the molecular response of patients diagnosed with CML. Since in numerous studies TTVs have been associated with bone marrow transplantation and blood transfusions, these anelloviruses seem not to be suitable as markers for immunosuppression or as prognostic markers for clinical events in patients after allogeneic transplantation (Shiramizu et al. 2002; Schmitz et al. 2020). Although there is not enough evidence to support the impact of TTV on health issues, multiple hypotheses suggest that TTVs represent a key factor in the pathogenesis of several diseases, such as acute respiratory and liver diseases, AIDS, autoimmune pathologies, and cancer (Vasilyev et al. 2009).

Due to the high variability of TTV genotypes, the distribution of TTV in different populations and pathologies is still a controversial topic (Vasilyev et al. 2009). Few studies in literature have assessed the distribution of TTVs in the global population. Most studies claim that the prevalence of TTVs depends on the PCR conditions used for detection and the amplified DNA fragment. Moreover, the prevalence of TTV genotypes might also vary based on the ethnic group of

the subjects. The TTMDV variant has also been estimated to occur with a prevalence of approximately 40% in the global population. Other research suggests that some TTV genotypes occur with higher frequency than others (Peng et al. 2015)(Spandole et al. 2015). Populational studies revealed that TTVs occurrence is higher (more than half) in Asia (China 93.3%(Peng et al. 2015); Pakistan 90% (Hussain et al. 2012); Qatar 75% (Al-Qahtani et al. 2016); India 72% (Magu et al. 2015) and Northern and Central European countries (Russia 94% (Vasilyev et al. 2009); Finland and Poland 84-88%, Czechia 52.6% (Saláková et al. 2004)). Also, it was observed a decrease in TTVs incidence in countries with warmer climates (Turkey 52% (Erensoy et al. 2002); Brazil 48% (Niel et al. 1999; Devalle and Niel 2004)) (Spandole et al. 2015; Spandole-Dinu et al. 2018; Giacconi et al. 2020).

Our data suggested that the overall incidence of TTVs in the Romanian population is higher than 90%. Considering that Romania belongs to the Central Europe region, this aspect follows the data obtained from the literature, according to which the incidence in this geographical area is higher (over 50%). Before our findings, other studies were conducted regarding the involvement of TTVs in various pathologies (2015 – diabetic nephropathy (Spandole et al. 2015); 2018 – breast cancer, chronic periodontitis, arterial hypertension, obesity, etc.(Spandole-Dinu et al. 2018)), but also regarding the incidence in the healthy population (2013)(Spandole et al. 2013). However, none of these included patients diagnosed with hematological diseases. A common term found in all these studies is the majority incidence of TTV compared to the other viruses. Also, the coexistence of all 3 viruses in the same patients was identified in high percentages. According to Figure 7, the distribution of the 3 viruses is proportional in previous studies on a group with different pathologies (TTV>TTMDV>TTMV). However, our new data suggest a different distribution of the viruses (TTV>TTMV>TTMDV), similar to the population of healthy subjects (2013) (Spandole et al. 2013). This may be due to both the differences in the pathologies studied, as well as the sizes of the groups.

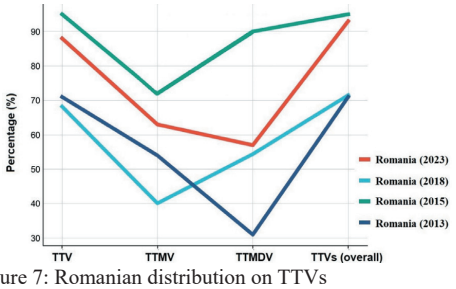

Figure 7: Romanian distribution on TTVs

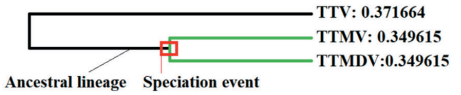

Figure 8:Phylogram of TTV viruses

Human TTV viruses (TTV, TTMDV, TTMV) are included in the Anelloviridae family. Due to the great genomic identity, the impact of these viruses on health is similar. However, it is assumed that the ancestor is TTV, from which, through evolution, TTMV and TTMDV emerged. Using Clustal X software, we obtain a phylogram, according to which, the hypothesis above is confirmed (Figure 8). Thus, the higher prevalence of TTV compared to TTMDV and TTMV (regardless of pathology and population) seems to be a matter of course. These field studies are important because they can provide answers and another perspective regarding the distribution of TTVs in different populations and pathologies.

Conclusions and future directions

Although the state of knowledge regarding the involvement of TVVs in various pathologies can be considered outdated, the impact of these viruses on hematological malignancies remains relevant.

Since our study group is represented by patients at CML diagnosis, the high prevalence of TTVs is in accordance with the high BCR-ABL load. In the future, we propose to develop new groups of patients that reflect optimal, warning and failure as responses to TKI in CML patients. According to the literature, TTVs could have an impact on therapeutic management and also on risk stratification, being able to become markers with clinical involvement in CML.

Further research and follow-up of patients with TTV are needed in the future, as well as the identification of new factors that can help to personalize treatment.

Author contributions: All authors have read and agreed on the final version of the article.

Conflict of interest: The authors have no conflict of interest to disclose.

Founding: This work received no funding.

Ethical approval: The study presented in this article was conducted following the recommendations of the Declaration of Helsinki of 1975, revised in 2013 and the Declaration of Taipei of 2016 (https://www.wma.net/what-we-do/medical-ethics/declaration-of-helsinki/). This study was approved by the Ethics Committee of the Fundeni Clinical Institute 54222/23.10.2020.

Informed consent: All participants included in the study signed the informed consent.

Disclaimer: The views expressed in the submitted article belong to the authors and are not an official position of an institution.

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