Exp Oncol 2022
44, 1, 83–86

METHODS AND TECHNIQUES

Experimental ONCOLOGY

RAPID LOW-COST DETECTION OF TYPE 2 *CALR* MUTATION BY ALLELE-SPECIFIC RT-PCR FOR DIAGNOSIS OF MYELOPROLIFERATIVE NEOPLASMS

*M.V. Dybkov[1, *], M.P. Zavelevich[2], D.F. Gluzman[2], G.D. Telegeev[1]*

[1]*Institute of Molecular Biology and Genetics, National Academy of Sciences of Ukraine, Kyiv 03143, Ukraine*

[2]*RE Kavetsky Institute of Experimental Pathology, Oncology and Radiobiology, National Academy of Sciences of Ukraine, Kyiv 03022, Ukraine*

***Background*: Approximately 15% to 24% of essential thrombocythemia (ET) and 25–35% of primary myelofibrosis cases carry a mutation in the calreticulin *(CALR)* gene. Sanger sequencing, qPCR, high resolution melt or targeted next generation sequencing usually used to detect these mutations are expensive and require costly equipment. Nevertheless, type 1 *CALR* mutations are detectable by using polymerase chain reaction (PCR) and agarose gel electrophoresis. *Aim*: To offer the use of the allele-specific reverse transcription (RT) PCR for rapid low-cost detection of the type 2 mutation in the *CALR* gene. *Materials and Methods*: Allele-specific primers designed for detecting type 2 mutation (5-bp insertion; c.1154_1155 ins TTGTC) of the *CALR* gene were used for allele-specific RT-PCR analysis of cDNA of the patient with *JAK2*-, *MPL*-negative ET, whose mutation in *CALR* gene has been identified by Sanger sequencing. RT-PCR samples were analyzed by agarose gel electrophoresis. *Results*: The type 2 mutation (K385fs*47 ins5) in *CALR* gene was detected by Sanger sequencing in *JAK2*- and *MPL*-negative ET patient. The cDNA obtained was then re-analyzed by using allele-specific RT-PCR with newly designed primers. Normal and type 2 mutation alleles of the *CALR* gene were detected by gel electrophoresis. The results of allele-specific RT-PCR were consistent with the data of Sanger sequencing. *Conclusion*: Allele-specific RT-PCR analysis may be used for the fast low-cost detection of the major type 2 mutation (ins 5) of the *CALR* gene in patients with MPNs.**

***Key Words*: calreticulin, essential thrombocythemia, allele-specific PCR, myeloproliferative neoplasms, mutation.**

DOI: 10.32471/exp-oncology.2312-8852.vol-44-no-1.17329

The myeloproliferative neoplasms (MPNs) are clonal hematopoietic stem cell disorders characterized by dysregulated proliferation and expansion of one or more myeloid lineages (erythroid, granulocytic, megakaryocytic, monocytic/macrophage, or mast cell) [1]. Most of the cases are initially diagnosed in a proliferative phase when counts of granulocytes, erythrocytes, and/or platelets in the peripheral blood increased because of the maturation of neoplastic cells in the bone marrow. In 2008, the World Health Organization (WHO) published a revised classification of the MPNs and altered the algorithms for their diagnosis [2]. The WHO diagnostic algorithms and classification of MPNs were further revised in 2016 [3]. According to this revision, WHO classification of MPNs includes chronic myeloid leukemia, BCR-ABL1-positive; chronic neutrophilic leukemia; polycythemia vera (PV); primary myelofibrosis (PMF); essential thrombocythemia (ET); chronic eosinophilic leukemia, not otherwise specified; MPN, unclassifiable. The revision of MPNs classification was influenced by recently discovered genetic abnormalities that play a role in the pathogenesis of these neoplasms and can be used as diagnostic parameters. The genetic abnormalities include mutations or rearrangements of genes that encode protein tyrosine kinases (but not only them) involved in a number of cellular signal transduction pathways. In chronic myeloid leukemia, the formation of the *BCR-ABL1* fusion gene results in constitutive activation of ABL-derived tyrosine kinase. This genetic abnormality is associated with such consistent clinical, laboratory, and morphologic findings that can be used as a major criterion for diagnosis. For Ph-negative MPNs (PV, ET, PMF), mutations in the Janus kinase 2 gene (*JAK2*), the thrombopoietin receptor gene (or myeloproliferative leukemia virus oncogene, *MPL*), calreticulin (*CALR*) gene, etc. are not specific for any single MPN, but their presence strictly indicates that the myeloid proliferation is neoplastic. The WHO diagnostic criteria for PV, ET, and PMF combine clinical and laboratory data with genetic information and histological features of the bone marrow biopsy.

ET involves primarily the megakaryocytic lineage. The disease can occur at any age, including children; but most cases occur after 60 years, or, less frequently, in 30 year-old individuals, especially women [4]. Approximately 35–50% of patients with ET are asymptomatic at diagnosis. The disease is usually discovered during routine screening or treatment of another illness. Some patients have symptoms that are related to microvascular occlusive events. The marked thrombocytosis is associated with abnormal platelet function and an increased risk of thrombosis and hemorrhage [2].

Submitted: August 12.

*Correspondence: E-mail: m.v.dybkov@imbg.org.ua

Abbreviations used: CALR – calreticulin; ET – essential thrombocythemia; JAK2 – Janus kinase 2; MPL – thrombopoietin receptor gene or myeloproliferative leukemia virus oncogene; MPNs – myeloproliferative neoplasms; PMF – primary myelofibrosis; PV – polycythemia vera; RT-PCR – reverse transcription polymerase chain reaction; WHO – World Health Organization.

PMF is Ph-negative MPN associated with formation of fibrous tissue within the bone marrow. PMF patients may be asymptomatic for many years. Eventually, fever, fatigue, frequent infections, night sweating, enlarged spleen and liver, and weight loss may occur.

Mutations in *JAK2, CALR* or *MPL* are among the main WHO diagnostic criteria for ET and PMF [3]. The *JAK2* gene mutations are found in 40–55% of ET patients and 35–50% of PMF patients [5–6]. The *MPL* mutations are present in up to 5% of patients with ET [7]. In 2013, mutations in *CALR* were identified in the majority of patients with non-mutated *JAK2* or *MPL* suggesting the role of mutant *CALR* in the pathogenesis of MPN [8–9]. 15–24% of ET patients and 25–35% of PMF patients had mutations in *CALR* gene. Mutations in this gene are not found in PV patients. Up to 10% of patients with ET or PMF were negative for *JAK2, MPL*, and *CALR* mutations [10].

CALR is a multifunctional Ca^{2+} binding protein mostly localized in the endoplasmic reticulum and involved in many biologic processes, including proliferation, apoptosis, and cell death. At present, more than 50 mutations (insertions and deletions) that cause a frameshift to an alternative reading frame in the *CALR* gene were detected. In ET patients, type 1 mutation (52-bp deletion; c.1092_1143del, also known as L367fs*46), and type 2 mutation (5-bp insertion; c.1154_1155 TTGTC, also known as K385fs*47) occur in 45–53% and 32–41% of all cases with the *CALR* gene mutations, respectively. Type 1 mutation occurs in 75% of all PMF cases with *CALR* gene mutations [11]. Other mutation types were observed at much lower frequencies. Mutations in the *CALR* gene are associated with younger age and male sex of ET patients. Patients with *JAK2* and *MPL* mutations have a higher risk of death than ET patients with *CALR* mutations [8, 10, 12].

The type 1 or 2 mutations are detected in up to 90% of all the cases with mutated *CALR* in ET. While type 1 mutation can be easily detected by using agarose gel electrophoresis, such approach encounters a problem in case of type 2 mutation due to the difficulties in separating the fragments that differ only slightly by their length. The aim of the study was to develop the modified method of allele-specific PCR for the detection of type 2 mutation of the *CALR* gene that could be useful for differential diagnosis of MPNs.

MATERIALS AND METHODS

cDNA samples of five ET patients who underwent molecular diagnostic tests at the Department of Molecular Genetics of the Institute of Molecular Biology and Genetics, the NASU, were used for the analysis. All the patients were referred to the Reference Laboratory of Immunocytochemistry and Oncohematology Department of RE Kavetsky Institute of Experimental Pathology Oncology and Radiobiology, the NASU for verifying the diagnosis. The design of the study was approved by the Ethics committees of both collaborating institutions. Mutation in *JAK2* and *MPL* genes were detected in three samples as described in [13–16]. Two samples with *JAK2*- and *MPL*-negative ET were further analyzed by Sanger sequencing [16]. A cDNA sample from a patient with type 2 mutation confirmed by Sanger sequencing was used to design and validate a mutation detection system using allele-specific reverse transcription polymerase chain reaction (RT-PCR). Primers for allele-specific RT-PCR were designed by using the Gene Runner 3.0.5 sequence of *Homo sapiens CALR* NM_004343.3. It's modification corresponding to the sequence of the type 2 mutation of the *CALR* (5-bp insertion TTGTC at c.1154_1155) was used as a template for analysis. Primers calr_ins_f — (F) 5'GAGGAGGCAGAGGACAATTGTC and calr2_r — (R) 5'AGGCCTCTCTACAGCTCGTC were selected to detect type 2 mutation. Primers calr2_f — (F) 5'GATCGACAACCCAGATTACAAG and calr2_r (R) 5'AGGCCTCTCTACAGCTCGTC were selected to detect type 1 mutation (52-bp deletion), normal allele, and were also used for Sanger sequencing. Sizes of the amplified fragments are 426 bp (normal allele), 431 bp (type 2 mutation) or 374 bp (type 1 mutation, del 52). Primer localization and size of the amplified fragments are shown in Fig. 1. All primers were tested by using MFE primer (https://mfeprimer3.igenetech.com/spec) and Primer-BLAST(http://www.ncbi.nlm.nih.gov/tools/primer-blast/).

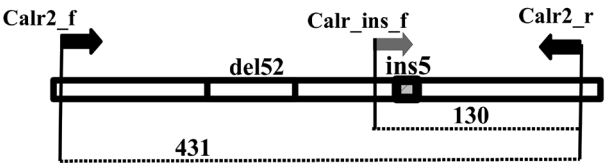

Fig. 1. Oligonucleotide primers localization on the sequence of the *Homo sapiens* CALR gene with type 2 mutation (ins5)

0.5 μM primers calr_ins_f and calr2_r, 2 mM $MgCl_2$, 0.2 mM dNTPs, 1 unit mix Taq DNA polymerase/Pfu polymerase (30:1) in a total volume of 20 μL were used to detect type 2 mutation of the *CALR* gene. The PCR conditions were as follows: initial denaturation step of 3 min at 95 °C, 35 cycles of 30 s at 94 °C, 15 s of annealing at 58 °C, and 20 s of extension at 72 °C. 0.5 μM primers calr2_f and calr2_r were used to detect normal *CALR* gene under the same conditions. The PCR products were resolved in 2.0–2.5% agarose gel in sodium borate buffer. After electrophoresis the gel was incubated with ethidium bromide and visualized under UV light.

RESULTS AND DISCUSSION

The cDNA samples of ET patients were re-analyzed to detect mutations of the *CALR* gene. Cases with *JAK2*- and *MPL*-negative ET were analyzed by Sanger sequencing to detect the mutation (Fig. 2, *a*). As can be seen from the sequencing data, TTGTC insertion in cDNA sample originated from 64 year old ET patient was confirmed. The patient was presented with the disease in 2008. The diagnosis of ET was suggested. According to the data of molecular-genetic analysis performed in 2010 at the Department of Molecular Genetics of the Institute of Molecular Biology

and Genetics NASU, no mutations in *JAK2* and *MPL* genes were revealed. Detection of type 2 mutation in the *CALR* gene confirmed finally the diagnosis of ET in accordance with the up-to-date WHO criteria [3].

Presently there are several techniques used for detecting *CALR* mutations. Some of them (Sanger sequencing, qPCR, or targeted next generation sequencing) are expensive and require costly equipment. High resolution melt (HRM) is of too low sensitivity and Sanger sequencing is needed for determining the genotype of the *CALR* variants. The method of real-time quantitative PCR with allele-specific primers [13] requires equipment for real-time PCR and expensive TaqMan primers. Fragment analysis PCR that could be used for the detection of the *CALR* gene mutations is simple, rapid and low cost but requires the use of high resolution gels and devices for capillary electrophoresis.

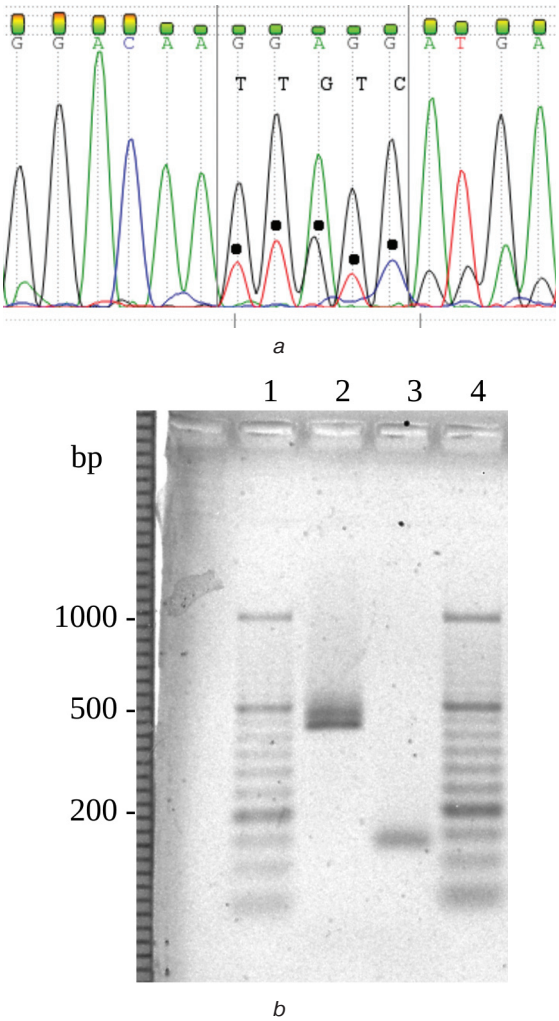

Fig. 2. *a* — Sanger sequencing of the mutation type 2 of the *CALR* gene in the ET patient (the region of the ins5 TTGTC location is marked by dots); *b* — Detection of mutation in *CALR* gene by using allele-specific RT-PCR in ET patient (lanes 2, 3). Line 1, 4 — DNA size markers O'Range Ruler 50 bp; lane 2 — normal and type 2 mutation alleles of the *CALR* gene (426 bp — N, 431 bp — ins5); lane 3 — type 2 mutation of the *CALR* gene (130 bp)

Here we present the fast and cost-effective detection method of allele-specific PCR to detect type 2 mutation of CALR gene that could be useful for differential diagnosis of MPNs. The result of the analysis of cDNA samples of ET patient is shown in Fig. 2, *b*. In lane 2, the separated products obtained by PCR using calr2_f and calr2_r primers are shown. Normal and type 2 mutation alleles of the *CALR* gene (426 bp — N, 431 bp — ins5) were detected. But as mentioned above, the separation of such fragments with similar size by agarose gel electrophoresis could not be always effective. The proposed allele-specific primers allowed us to solve this problem. After PCR using calr_ins_f and calr2_r primers, the mutant allele was detected (Fig. 2, *b*, lane 3). This is completely consistent with the data obtained by Sanger sequencing.

Thus, the results presented allow us to suggest the use of the allele-specific RT-PCR analysis for fast low-cost detection of the major mutation type 2 (ins 5) in *CALR* gene in ET and PMF patients for differential diagnosis.

REFERENCES

1. **Verstovsek S, Tefferi A.** Myeloproliferative Neoplasms: Biology and Therapy (Contemporary Hematology). Humana Press, 2011. 230 p. ISBN: 978-1-60761-266-7

2. **Swerdlow SH, Campo E, Harris NL, *et al.*** WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue (IARC WHO Classification of Tumours), Fourth Edition. Lyon: IARC Press, 2008. 439 p. ISBN-13 9789283244943

3. **Arber DA, Orazi A, Hasserjian R, *et al.*** The 2016 revision to the World Health Organization classification of myeloid neoplasms and acute leukemia. Blood 2016; **127**: 2391–405. https://doi.org/10.1182/blood-2016-03-643544

4. **Finazzi G, Harrison C.** Essential thrombocythemia. Semin Hematol 2005; **42**: 230–8. https://doi.org/10.1053/j.seminhematol.2005.05.022

5. **Baxter EJ, Scott LM, Campbell PJ, *et al.*** Acquired mutation of the tyrosine kinase JAK2 in human myeloproliferative disorders. Lancet 2005; **365**: 1054–61. https://doi.org/10.1016/s0140-6736(05)71142-9

6. **Levine RL, Wadleigh M, Cools J, *et al.*** Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis. Cancer Cell 2005; **7**: 387–97. https://doi.org/10.1016/j.ccr.2005.03.023

7. **Pardanani A, Levine R, Lasho T, *et al.*** MPL515 mutations in myeloproliferative and other myeloid disorders: a study of 1182 patients. Blood 2006; **108**: 3472–6. https://doi.org/10.1182/blood-2006-04-018879

8. **Klampfl T, Gisslinger H, Harutyunyan AS, *et al.*** Somatic mutations of calreticulin in myeloproliferative neoplasms. N Engl J Med 2013; **369**: 2379–90. https://doi.org/10.1056/nejmoa1311347

9. **Nangalia J, Massie CE, Baxter EJ, *et al.*** Somatic CALR mutations in myeloproliferative neoplasms with nonmutated JAK2. N Engl J Med 2013; **369**: 2391–405. https://doi.org/10.1056/nejmoa1312542

10. **Tefferi A, Barbui B.** Polycythemia vera and essential thrombocythemia: 2019 update on diagnosis, risk-stratification and management. Am J Hematol 2019; **94**: 133–43. https://doi.org/10.1002/ajh.25303

11. **Jones AV, Ward D, Lyon M, *et al.*** Evaluation of methods to detect CALR mutations in myeloproliferative neoplasms. Leukemia 2015; **39:** 82–7. https://doi.org/10.1016/j.leukres.2014.11.019

12. **Cabagnols X, Defour JP, Ugo V, *et al.*** Differential association of calreticulin type 1 and type 2 mutations with myelofibrosis and essential thrombocytemia: relevance for disease evolution. Leukemia 2015; **29**: 249–52. https://doi.org/10.1038/leu.2014.270

13. **Zinke M, Nageswaran V, Reinhardt R, *et al*.** Rapid and sensitive detection of calreticulin type 1 and 2 mutations by real-time quantitative PCR Mol Diagn Ther 2015; **19**: 329–34. https://doi.org/10.1007/s40291-015-0162-3

14. **Dybkov MV, Gartovska IR, Maliuta SS, *et al*.** Detection of V617F mutation of gene jak2 at patients with chronic myeloproliferative neoplasms. Biopolym Cell 2010; **26**: 214–7. http://dx.doi.org/10.7124/bc.000159

15. **Gluzman DF, Sklyarenko LM, Zavelevich MP, *et al*.** Complex diagnosis of myeloid neoplasms in regional oncological centers. Science and Innovation (Nauka Innov) 2013; **9:** 61–72. http://dx.doi.org/10.15407/scin9.06.061

16. **Gluzman DF, Sklyarenko LM, Telegeev GD, *et al*.** Diagnostics of acute leukemia and myeloproliferative neoplasms Scientific-practical guide. Kyiv: DIA, 2016. 124 p (in Russian). ISBN 978-617-7015-40-5

ШВИДКИЙ ТА ДЕШЕВИЙ СПОСІБ ВИЯВЛЕННЯ МУТАЦІЇ 2-ГО ТИПУ ГЕНА *CALR* ЗА ДОПОМОГОЮ АЛЕЛЬ-СПЕЦИФІЧНОЇ ЗТ-ПЛР ДЛЯ ДІАГНОСТИКИ МІЄЛОПРОЛІФЕРАТИВНИХ НОВОУТВОРЕНЬ

***М.В. Дибков*[1,*], *М.П. Завелевич*[2], *Д.Ф. Глузман*[2], *Г.Д. Телегєєв*[1]**

[1]Інститут молекулярної біології і генетики НАН України, Київ 03143, Україна

[2]Інститут експериментальної патології, онкології і радіобіології ім. Р.Є. Кавецького НАН України, Київ 03022, Україна

Близько 15–24% випадків есенціальної тромбоцитемії (ЕТ) і 25–35% випадків первинного мієлофіброзу (ПМФ) характеризуються наявністю мутації в гені кальретикуліну (*CALR*). Секвенування за Сенгером, кількісна полімеразна ланцюгова реакція (ПЛР), аналіз плавлення ДНК з високою роздільною здатністю (high resolution melt — HRM) або повногеномне секвенування наступного покоління (next generation sequencing — NGS), які зазвичай використовуються для виявлення цих мутацій, є дорогими та вимагають високовартісного обладнання. Однак, мутацію *CALR* 1-го типу можна виявити за допомогою ПЛР та електрофорезу в агарозному гелі. ***Мета:*** Використання алель-специфічної ПЛР зі зворотною транскрипцією для швидкого та недорогого виявлення мутації 2-го типу в гені *CALR*. ***Матеріали та методи:*** Алельспецифічні праймери, призначені для виявлення мутації 2-го типу (вставка 5-bp; c.1154_1155 ins TTGTC) гена *CALR* були використані для алельспецифічного аналізу методом ПЛР зі зворотною транскрипцією кДНК пацієнта з мієлопроліферативним новоутворенням, у якого не було виявлено мутацій в генах *JAK2* та *MPL* і визначено мутацію гена *CALR* секвенуванням за Сенгером. Зразки методом ПЛР зі зворотною транскрипцією аналізували електрофорезом у агарозному гелі. ***Результати:*** Для аналізу використано випадок з *JAK2*- та *MPL*-негативною ЕТ. Мутацію 2-го типу (K385fs*47 ins5) було виявлено секвенуванням за Сенгером. Цю кДНК повторно аналізували за допомогою алель-специфічної ПЛР зі зворотною транскрипцією. Нормальний алель та алель з мутацією 2-го типу гена *CALR* виявляли за допомогою гель-електрофорезу. Отримані результати повністю узгоджуються з даними, отриманими за допомогою секвенування за Сенгером. Виявлення мутації 2-го типу (K385fs*47 ins5) остаточно підтвердило діагноз «есенціальна тромбоцитемія» згідно з сучасними критеріями ВООЗ. ***Висновки:*** Алель-специфічний аналіз ПЛР зі зворотною транскрипцією може бути використано для швидкого та недорогого виявлення основної мутації 2-го типу (ins 5) гена *CALR* у пацієнтів з мієлопроліферативними новоутвореннями.

Ключові слова: CALR (кальретикулін), есенціальна тромбоцитемія (ЕТ), алельспецифічна ПЛР, мієлопроліферативні новоутворення, мутація.