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Rapid detection of CALR type 1 and type 2 mutations using PNA-LNA clamping loop-mediated isothermal amplification on a CD-like microfluidic chip

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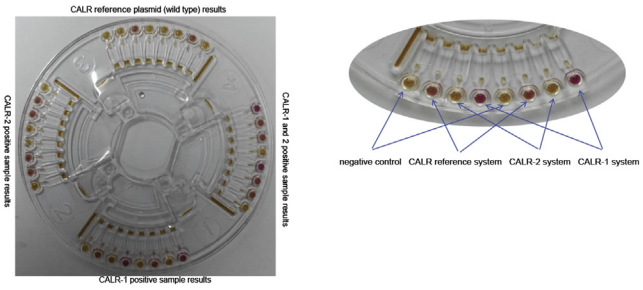
HIGHLIGHTS

- The LAMP microfluidic platform established greatly facilitated the rapid diagnosis of MPNs.
- This microfluidic device has the characteristic of less dependence on ancillary devices.
- PNA and LNA were used to enhance the performance of the LAMP methods.

GRAPHICAL ABSTRACT



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ABSTRACT

Bleeding and thrombosis represent common complications in myeloproliferative neoplasms (MPN) and significantly contribute to morbidity and mortality. Molecular markers, including CALR mutations, were considered not only as diagnostic markers, but also as risk factors for bleeding and thrombosis associated with MPN, especially for patients in remote primary hospitals. We sought to develop an easy-to-use assay for the rapid detection of CALR type 1 (CALR-1) and type 2 (CALR-2) mutations in Philadelphia chromosome-negative MPN patients. Peptide nucleic acid-locked nucleic acid (PNA-LNA) clamping loop-mediated isothermal amplification (LAMP) assays were established, which were integrated into a centrifugal compact disc (CD) microfluidic platform. A total of 158 clinical blood samples were tested simultaneously by this microfluidic platform and an in-house real time PCR assay. The detection performance of the LAMP arrays was validated and conflicting results were identified by Sanger sequencing. The results suggested that the LAMP methods we developed exhibited good sensitivity, specificity, and precision. By real time fluorescence assay the detection limit for CALR-1 and CALR-2 mutations could reach as low as 1% and 0.5% respectively, and 10% and 5% respectively by visual method. There were no nonspecific background amplifications among different detection systems. For the CALR-1 and CALR-2 LAMP detection systems, intra-batch CV values of 1% mutated plasmid were 10.56% and 10.51%

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respectively, and the inter-batch CV values were 19.55% and 18.39%, respectively. The products were all analyzed by melting curve analysis and electrophoresis followed by Sanger sequencing analysis, which were consistent with the database sequences. The microfluidic platform could complete rapid detection of CALR-1/2 mutations within 60 min. The results of clinical samples detected by our CD-like microfluidic chipLAMP assay and rtPCR assay suggested that 133 samples were CALR wild type, 15 were CALR-1 mutation type, and 9 were CALR-2 mutation type. The correlation coefficient value (Kendall's tau_b) of the two assays was 0.99. Interestingly, by the newly established detection platform, we were surprised to find that one patient of Chinese origin harbored both CALR-1 and CALR-2 mutations. This result was verified by Sanger sequencing analysis. The LAMP detection systems developed herein displayed good sensitivity, specificity, and stability. Additionally, the detection results could be directly judged by color changes of the reaction systems without any auxiliary equipment. Thus, the platform we developed has the potential of being widely used in remote and economically undeveloped areas in the future.

1. Introduction

Philadelphia chromosome-negative myeloproliferative neoplasms (MPNs) are a group of clonal hematopoietic diseases characterized by aberrant proliferation of one or more myeloid lineages and progressive bone marrow fibrosis [1]. MPNs are a type of chronic myeloid neoplasms with the potential for transformation to acute leukemia. According to the 2016 revised World Health Organization (WHO) classification of hematologic tumors [2,3], classic Philadelphia chromosome-negative MPNs include essential thrombocytosis (ET), polycythemia vera (PV), and primary myelofibrosis (PMF). The biological confirmations of these pathologies mainly rely on the detection of the mutations of three genes: JAK2 (Janus kinase 2), MPL (myeloproliferative leukemia virus oncogene), and CALR (calreticulin). JAK2 and MPL gene mutations are the most common in patients with Philadelphia chromosome negative MPNs. However, it was recently found that 56–88% of JAK2/MPL-negative patients harbor CALR gene mutations (4). CALR mutation analysis could improve the accuracy of clinical diagnosis in patients without other molecular markers and was included as diagnostic criteria for ET and PMF in the 2016 revision to the WHO classification of myeloid neoplasms including MPNs [4]. Compared with the research work on activating JAK2 or MPL mutation detection in Ph negative patients, there have been relatively few studies for the rapid detection of CALR mutations, because of the complexity of their structure. CALR consists of three domains and is a highly conserved gene among different species. At present about 55 kinds of CALR mutants [5] have been found, which has presented a significant impediment to their detection, especially for conventional molecular testing methods. The most common mutations in the CALR gene are type-1 (c.1092_1143del in exon 9, hereafter called CALR-1) mutation and type-2 (c.1154_1155insTTGTC in exon 9, hereafter called CALR-2) mutation, which are the most prevalent CALR mutations accounting for more than 85% of the all CALR mutations [5,6]. Either of these mutations cause a +1 frameshift in the reading frame of the CALR carboxyl-terminus, resulting in the generation of a new amino acid sequence at the C terminus of CALR-mutated proteins in MPN patients, which is believed to be a gain-of-function mutation [7,8].

CALR takes part in numerous cell surface intracellular and extracellular functions, such as calcium metabolism, phagocytosis, cell adhesion, immune response, etc. [9]. CALR mutations could impair calcium binding activity. It has been reported that clinical phenotype and outcomes are different between patients with CALR-1 mutation and those with CALR-2 mutation [10]. The CALR-1 positive mutations were mainly associated with a myelofibrosis phenotype and a significantly higher risk of myelofibrotic transformation, while the CALR-2 positive mutations were preferentially associated with a thrombocythemic phenotype, low risk of thrombosis and indolent clinical course. Thus, the mutation subtype contributes strongly to determine the clinical phenotype and outcomes in CALR mutation-positive MPNs.

Bleeding and thrombosis represent common complications in MPN and significantly contribute to morbidity and mortality. Various molecular markers including CALR mutation were recently considered to evaluate the risk of bleeding and thrombosis associated with MPN, especially for patients in remote primary hospitals [11,12]. Because thrombosis is the initial presenting symptom of most MPN patients, it is necessary to distinguish them from the ordinary thrombosis patients, which is critical for the formulation of treatment prescription and minimal residual disease monitoring in patients during or after therapy. Thus, in a resource-limited setting, there is an urgent need for an assay integrated with a sample-in/answer-out chip for CALR mutations for quantitative POCT.

There are many techniques available for gene mutation detection including real-time PCR [7], high-resolution melting (HRM) analysis [13], sequencing analysis, amplification refractory mutation system (ARMS)-PCR method, etc. [14]. However, each of the aforementioned methods have different drawbacks, such as low detection sensitivity, low accuracy, high requirements for laboratory resources and equipment, complex follow-up analysis, laborious interpretations, long reporting time, etc. Peptide nucleic acid-locked nucleic acid (PNA-LNA) clamping LAMP is a sensitive and specific method for rapid mutations detection [15]. Therefore, in this study our goal was to develop a kind of PNA-LNA clamping loop-mediated isothermal amplification for the rapid and convenient detection of CALR 1/2 mutations. Our methods were integrated into a CD-like microfluidic chip for simultaneous multiplex detection and quality control, which is useful for the early diagnosis of MPNs and the rapid screening of MPN-associated thrombotic events, especially for patients in remote primary hospitals. The microfluidic chip method was further validated for precision, accuracy and sensitivity, establishing its potential for application to POC diagnostics.

2. Materials and methods

2.1. Clinical samples and nucleic acid extraction

The sample screening criteria were as follows: hemoglobin >20 g/dl, or platelet $\geq 500 \times 10^9$/l, or megakaryocyte proliferation and atypia accompanied. All 158 clinical blood samples were collected from Huashan Hospital, Fudan University after patients provided written informed consent. Institutional approval for