A triplex probe-based TaqMan qPCR assay for Calreticulin type I and II mutation detection

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ABSTRACT

Background: Calreticulin (CALR) exon 9 frameshift mutations have recently been identified in 30–40% of patients with essential thrombocythemia (ET) and primary myelofibrosis (PMF) without JAK2 or MPL mutations. We aimed to develop a qPCR assay to screen type I and II mutations of CALR.

Methods: Three different fluorescent-labeled hydrolysis probes and one pair of primers in a closed-tube system were developed to detect CALR type I and II mutations and distinguish them from wild-type. The sensitivity and specificity were validated using TA-cloning plasmids containing CALR wild-type and type I and II mutants, respectively. Fifty-nine ET and PMF specimens were screened by TaqMan qPCR and sequenced by Sanger sequencing. For intra-assay validation, 20 replicates of each sample were performed and repeated continuously for 5 days. Genotyping results obtained from TaqMan qPCR were 100% concordant with Sanger sequencing. The intra- and inter-assay CVs of TaqMan qPCR were less than 3%, respectively.

Results: We found that triplex probe-based TaqMan qPCR was reliable in detecting CALR type I and II mutants within DNA that was diluted to 1% of total DNA with the wild-type DNA as background. In 59 patient specimens, six of the observed mutations of CALR were type I and five were type II. Genotyping results obtained from TaqMan qPCR were 100% concordant with Sanger sequencing.

Conclusions: Triplex probe-based TaqMan qPCR is an accurate and sensitive method for screening ET or PMF patients with type I and II mutations in CALR.

KEYWORDS

CALR; essential thrombocythemia; primary myelofibrosis; TaqMan; myeloproliferative neoplasms

Introduction

The classical Philadelphia chromosome-negative (Ph−) myeloproliferative neoplasms (MPNs), namely polycythemia vera, essential thrombocythemia (ET) and primary myelofibrosis (PMF), are a set of disorders featuring hyper-proliferation of a specific subgroup of blood cells [1]. Genetic mutations in JAK2 and MPL have been identified as the driving force of disease development and the canonical molecular signatures for diagnosis. However, there are still approximately 40% of Ph− MPN patients who remain JAK2 and MPL mutation-free. Recently, insertions and deletions in calreticulin (CALR) exon 9 that lead to the gene open reading frame (ORF) shifting and malfunction of the CALR protein have been identified in approximately 60% of ET and PMF patients lacking JAK2 and MPL mutations [2,3].

CALR is a Ca2+ binding protein mainly localized in the endoplasmic reticulum (ER) lumen of most human cells. It functions as a key component in controlling the quality of glycoprotein synthesis and folding [4], and also mediates a variety of cellular processes including apoptotic cell clearance, cell adhesion, migration and autoimmune response. Given the primary effect of CALR exon 9 mutations on JAK-STAT pathway dysregulation and platelet production, these high-prevalence genetic variations are now considered as the driving cause of disease development [2,3]. CALR has been included in the recommended ET and PMF diagnostic criteria. Furthermore, significant differences in clinical phenotype have been reported that distinguish CALR mutant patients from CALR wild-type patients [1,3]. The CALR-mutant patients are now considered to be younger in age, male sex...
dominant, and have higher platelet counts, lower thrombosis and bleeding risks, etc. [5]. Additionally, many studies have confirmed that CALR exon 9 mutations exert a unique effect to the clinical phenotype of the disease, have a positive impact on patients’ prognosis and facilitate options for therapeutic approaches [6].

CALR mutations were initially identified in ET and PMF patients by two groups using whole-exome sequencing [2,3] _ENREF_2. Almost all mutations are insertions, deletions and complex insertion/deletions in exon 9 of CALR. All CALR mutations cause a shift of one base pair in the ORF and after translation a novel C-terminal acidic domain, resulting in a loss of multiple calcium binding sites and KDEL ER-retention sequence. So far there are over 50 types of indel variants reported in CALR exon 9, with a growth in numbers enriching the mutation diversity. Many clinical laboratories are currently working to detect CALR exon 9 mutations using PCR fragment analysis and Sanger sequencing, however, time consuming and costly. As recently reported, two well-defined CALR type I (52-bp deletion; c.1092_1143del) and type II (5-bp insertion; c.1154_1155insTTGTC) mutations account for nearly 85–90% of all the mutant genotypes [6,7]. There is emerging evidence supporting that these two kinds of mutations have a favorable prognostic impact. Identification of these mutations could also help in the stratification of MPNs patients to alternative therapeutic approaches [8].

Given the high prevalence of these genetic alterations and the importance for the diagnosis of Ph− MPNs, there is an urgent need for a simple and accurate system that is capable of discriminating CALR type I and II mutations and wild-type CALR from each other. Although there are several reported systems for CALR mutation screening [9,10]. Here we describe our development of a closed tube triplex-probe TaqMan qPCR assay for detection of CALR type I and II mutation that addresses all the aforementioned demands, as well as our completion of a full set of analytical performance validation.

**Materials and methods**

**Specimens**

We obtained samples from patients with ET (n = 45) and PMF (n = 14) from December 2015 to January 2017 at the Department of Hematology in Huashan Hospital of Fudan University and Shanghai Fifth People’s Hospital. All these patients were diagnosed with Ph− MPNs according to the WHO Classification of Tumors of Hematopoietic and Lymphoid Tissue (2008). Additionally, 40 blood donors who did not have hematological diseases, and who were of comparable composition in age and sex with the patients group, were used as negative control. The study protocol was approved by the Ethics Committee of the Huashan hospital.

**DNA extraction**

DNA was extracted from peripheral blood (200 μl) collected in ethylenediaminetetraacetic acid anticoagulant with QiAamp DNA Blood Mini kit (Qiagen, Holden, German), and photometrically analyzed with a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). Then the DNA was diluted with nuclease-free water to get a final concentration of 20 ng/μl and stored at −20°C. All DNA used for PCR had 260 nm:280 nm absorbance ratio more than 1.8.

**Construction of control plasmids for CALR type I and II mutations and wild-type CALR genotyping**

PCR was performed to generate fragments (584 bp for CALR exon 9 wild-type, 532 bp for type I mutation and 589 bp for type II mutation) flanking exons 8 and 9 of the CALR gene as previously described in Ref. [11]. The PCR products were then purified by a QIAquick Gel Extraction kit (Qiagen). The purified DNA was ligated to the PMD19-T simple vector (Takara, Dalian, China), and then transformed into DH5α competent _Escherichia coli_ cells. The bacteria were propagated in Luria–Bertani broth and spread onto IPTG-Xgel (Invitrogen, Carlsbad, CA, USA) coated ampicillin-LB agar dishes for blue/white selection. Plasmids were extracted from the enriched white isolates with QiaAmp Plasmid Mini Kit (Qiagen) and the genotypes were again validated by bidirectional Sanger sequencing, which were used as standards for the triplex probe TaqMan qPCR. The copy numbers of these three recombinant plasmids were calculated based on the density value and serially diluted 10-fold from 10E2 to 10E7 copies/l.

**Primers and probes**

The primers were designed with Primer5 software, for amplifying both wild-type and the two mutant types of CALR exon 9. The span of the wild-type probe starts in the site of 52 bp deletion and ends close 3 of the 5 bp insertion, the type I probe bridges the site of the 52 bp deletion and ends before the 5 bp insertion, and the type II probe starts in the site of 52 bp deletion and ends at the 5 bp insertion (Figure 1). The three TaqMan probes were designed for distinguishing CALR variants from wild-type in Table 1.

**Development of triplex probe TaqMan qPCR for CALR type I and II mutant detection**

qPCR reactions were developed and standardized for two real-time PCR platforms, Cobas Z480 (Roche...
Diagnostics, Basel, Switzerland) and ABI 7500 (Applied Biosystems, Foster City, CA, USA), using the control plasmids harboring CALR type I or II mutation or wild-type genotype, respectively. Each reaction was performed in 10 μl volume with TaqMan® Universal Master Mix II (Applied Biosystems), 100 nM of each TP-qPCR primer, 100 nM of each TP-TaqMan probe and 10 pg plasmid DNA. The sequence of primers and probes was listed in Table 1. Each qPCR assay was carried out at 95°C for 10 minutes followed by 40 cycles at 95°C for 15 seconds and annealing/extension at 60°C for 60 seconds. Detection took place in the extension step of qPCR. The qPCR running data were collected by either LightCycler-480 software Version 1.5 (for Roche Cobas Z480 platform) or 7500 system Version 1.4.0 (for Applied Biosystems ABI 7500 platform). For every batch of assay, the serial standards for each mutation type were amplified along with samples with the unknown genotype.

Mutation scanning of CALR type I and type II mutations with triplex probe TaqMan qPCR

All 99 DNA samples were assessed for detection of CALR wild-type, type I and type II mutations by being subjected to triplex probe-based TaqMan qPCR. The qPCR procedure was carried out as mentioned above, and data analysis was performed with the 7500 system Version 1.4.0 (Applied Biosystems).

Repeatability of triplex probe TaqMan qPCR

Repeatability of the triplex probe TaqMan qPCR was validated by intra- and inter-assay runs. Three samples with CALR exon 9 wild-type, type I and type II mutant genotype were pulled out for the validation. For intra-assay validation, 20 replicates of the assay were performed with each sample. For inter-assay validation, 4 replications of each sample were carried out and the procedure was repeated continuously for 5 days. Raw Cq values of each run were exported. Imprecision of assay was assessed by coefficient of variation (CV) of either Cq value.

Sanger sequencing of CALR exon 9

To validate the exact genomic sequence of CALR exon 9 for all the enrolled cases, peripheral blood genomic DNA was further PCR amplified using the primers listed in Table 1. Agarose electrophoresis-purified amplicons were then subjected to bi-directional Sanger sequencing. All DNA sequencing assays were performed on an ABI XL3730 genetic analyzer in the BGI Shanghai laboratory. For each mutation-susceptive sample, T-A cloning was used to confirm the mutation with pure mutant subclones.

Statistical analysis

All statistical analysis was performed using Stata 5.0 software and Microsoft Excel.

Results

Analytical sensitivity

In this study, the sensitivity of the triplex probe TaqMan qPCR system to detect CALR type I and II mutations was...
thus considered to be 1% (i.e. 1% of total DNA in a background of 99% wild-type DNA; Figure 2).

Repeatability

Both the intra- and inter-assay runs produced an expected amplification plot for the non-mutant and type I/II mutant specimens consistently in all replicates. The CV of Cq value determined by each amplification was taken as the parameter to measure the imprecision of the system. For intra-assay, the CV for 20 replicates of each sample for all 3 genotypes (wild-type, 1.06%; CALR type I mutant, 1.86%; CALR type II mutant, 1.15%) was lower than 3%. In comparison, the CV for the inter-assay runs (20 replicates per specimen) for all genotypes (wild-type, 1.99%; CALR type I mutant, 1.58%; CALR type II mutant, 1.35%) was lower than 3% (Table 2). Overall, the imprecision of the assay as determined by CV was low for Cq value, revealing the desired reproducibility of our system.

Different CALR variants detected by triplex probe TaqMan qPCR

Different fluorescence signals of CY5, FAM and TAMRA were specific for CALR wild-type, CALR type I (52 bp deletion) and CALR type II mutations (5 bp insertion), respectively. Based on fluorescence signals, CALR variants could be distinguished as expected. No interference signal was observed in the controls. The sequencing results were consistent with those of the assay. Different CALR variants could also be distinguished simultaneously (Figure 3).

CALR exon 9 Sanger sequencing

These results were further confirmed by bidirectional Sanger sequencing. Forty normal specimens did not carry either type I or type II mutations. Of the 99 patient samples, type I mutation was detected in 6 samples, and type II mutation was identified in 5 samples (Table 3). All these samples were verified by target DNA cloning and bidirectional Sanger sequencing.

Discussion

Since CALR mutations were identified in a substantial proportion of patients with ET and PMF without JAK2 V617F or MPL mutations, these mutations have recently been included in the 2016 WHO diagnostic criteria for ET and PMF [12]. Although the precise mechanism by which mutant CALR can induce myeloproliferative diseases remains to be elucidated, mouse models have suggested a primary effect on platelet production [11,13]. As with the mutant CALR phenotype in ET patients (younger age, male sex, higher platelet count, lower hemoglobin level, lower leukocyte count, and lower incidence of thrombotic events), CALR mutant PMF patients also exhibited a phenotype of younger age, higher platelet count, and lower risks of anemia, leukocytosis, and splicesome mutations.

CALR mutation is of great importance for the diagnosis of MPNs; furthermore, identification of these mutations can facilitate the stratification of MPN patients during the treatment decision-making process. There is now burgeoning evidence suggesting that the two major CALR exon 9 indel mutations, type I (52-bp deletion; c.1092_1143del) and type II (5-bp insertion; c.1154_1155insTTGTC), which account for

Table 2. Intra- and inter-assay precision of triplex-probe TaqMan qPCR system.

| Specimen          | Intra-assay | Inter-assay |
|-------------------|-------------|-------------|
|                   | Mean Cq SD  | SD % CV     |
| CALR Wild-type    | 24.77 0.2634| 0.4936 1.99 |
| CALR Type I mutant| 27.26 0.5069| 0.4321 1.58 |
| CALR Type II mutant| 27.07 0.3101| 0.4267 1.36 |
over 90% genetic alteration in this locus, can lead to better clinical outcomes [14]. In ET, the type II CALR mutation was associated with significantly higher platelet counts; likewise, in PMF, with higher Dynamic International Prognostic Scoring System (DIPSS)-plus scores, circulating blast percentage, and leukocyte counts and superior survival [15,16].

Several assays have been developed to differentiate CALR exon 9 indel mutations from wild-type, including Sanger sequencing [2,3], high-resolution melting (HRM) [17], PCR followed by fragment analysis [18] and targeted next generation sequencing (NGS) [19]. Sanger sequencing was incapable of detecting the mutations below an approximately 10% mutated allele in a wild-type background [20]. Various studies have reported sensitivity of HRM for the detection of CALR mutations around 2.5–5% [10], followed by fragment analysis at 5–10% [9], and the most sensitive method was targeted NGS, which detected mutations down to a 1.25% mutant load [21]. Although sensitive, effective, and accurate methods, HRM, fragment analysis and targeted NGS are very costly, requiring additional reagents and expensive instrumentation; moreover, some samples that were detected as indeterminate by HRM required confirmation of mutation type via target DNA T-A cloning [14].

In this study, we have developed a qPCR-based TaqMan assay for distinguishing CALR exon 9 indel mutations from wild-type, which could be used in a routine diagnostic setting on Roche Cobas Z480 and Applied Biosystems ABI 7500 platform. According to Chi’s report [18], CALR type I and type II mutations account for about 90% of CALR genetic alterations, and there are few studies on the relationship between other types of CALR mutation and clinical symptoms. We strategically designed three probes at the region neighboring CALR exon 9 indel mutations and a pair of primers for amplification. Then we developed a triplex probe TaqMan qPCR assay to distinguish the type I and II CALR exon 9 mutations from wild-type background, focusing on the vast majority of patients.

Table 3. Mutation identified in CALR using triplex-probe TaqMan qPCR, and Sanger sequencing.

| Patient ID | Diagnosis | Detecting method          |
|-----------|-----------|---------------------------|
| Triplex-probe TaqMan qPCR | Sanger sequencing |
| M5 | ET | Type II | Type II |
| M9 | ET | Type II | Type II |
| M25 | ET | Type II | Type II |
| M29 | PMF | Type I | Type I |
| M33 | ET | Type I | Type I |
| M55 | ET | Type I | Type I |
| M88 | ET | Type II | Type II |
| M129 | PMF | Type I | Type I |
| M134 | PMF | Type I | Type I |
| M140 | PMF | Type I | Type I |
| M146 | ET | Type II | Wild |

Figure 3. Different CALR variants from three disparate patients detected by triplex-probe TaqMan qPCR. Panel A shows a schematic representation of triplex probe TaqMan qPCR, panel B shows triplex probe TaqMan qPCR, panel C shows Sanger sequencing of CALR exon 9.
the probes. This method achieved 100% accuracy compared with T-A DNA cloning. We evaluated this method for sensitivity by a serial dilution panel of CALR type I- and type II-mutant T-A plasmids at the proportion of 1%, 3%, 5%, 10%, 25% and 50% within a background of wild-type DNA. Our laboratory defined a Cq value at 1% as a weak positive result, while the threshold for a weak positive result may differ between laboratories. Each reaction type, which was performed in 10 μl volume, saves considerable detection cost, and the laboratory testing only requires fewer than 3 hours from DNA extraction to completion.

In an agreement test, one sample tested wild-type by Sanger sequencing was type II mutation by triplex probe TaqMan qPCR. T-A DNA cloning was applied for further verification, and confirmed the results of the triplex probe assay. We speculated that this sample had a low allele fraction mutation, which was below the limits of detection (LOD) of Sanger sequencing.

In conclusion, we developed a closed-tube triplex probe TaqMan qPCR assay to define the two most common mutations of CALR exon 9. The limitation of the present assay is that it can only distinguish the two most common mutations from the wild-type background, the triplex probe TaqMan qPCR assay could become a routine diagnostic tool for detecting CALR indel mutations because of its rapidity, ease of use, economy, and high sensitivity. However, this procedure cannot fully replace existing CALR mutational analysis as cases testing negative using this novel procedure will still require analysis using an alternative technique that is still capable of detecting patients harboring rare CALR variants.

Disclosure statement

No potential conflict of interest was reported by the authors.

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