Leukemia Research 39 (2015) 273–278

Contents lists available at ScienceDirect

Leukemia Research

journal homepage: www.elsevier.com/locate/leukres

The impact of sensitive *KIT* D816V detection on recognition of Indolent Systemic Mastocytosis

Giovanna De Matteis[a,b,*], Roberta Zanotti[b,c], Sabrina Colarossi[d], Caterina De Benedittis[d], Andrès Garcia-Montero[e,f], Massimiliano Bonifacio[b,c], Marta Sartori[a], Fiorenza Aprili[a], Beatrice Caruso[a], Elisa Paviati[a], Giuseppe Carli[b,c], Omar Perbellini[b,c], Alberto Zamò[g], Patrizia Bonadonna[b,h], Giovanni Pizzolo[b], Giancesare Guidi[a], Giovanni Martinelli[d], Simona Soverini[d]

[a] *Section of Clinical Biochemistry, Department of Life and Reproduction Sciences, University of Verona, Verona, Italy*
[b] *Multidisciplinary Outpatients Clinics for Mastocytosis, Verona, Italy*
[c] *Section of Haematology, Department of Medicine, University of Verona, Verona, Italy*
[d] *Department of Haematology/Oncology, L. and A. Seragnoli, University of Bologna, Bologna, Italy*
[e] *Spanish Network on Mastocytosis (REMA), Toledo, Salamanca, Spain*
[f] *Centro de Investigacion del Cancer/IBMCC (USAL/CSIC) and IBSAL, Departamento de Medicina and Servicio General de Citometria, University of Salamanca, Salamanca, Spain*
[g] *Section of Pathological Anatomy, University of Verona, Verona, Italy*
[h] *Allergy Unit, Azienda Ospedaliera Universitaria Integrata, Verona, Italy*

ARTICLE INFO

Article history:
Received 14 August 2014
Received in revised form
24 November 2014
Accepted 29 November 2014
Available online 6 December 2014

Keywords:
Systemic mastocytosis
KIT
D816V
Real time PCR
D-HPLC
Sequencing

ABSTRACT

Patients with Systemic Mastocytosis (SM) need a highly sensitive diagnostic test for D816V detection of the *KIT* receptor gene.

Along with histology/cytology and flow cytometry evaluation, bone marrow (BM) from 110 consecutive adult patients referred with a suspicion of SM to Multidisciplinary Outpatient Clinic for Mastocytosis in Verona were tested both by Amplification Refractory Mutation System Reverse Transcriptase quantitative real time Polymerase Chain Reaction (ARMS-RT-qPCR) and RT-PCR + Restriction Fragment Length Polymorphism (RFLP) followed by Denaturing-High Performance Liquid Chromatography (D-HPLC) and Sanger sequencing.

ARMS-RT-qPCR identified D816V mutation in 77 patients, corresponding to 100% of cases showing $CD25^+$ mast cells (MCs) whereas RT-PCR + RFLP/D-HPLC + sequencing revealed D816V mutations in 47 patients.

According to the 2008 WHO criteria 75 SM, 1 Cutaneous Mastocytosis (CM), 1 monoclonal MC activation syndrome (MMAS), and 1 SM Associated with Haematologic Non-Mast Cell Disorder (SM-AHNMD) were diagnosed. Seventeen out 75 SM patients (23%) would have not satisfied sufficient WHO criteria on the basis of the sole RT-PCR + RFLP: these patients had significantly lower serum tryptase levels and amount of $CD25^+$ MCs.

Therefore, ARMS-RT-qPCR might result particularly useful, in patients that do not fulfil major BM histological criterion, for the recognition of indolent SM with a very low MC burden.

1. Introduction

Mastocytosis is a heterogeneous group of clonal diseases, with different clinical features and prognosis, characterized by proliferation and accumulation of mast cells (MCs) in different tissues, mainly in the skin and bone marrow [1]. Cutaneous Mastocytosis (CM) is the most frequent form in the childhood, while adults are almost invariably affected by systemic disease (SM), which is characterized by the involvement of at least one or more extra-cutaneous (EC) organs (bone marrow, gastrointestinal tract, lymph nodes and spleen), with or without skin lesions. The majority of cases of SM show a somatic 'autoactivating' point mutation [2,3] at codon 816 of *KIT* receptor gene; the most common mutation causes an adenine by thymine substitution at 2447 position leading

* Corresponding author at: Section of Clinical Biochemistry, Department of Life and Reproduction Sciences Policlinico G.B. Rossi, P.le L.A. Scuro, 10 Verona 37134 Italy. Tel.: +39 045 8124266; fax: +39 045 8027484.
E-mail addresses: giovanna.dematteis@ospedaleuniverona.it, gnndmt68@gmail.com (G. De Matteis).

http://dx.doi.org/10.1016/j.leukres.2014.11.029
0145-2126/

to D816V aminoacid substitution and auto-activation of KIT independently from stem cell factor binding [4]. This mutation is found in bone marrow (BM) MCs from more than 90% of adults with SM [3]. The same mutation has been also detected in skin lesions of 42% infant forms of mastocytosis [5]. Infant CM may be also associated to other *KIT* mutations (D816F, D816Y, V560L, V560G, del419D, K509I, M541L, E839K) [2,6,7].

According to the World Health Organization (WHO) classification, diagnosis of SM requires the presence of either the major and one minor criterion, or three minor criteria. The major criterion is represented by the histological finding of multifocal dense infiltrates of MCs in either BM or other EC organs. The minor criteria include the abnormal morphology of EC MCs, tryptase serum levels (sT) > 20 ng/mL, detection of a *KIT* mutations at codon 816 in BM or EC tissues, and the expression of CD2 and/or CD25 on BM MCs [2,8–11].

The term Monoclonal MC Activation Syndrome (MMAS) has been proposed to identify the subjects with unexplained or recurrent anaphylaxis without skin lesions and in the absence of the major criterion, but with documented MC clonality markers [12–15].

In indolent SM the neoplastic MC burden is frequently very low, particularly in those patients without skin involvement, in which normal MCs are prevailing [15,16] making sometimes difficult to analyze the mutational status of *KIT* on the aberrant $CD25^+$ MC population. In any case the specimens are characterized by a heterogeneous population of cells so the demonstration of *KIT* point mutations in mastocytosis patients may be a tricky issue [17]. Direct sequencing by Sanger method was generally considered as the 'gold standard' for sequence analysis [18] but it has remarkable limits of sensitiveness since a mutation must be present at least in a 10–20% of the alleles to be readily detected [19,20], and such a percentage is rarely found in the BM MCs of SM patients [21]. Recent guidelines [17] admit the use of Reverse-Transcriptase Polymerase Chain Reaction with Restriction Fragment Length Polymorphism (RT-PCR + RFLP) [22], peptide nucleic acid PCR (PNA-PCR) that identifies all codon 816 mutations [23] and allele-specific PCR [24,25,26].

We evaluated in patients with Systemic Mastocytosis the impact of mutation detection with allele-specific ARMS-RT-qPCR and with conventional RT-PCR + RFLP followed, in negative cases, by D-HPLC plus direct Sanger sequencing to exclude other mutations.

2. Material and methods

2.1. Patients

We tested BM samples from 110 consecutive adult patients referred with a suspicion for SM to our Multidisciplinary Outpatient Clinic for Mastocytosis in Verona from May 2009 to July 2011. Patients were referred for Urticaria Pigmentosa (25), episodes of anaphylaxis and persistent raised basal serum tryptase (67), unexplained mediator-related symptoms (11), haematological alteration (1), unexplained osteoporosis (6). All patients underwent BM evaluation with histology/cytology and flow cytometry, as described elsewhere [12]. An informed consent was obtained from all patients before the inclusion in the study. This study was approved by the local institutional review board of the Azienda Ospedaliera Universitaria Integrata of Verona.

2.2. Mutation analyses

2.2.1. Cell selection, RNA isolation and cDNA synthesis

Mononuclear cell (MNC) fraction was isolated from EDTA anticoagulated BM and PB samples, within 24 h after collection, by Ficoll/Hypaque density gradient centrifugation (Ficoll-Paque™ PLUS, Amersham Biosciences, Uppsala, Sweden). The HMC-1 mast cell line (kindly provided by Joseph Butterfield, Mayo Foundation for Medical Education and Research), which is heterozygous for the *KIT* D816V mutation, was used as positive control sample. Total RNA was extracted from BM, PB and HMC-1 cell line by Trizol Reagent (Invitrogen, Life Technology, UK) and quality and quantity were evaluated by gel electrophoresis and spectrophotometry. cDNA was synthesized from 1 μg of total RNA using M-MLV Reverse Transcriptase (Invitrogen, Life Technology, UK).

ARMS-RT-qPCR and PCR + RFLP/D-HPLC + sequencing were conducted in Verona and Bologna laboratories, respectively. ARMS-RT-qPCR in PB samples of 61/110 patients was also performed.

Table 1
Primer and probe sequence.

Primer name	Primer sequence
816armsWT	Ttgtgattttggtctagccagaca
816armsMUTmod	Tgattttggtctagccagact
816armsRE	Aaaaatcccataggaccagac
816asoPROBE	FAM-tggcacctgaaagcattttc-BQ1

2.2.2. KIT D816V mutation detection by ARMS-RT-qPCR

Mismatched forward primer for ARMS-RT-qPCR was designed to preferentially amplify the mutated D816V sequence which was detected by Real Time PCR using Taqman probe. To minimise amplification of the unwanted wild-type (WT) allele a mismatched forward primer for the mutated (MUT) allele was used. In addition, WT sequence was amplified as internal control to allow a RNA quality and quantity comparison between samples.

In the same laboratory of Verona two Real Time instruments were used sequentially.

First amplification reactions were developed on a Rotor Gene 2000 (Corbett Research, Australia) using Taqman Universal Master Mix (Fermentas, Germany) and final concentrations of 900 nM for each primer and 200 nM for the probe. Reactions were performed with the following thermal cycling protocol: an initial cycle of 50 °C for 2 min and 95 °C for 10 min followed by 50 cycles of 95 °C for 15 s and 60 °C for 1 min; all samples were analyzed as duplicate in two independent experiments. The method was validated by analyzing 6 BM samples positive at RT-PCR + RFLP D816V detection and other 75 consecutive BM samples of patients expected not to carry D816V because not fulfilling any other WHO minor criteria for SM and with a final diagnosis of anaphylaxis (29), mast cell activation syndrome MCAS (20), hypereosinophilic syndrome (4), unexplained osteoporosis (11), and chronic myeloproliferative neoplasm (2). The standard curves to establish the sensitivity and the cut-off for positivity were generated by serial 10-fold dilutions (from 10% to 0.0001%) of RNA from mutated HMC-1 cell line in a background of RNA pool obtained from PB MNCs of 5 healthy volunteers: these curves were included in every run. Taking into account that PB cells expresses a low amounts of KIT gene transcripts, we compared the PB MNCs background with a healthy donor BM MNCs background, where higher amounts of non-mutated KIT gene transcripts are likely to be present. For contamination checking of PCR products, in every run at least one RNA obtained from normal PB and one aliquot of water were included as No Amplification Control (NAC) and No Template Control (NTC), respectively. Primers and probe sequences are shown in Table 1.

Due to the availability of a new machine (ABI 7500 Real-Time PCR System, Applied Biosystems) in the same laboratory, ARMS-RT-qPCR from July 2010 (60 samples) was conducted with primers proposed by Lawley [24] (Table 2) but with the same WT primer used on Rotor Gene 2000 and 5 μl of 1:2.5 dilution of cDNA.

The sensitivity and the validation for false positives and contamination checking were conducted as for Rotor Gene 2000 method.