Human Pathology (2014) **45**, 573–582

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Human PATHOLOGY

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Original contribution

High-resolution melting analysis is a sensitive diagnostic tool to detect imatinib-resistant and imatinib-sensitive *PDGFRA* exon 18 mutations in gastrointestinal stromal tumors☆

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Received 10 June 2013; revised 10 October 2013; accepted 19 October 2013

Keywords:
Gastrointestinal stromal tumor;
GIST;
PDGFRA;
Imatinib;
High-resolution melting

Summary The mutational status of *KIT* and *PDGFRA* is highly relevant for prognosis and therapy prediction in gastrointestinal stromal tumors (GIST). *PDGFRA* exon 18 mutations have direct therapeutic implications since it is crucial to distinguish mutations associated with sensitivity to tyrosine kinase inhibitors from those causing primary resistance, eg, the most common exon 18 mutation p.D842V. In response to a growing demand for reliable, faster and more sensitive methods we established and validated a high-resolution melting (HRM) assay for *PDGFRA* exon 18. A total of 159 GIST samples were comparatively analyzed by HRM and direct Sanger sequencing. We demonstrate that HRM provides highly reliable mutational results with higher sensitivity and shorter time to diagnosis compared to Sanger sequencing. We determined the sensitivity threshold of HRM at 6% of mutated alleles. *PDGFRA* exon 18 wild-type status and the most common p.D842V resistance mutation (together representing >90% of the cases) can be detected specifically by HRM. Other rare mutations can be pre-screened by HRM and afterwards determined precisely by DNA sequencing. In this way we detected four novel mutations in *PDGFRA* exon 18, two of which were associated with an aggressive clinical course. Including these new mutations, we provide a comprehensive overview of all 60 currently known subtypes of *PDGFRA* exon 18 mutations in GIST. Seven of them (accounting for about 75% of all exon 18–mutated GISTs) are reported to be resistant to imatinib. However, there are at least 10 other mutations which are regarded as sensitive to tyrosine kinase inhibitors.

☆ Disclosures: This study was supported by a research grant from Novartis Oncology to H.U.S. E.W., S.M.B. and H.U.S. received research grants and honoraria from Novartis Oncology (out of this study). E.W. received honoraria and reimbursements from Qiagen, Menarini, and M.S.D. (out of this study). H.U.S. has received honoraria and reimbursements from Pfizer Oncology, Roche, Novartis Oncology, Abbott Molecular and Zytomed Systems (out of this study). R.B. has received honoraria from and serves on advisory boards for Novartis Oncology, Roche, Boehringer Ingelheim, Pfizer, Lilly, Merck and Qiagen (out of this study).

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0046-8177/$ – see front matter
http://dx.doi.org/10.1016/j.humpath.2013.10.025

1. Introduction

Gastrointestinal stromal tumors (GIST) harbor activating mutations in *KIT* or *PDGFRA* (platelet-derived growth factor receptor alpha) in more than 85% of all cases [1]. Mutational status has become a well-established prognostic and predictive factor and current guidelines recommend mutational analysis of *KIT* and *PDGFRA* as standard practice in the diagnostic processing of GIST samples at least in tumors with intermediate or high risk of recurrence [2–4]. Especially in metastatic GIST or in locally advanced tumors it is essential to obtain the result of mutational testing rapidly after diagnosis in order to initiate appropriate drug treatment immediately.

Nearly 16% of GIST carry a mutation in the *PDGFRA* gene, and these mutations are associated with gastric primary site and epithelioid or mixed cell morphology [5–7]. The detection of mutations in exon 18 of *PDGFRA* is highly relevant because several mutational subtypes such as p.D842V are associated with primary resistance to imatinib which is the gold standard in first-line treatment of GIST [8,9]. However, the mutational spectrum of exon 18 also comprises other less frequent point mutations, several deletions and complex deletion/insertion mutations which differ in their sensitivity to imatinib. It is therefore important to distinguish as quickly as possible between resistant and sensitive mutations. At present, mutational testing of GIST is routinely carried out by Sanger sequencing, a technique that is the method of choice for highly variable DNA regions like exon 11 of *KIT* [10]. However, as Sanger sequencing is very labor-intensive and time consuming as well as rather expensive, we aimed at developing an alternative method for fast and cost-efficient mutational analysis of *PDGFRA* exon 18.

In high-resolution melting (HRM) analysis the different melting curves of homo- and heteroduplexes from wild-type and mutated DNA are detected and can be discriminated from each other. Therefore, that method can serve as a post–polymerase chain reaction (PCR) mutation screening tool.

In this study we established and validated a robust HRM assay for *PDGFRA* exon 18 with special regard to high sensitivity and the capability to discriminate between imatinib-sensitive and imatinib-resistant mutations. Furthermore, we describe four GIST cases with novel mutations and provide a comprehensive list of all known *PDGFRA* exon 18 mutations in GIST. Finally, we discuss the therapeutic consequences of different mutational subtypes with special attention to tyrosine kinase inhibitor treatment.

2. Materials and methods

2.1. Cases and immunohistochemistry

A total of 159 GIST samples were selected from our consultation files. On the basis of their known *PDGFRA* status 101 cases were selected, retrospectively; 58 lesions were randomly selected and analyzed prospectively, ie, in parallel to Sanger sequencing. Mutated cases were enriched to nearly 50% in the cohort to ensure validation (see below). Immunohistochemistry was carried out in all cases as described earlier [7,11,12].

2.2. High-resolution melting analysis

After DNA extraction, HRM analyses were performed using the LightCycler 480 platform (Roche Diagnostics, Mannheim, Germany) as described earlier [12]. Each run included *PDGFRA*-mutated (p.D842V) and wild-type DNA as controls. Ten nanograms of DNA were amplified with specific primers for the relevant region of *PDGFRA* exon 18 (For 5′-GCACAAGGAAAAATTGTGAAGAT-3′, Rev 5′-AGGGAAGTGAGGACGTACACTG-3′). Different primer pairs and PCR conditions were tested. Optimal results were achieved with the mentioned primer pair and an annealing temperature of 60°C. The expected size of wild-type PCR product was 102 bp. All samples were tested in duplicate.

After normalization and temperature-shifting, melting curves in samples with mutated DNA differed clearly from those of wild-type DNA. Additionally, different types of mutations could be distinguished based on the course of the corresponding melting curves (Fig. 1). Differential plots of HRM assays were easy to interpret if negative, and positive p.D842V controls were used in each run. The evaluation with our method is further facilitated by the specific primer design which excludes the common p.V824V polymorphism (SNP rs2228230) from the amplicon.

In order to determine the detection limit of the HRM assay we performed a dilution series with a sample carrying a defined proportion of mutated alleles (35% p.D842V) diluted with increasing amounts of a wild-type DNA sample. We used pyrosequencing for the detection of the allele frequency of the DNA stocks. A frequency of 6% mutated alleles was reliably detectable by HRM analysis (Fig. 2).

The HRM assay was validated by screening of a total of 159 GIST samples. We analyzed 101 cases retrospectively with prior Sanger sequencing, among them four cases which were not evaluable by Sanger sequencing. A further 58 samples were tested prospectively in parallel to Sanger sequencing. Mutated samples were enriched in the retrospective group resulting in nearly 50% of the cases with exon 18 mutations in the entire cohort.

2.3. Sanger sequencing

PDGFRA exon 18 DNA was amplified by PCR with specific primers (For 5′-CAGCTACAGATGGCTTGATC-3′, Rev 5′-GAAGGAGGATGAGCCTGAC-3′) and an annealing temperature of 60°C. PCR products were checked for the expected fragment length of 213 bp and were purified using Exonuclease I and Fast-AP (Thermo Fisher Scientific,