Unlabeled-probe high-resolution melting to detect KRAS codon 12 and 13 mutations in pancreatic adenocarcinoma tissues

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Abstract

Background: The aim of our study was to establish an unlabeled-probe high-resolution melting (HRM) approach to the detection of Kirsten Ras (KRAS) codon 12 and 13 mutations in pancreatic adenocarcinoma (PA) tissues as a novel and effective diagnostic technique.

Methods: We tested the sensitivity and specificity of this genotyping approach in cell lines with known KRAS mutations using 166 bp amplicons and 37 bp wild-type probe to detect KRAS codon 12 and 13 mutations. We screened 49 PA tissues to be subsequently sequenced to confirm the mutations. Simultaneously, we tested the specimens using Sanger sequencing and then used target-DNA cloning and sequencing for verification.

Results: It was found that unlabeled-probe HRM was reliable in detecting 3% of mutant cell lines DNA diluted with that of the wild-type, whereas Sanger sequencing could only discriminate 20% mutant cell ratios. In detecting 49 specimens, the former was capable of detecting 23 mutations (46.9%); and the latter could observe 15 (30.6%). For further verification, T-A DNA cloning and sequencing was applied to the differences, with the results matching those of the unlabeled-probe HRM.

Conclusions: It was concluded that the unlabeled-probe HRM approach can be a sensitive and accurate screening technique to detect KRAS codon 12 and 13 mutations in diagnosing and treating PA.

Keywords: high-resolution melting (HRM); KRAS gene mutations; pancreatic adenocarcinoma (PA); unlabeled-probe.

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Received September 1, 2011; accepted January 1, 2012; previously published online January 31, 2012

Introduction

Pancreatic adenocarcinoma (PA), one of the most common alimentary system cancers, produces a high rate of malignancy and poor prognosis, the incidence rate of 8th and 7th (1) for males and females, respectively, and presents a growing trend in the world. KRAS gene mutations have been reported to be one of the early molecular changes in PA, the most common mutations found in codons 12 and 13 (Figure 1) (2–4), but rarely in codons 59 and 61 (5). The mutations alter the conformation of KRAS, causing impaired GTPase activity resulting in constitutive activation of the ras protein and tumor cell proliferation and protection against apoptosis, and increased invasion and metastasis (6, 7).

Except for PA, KRAS mutations are also a predictive and prognostic marker in other tumor types (8). Patients with KRAS gene mutations are correlated with poor prognosis (9). A review of the literature has indicated that KRAS, but not p53, mutations are associated with poorer overall survival in colorectal cancer patients, especially in PA (10). In a recent study of metastatic colorectal cancer treated with the monoclonal antibody cetuximab, patients with KRAS mutations were resistant to the drug, with a poorer prognosis compared with those without mutations. Therefore, an accurate detection of KRAS mutations is vital to the molecular diagnosis of PA on the part of physicians.

Various approaches have been described for the detection of KRAS gene mutations, such as restriction fragment length polymorphism polymerase chain reaction (PCR-RFLP) (11), mutagenic PCR analysis (12), pyrosequencing (13), real-time PCR (14) and Sanger sequencing. In particular, Sanger sequencing has been well recognized as the gold standard for detecting gene mutations for its accuracy (15). However, Sanger sequencing can only detect mutations at a ratio as low as 20% due to its limited insensitivity (16); moreover, it is expensive and time-consuming (17), hence considered impractical in clinical settings.

High-resolution melting (HRM) was reported to be a novel technique developed in 2003 (18). Since then, HRM has been widely recognized and employed for its high sensitivity, high throughput and easy operation, and used to detect KRAS gene mutations (19). And for the first time, asymmetric PCR with unlabeled-probe melting curve was applied to the detection of cystic fibrosis mutations (20). In the current study, an unlabeled-probe HRM technique, an integration of the previous approaches, was developed for detecting KRAS codon 12 and 13 mutations in PA tissues, thereby expanding the scope of HRM application in the clinic (21).
Materials and methods

Specimens and cell lines

We obtained PA tissues (29 formalin-fixed tissues and 20 paraffin-embedded tissues) from 49 patients under surgery, 27 males and 22 females aged 35 to 80, from December 2008 to December 2010 at Zhongshan Hospital of Fudan University, and made 36 pathological diagnoses of duct adenocarcinoma, four of adenosquamous carcinoma, three of poorly differentiated adenocarcinoma, three of endocrine tumor, two of pseudopapilloma, and one of serous cystic carcinoma.

KRAS wild-type cell lines (SW480 colon carcinoma cells) and the KRAS mutant cell lines (AsPC-1 metastatic pancreatic carcinoma cells) were obtained from the Cell Bank of the Chinese Academy of Sciences.

Extraction of tissues DNA

The formalin-fixed tissues were washed away with PBS and the paraffin-embedded tissues were dewaxed with xylene and dehydrated with alcohol to be extracted for DNA with a QIAamp DNA Mini Kit based on the instructions (Qiagen Corp., Hilden, Germany). A colorimetric assay was performed to determine the concentration and purity (A260/A280 ratio) of the DNA using an ultraviolet spectrometer (U-3010, HITACHI, Japan), its concentration adjusted to 20 ng/mL and stored at –20°C.

Unlabeled-probe HRM analysis

The primers for the amplification of KRAS codons 12 and 13 were designed with Primer3 software (http://frodo.wi.mit.edu/primer3), the sequences for KRAS amplimer as follows: forward-CTGAAATAAAACCTTGTGATTGGA and reverse-TATCGTCAAGGCACTCTTGC (166 bp); the sequences for each of the following: forward-AAAAGGTACTGGTGGAGTATTTGA and reverse-TGAAACCCAAGGTACATTTCAG.

PCR was performed in a 20 μL volume containing TaKaRa Taq (5 U/μL) 0.1 μL (TaKaRa, Dalian, China), 10×PCR Buffer (Mg2+ Plus) 2 μL, dNTP Mixture (2.5 mM) 1.6 μL, 10 μmol/L forward primer (1:20 dilutions) 1 μL, 10 μmol/L reverse primer 1 μL, probe 1 μL, SYTO 9 fluorochrome 0.6 μL, PCR grade water and DNA templates. KRAS wild-type and KRAS mutation-type cell lines as DNA templates were used, respectively.

The PCR and HRM was run according under the following conditions: one cycle of 95°C for 10 min (pre-denaturation), 55 cycles of 95°C for 10 s, 56°C for 15 s, 72°C for 25 s (amplification), one cycle of 95°C for 2 min, 40°C for 2 min and a melt from 65°C to 85°C rising at 0.2°C/s. PCR and HRM analysis was performed on the Rotor-Gene Q PCR Amplification Instrument (Qiagen Corp.).

Sensitivity of unlabeled-probe HRM

A KRAS wild-type cell lines were mixed in various ratios with the KRAS mutant cell lines to obtain dilutions of 0%, 3%, 8%, 20%, 50%, and 100%, respectively, and all the dilutions were tested after DNA extraction.

Repeatability of unlabeled-probe HRM

KRAS wild-type cell lines were mixed in random ratios with the KRAS mutant ones to obtain a mixture. Following DNA extraction, the same procedures were employed to examine the same DNA specimen 20 times in one run to calculate the within-run coefficient of variation (CV) and average, and then repeated in 20 runs to calculate the between-run CV and average.

Examinations of mutations

The same procedures were applied to the examining of KRAS codon 12 and 13 mutations in DNA of 49 PA tissues.

Sanger sequencing

Upon HRM analysis, all the DNA of 49 PA tissues were applied to sequence (Sangon, Shanghai, China), the sequences of the primers as follows: forward-AAAAGGTACTGGTGGAGTATTTGA and reverse-TGAAACCCAAGGTACATTTCAG.

Target DNA cloning and sequencing

The amplified DNA was purified through Gel Extraction Kits before combined with pMD19-T vector (TaKaRa), transformed into Escherichia coli DH5α competent cells (Tiangen Biotech Corp., Beijing, China), and spread on culture medium containing Isopropyl β-D-1-thiogalactopyranoside (IPTG), 5-Bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) and ampicillin Lysogeny Broth (AMP-LB) (HORA Biological Technology Co., Ltd, Shanghai, China), to be cultured at 37°C overnight. The monoclonal colonies collected were cultured in the AMP-LB liquid medium so that the plasmid DNA was extracted via a corresponding kit (Bio Basic, Toronto, ON, Canada) before sequencing.

Statistical analysis

All statistical analyses were performed using Stata 7.0 software and Microsoft Excel. The difference between the melting temperatures (Tm) of wild-type and mutant cell lines was used paired t-test. The difference of the positive ratios in detecting mutations between unlabeled-probe HRM and Sanger Sequencing was examined using...
the κ-test. The correlation between KRAS mutation ratio and pancreatic tumor pathological type analyzed was examined using χ²-test. p<0.05 was considered statistically significant.

Results

Sensitivity of unlabeled-probe HRM

The unlabeled-probe HRM was found to have detected 3% of the mutations, however only 20% were detected using Sanger sequencing in the DNA of the mutant cell lines diluted with that of the wild-type (Figures 2 and 3).

Repeatability of unlabeled-probe HRM

A significant difference between Tm of wild-type and that of mutant cell lines (t=54.63, 43.7; p<0.05, 0.05, respectively) were observed in the within-run CV and average and the between-run CV and average (Table 1).

Unlabeled-probe HRM analysis

The amplified DNA of KRAS wild-type cell lines were found to have matched the probe completely, which formed a peak at higher Tm (76.23°C±0.42°C), while that of KRAS mutant ones failed to do so, which produced a peak at lower Tm (73.45°C±0.56°C), the combination of both presenting double peaks (Figure 4).

Unlabeled-probe HRM to detect DNA

Out of 49 pancreatic cancerous tissues 23 KRAS mutations (46.9%) were observed (Table 2). Compared with duct adenocarcinoma, the p-value of adenosquamous carcinoma, poorly differentiated adenocarcinoma, endocrine tumor, pseudopapilloma, serous cystic carcinoma was 0.613, 0.615, 0.231, 0.486 and 0.486, respectively. Statistically, there was no significant correlation between KRAS gene mutations and pathological diagnoses (Table 3).

Sanger sequencing to detect DNA

Fifteen KRAS mutations (30.6%) were observed in 49 pancreatic cancerous tissues (Table 2). The difference was found to be significant between unlabeled-probe HRM and Sanger sequencing in terms of capability (κ=0.668, p<0.05) (Table 4).

Target DNA cloning and sequencing for verification

The results, as indicated by the verification by the target DNA cloning and sequencing, were found to be consistent with those of the unlabeled-probe HRM (Table 2).

Discussion

The ras family genes were originally identified as oncogenes in acutely transforming retroviruses (19). Three highly homologous ras proteins are encoded by the KRAS, HRAS and NRAS genes. A high frequency of ras mutations has been found in many tumor types; approximately 30% of all human cancers harbor a mutation in a ras gene with mutations most frequently occurring in KRAS.

KRAS gene mutation, capable of being detected in pancreatic intraepithelial neoplasia (PanIN), is well known as one of the most important causes of inducing the PA (2); and there is an association between KRAS gene status and the therapeutic effect of epidermal growth factor receptor (EGFR) inhibitors (22). Thus, its detection could be of clinical importance to the clinician in diagnosing and treating PA.

Figure 2  Sensitivity of unlabeled-probe HRM.
The melt curve, firstly carried out in 1977 (23, 24), was used to examine mutations by observing through a real-time monitor fluorochromes dye intercalated into the double-stranded DNA during the warming-up period. The existence of a point of mutation can be determined when the fluorescence signal and time curve are analyzed.

The novel saturating fluorochromes and HRM instrument, developed in 2003, are specifically applied to HRM analysis, thereby greatly expanding the application of the melting curve technique.

HRM, one of the mutation screening techniques, is used to detect the presence of genetic mutations, but incapable of verifying mutating sites. In addition, HRM can be affected by such factors as sequence length and mutation type, and sometimes, HRM is incapable of detecting homozygous mutations with a small difference in Tm. Therefore, HRM is characterized by poor sensitivity and specificity, even though the technique helps determine KRAS mutations through the analysis of melting peaks.

The unlabeled-probe HRM technique was developed as a novel approach by integrating HRM with asymmetric PCR using an unlabeled fluorochromes probe and known mutation sequence, which facilitated us to detect mutation sites without being affected by sequence length and mutation type.

In our study, we designed an unlabeled-wild probe to detect KRAS gene codon 12, 13 mutations in PA tissues. The within-run CV and between-run CV of the mutation-peak’s Tm and that of the wild-peak were found to be <0.5%, SD <0.30°C. Their peaks were observed at 73.3°C and 76.2°C, respectively, and the difference was about 3°C, so significant that it was easy to distinguish mutant-peaks from wild-peaks via the HRM curve, suggesting its reliability for clinical gene mutation detection.

The KRAS mutant cell lines dilutions to evaluate the sensitivity of unlabeled-probe HRM were found to be reliable in detecting even 3% of mutant cell line DNA diluted with that of the wild-type, whereas Sanger sequencing known as the acknowledged gold standard for detecting gene mutation can only discriminate 20% of the mutant cells, the comparison indicating the high sensitivity of our unlabeled-probe HRM.

Our technique analyzed the mutations using the high-resolution melting curve. The sense-strand in asymmetric PCR was found to be capable of matching the KRAS wild-type probe, suggesting that the DNA strand was of wild-type. At the point of mutation, the unmatched bases made it possible for the double-stranded DNA to melt earlier during the warming-up period and for fluorochromes dye to be released from the DNA molecule. The change of the fluorescence signal facilitated the determination of the existence of mutation (20).

In our study, we adjusted the proportion of primer to run asymmetric PCR. In the asymmetric PCR system, only the

Table 1 Repeatability of unlabeled-probe HRM (n=20).

<table>
<thead>
<tr>
<th></th>
<th>Within-run</th>
<th></th>
<th></th>
<th>Between-run</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>x±s, °C</td>
<td>CV, %</td>
<td></td>
<td>x±s, °C</td>
<td>CV, %</td>
</tr>
<tr>
<td>Mutation peak Tm</td>
<td>73.33±0.19a</td>
<td>0.25</td>
<td></td>
<td>73.45±0.28b</td>
<td>0.39</td>
</tr>
<tr>
<td>Wild-peak Tm</td>
<td>76.18±0.19a</td>
<td>0.25</td>
<td></td>
<td>76.23±0.21b</td>
<td>0.28</td>
</tr>
<tr>
<td>Main peak Tm</td>
<td>80.91±0.09a</td>
<td>0.11</td>
<td></td>
<td>81.17±0.21</td>
<td>0.26</td>
</tr>
</tbody>
</table>

*Paired t-test. The difference of within-run between Tm of wild-type and that of mutant cell lines was significant, t=54.63, p<0.05. *Paired t-test. The difference of between-run between Tm of wild-type and that of mutant cell lines was significant, t=43.7, p<0.05. s, standard deviation.
reverse primer remained when the forward primer had been exhausted, hence more sense-strand, which was then combined with the unlabeled-probe. During the heating process, the strand in combination with the unlabeled-probe began to melt at a lower temperature, forming a melting peak. Since the unlabeled-probe was limited to the length of 40 bp, it was easier to distinguish the differences between the Tm of the mutation-peak and that of the wild-peak.

In the reaction system, there were two different products (Figure 3), 166 bp amplimer and 37 bp probe combined with the strand. Therefore, two melting peaks, main-peak and probe-peak, were developed in the HRM analysis. Since its Tm was lower than that of 166 bp amplimer, the combined probe tended to melt earlier, forming a probe-peak. The wild-type, likely to match the probe firmly, formed a probe-peak at a higher Tm; homozygous mutants, whose Tm was lower than that of the wild-type, formed a peak to its left side; and heterozygous mutants, whose trends could be affected by the proportion of mutations, produced double probe-peaks. Therefore, we can confirm the existence of the mutations based on the melting curve.

Of the 49 PA tissues, the unlabeled-probe HRM was capable of detecting 23 mutations (46.9%), while the Sanger sequencing could detect only 12 (30.6%). For further verification, T-A DNA cloning and sequencing was applied to the differences, with the results matching those of the unlabeled-probe HRM. Compared with the former, the latter was characterized by quick examination (within 2 h), test by batch, easy operation, accuracy and sensitivity.

Table 2 Unlabeled-probe HRM and Sanger-based sequence examining DNA of 49 pancreatic carcinoma tissues.

<table>
<thead>
<tr>
<th>Specimen number</th>
<th>Unlabeled-probe HRM</th>
<th>Sanger-based sequence</th>
<th>TA-DNA clone and sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>Mutation</td>
<td>35G&gt;A</td>
<td>–</td>
</tr>
<tr>
<td>4</td>
<td>Mutation</td>
<td>35G&gt;T</td>
<td>–</td>
</tr>
<tr>
<td>2</td>
<td>Mutation</td>
<td>34G&gt;C</td>
<td>–</td>
</tr>
<tr>
<td>3</td>
<td>Mutation</td>
<td>Wild</td>
<td>35G&gt;A</td>
</tr>
<tr>
<td>4</td>
<td>Mutation</td>
<td>Wild</td>
<td>34G&gt;C</td>
</tr>
<tr>
<td>1</td>
<td>Mutation</td>
<td>Wild</td>
<td>35G&gt;T</td>
</tr>
<tr>
<td>26</td>
<td>Wild</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A total of 49 pancreatic adenocarcinoma tissue samples were examined via both unlabeled-probe HRM and Sanger sequencing, T-A DNA cloning and sequencing used to verify the different results of the two methods.

Table 3 Relation of mutation ratio between unlabeled-probe HRM and pathological diagnoses.

<table>
<thead>
<tr>
<th>Pathological diagnoses</th>
<th>KRAS, n</th>
<th>Mutation ratio, %</th>
<th>p-Value</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mutation</td>
<td>Wild</td>
</tr>
<tr>
<td>Duct adenocarcinoma</td>
<td>19</td>
<td>17</td>
<td>52.8</td>
</tr>
<tr>
<td>Adenosquamous carcinoma</td>
<td>3</td>
<td>1</td>
<td>75</td>
</tr>
<tr>
<td>Poorly differentiated adenocarcinoma</td>
<td>1</td>
<td>2</td>
<td>33.3</td>
</tr>
<tr>
<td>Endocrine tumor</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Pseudopapilloma</td>
<td>0</td>
<td>2</td>
<td>0</td>
</tr>
<tr>
<td>Serous cystic carcinoma</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

*Compared with duct adenocarcinoma.
The present study had limitations. There were not enough PA tissue specimens to develop a correlation between histological type and KRAS mutation. This may have resulted in no significant correlation between KRAS gene mutation and histological type of PA in our study.

In conclusion, our findings confirmed that the unlabeled-probe HRM approach could increase the sensitivity of KRAS codon 12, 13 mutations detection in cancerous tissues. With this improvement, the combination of unlabeled-probe and HRM could become an important routine screening tool for mutations at very low abundance because of its speed, ease of use, and low cost. Thus, this holds promise as an effective approach to diagnosing cancers such as colorectal carcinomas and lung adenocarcinomas. We believe that the novel approach can lay a basis for the future research on the screening of peripheral blood KRAS codon 12, 13 mutations.

**Conflict of interest statement**

**Authors’ conflict of interest disclosure:** The authors stated that there are no conflicts of interest regarding the publication of this article. Research funding played no role in the study design; in the collection, analysis, and interpretation of data; in the writing of the report; or in the decision to submit the report for publication.

**Research funding:** The study was supported by the fund of Shanghai Municipal Public Health Bureau (2008136).

**Employment or leadership:** None declared.

**Honorarium:** None declared.

**References**


**Table 4**

<table>
<thead>
<tr>
<th></th>
<th>Sanger sequencing (Mutation)</th>
<th>Sanger sequencing (Wild)</th>
<th>Total</th>
</tr>
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<tbody>
<tr>
<td>Unlabeled-probe HRM (Mutation)</td>
<td>15</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td>Unlabeled-probe HRM (Wild)</td>
<td>0</td>
<td>26</td>
<td>26</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>34</td>
<td>49</td>
</tr>
<tr>
<td>k-test</td>
<td>0.668, p&lt;0.05</td>
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