RESEARCH ARTICLE

Co-amplification at Lower Denaturation-temperature PCR Combined with Unlabled-probe High-resolution Melting to Detect *KRAS* Codon 12 and 13 Mutations in Plasma-circulating DNA of Pancreatic Adenocarcinoma Cases

Jiong Wu[1&], Yan Zhou[1&], Chun-Yan Zhang[1], Bin-Bin Song[1], Bei-Li Wang[1], Bai-Shen Pan[1], Wen-Hui Lou[2], Wei Guo[1*]

Abstract

Background: The aim of our study was to establish COLD-PCR combined with an unlabeled-probe HRM approach for detecting *KRAS* codon 12 and 13 mutations in plasma-circulating DNA of pancreatic adenocarcinoma (PA) cases as a novel and effective diagnostic technique. Materials and Methods: We tested the sensitivity and specificity of this approach with dilutions of known mutated cell lines. We screened 36 plasma-circulating DNA samples, 24 from the disease control group and 25 of a healthy group, to be subsequently sequenced to confirm mutations. Simultaneously, we tested the specimens using conventional PCR followed by HRM and then used target-DNA cloning and sequencing for verification. The ROC and respective AUC were calculated for *KRAS* mutations and/or serum CA 19-9. Results: It was found that the sensitivity of Sanger reached 0.5% with COLD-PCR, whereas that obtained after conventional PCR did 20%; that of COLD-PCR based on unlabeled-probe HRM, 0.1%. *KRAS* mutations were identified in 26 of 36 PA cases (72.2%), while none were detected in the disease control and/or healthy group. *KRAS* mutations were identified both in 26 PA tissues and plasma samples. The AUC of COLD-PCR based unlabeled probe HRM turned out to be 0.861, which when combined with CA 19-9 increased to 0.934. Conclusions: It was concluded that COLD-PCR with unlabeled-probe HRM can be a sensitive and accurate screening technique to detect *KRAS* codon 12 and 13 mutations in plasma-circulating DNA for diagnosing and treating PA.

Keywords: Plasma-circulating DNA - panreatic adenocarcinoma - *KRAS* gene - unlabled-probe - high-resolution melting - mutations

Asian Pac J Cancer Prev, **15 (24)**, 10647-10652

Introduction

Carcinoma of the pancreas continues to be a major and depressingly difficult cancer problem, currently rating the eighth most commom cancer and the fifth most frequent cause of cancer death (Pitman et al., 2010), with the median length of survival 3.3 months, and a one-year overall surival rate of 11.9% (Robert et al., 1981). Generally, it requires a high level of clinical suspicion to ensure an early diagnosis of Panreatic adenocarcinoma (PA) (Gary et al., 2005).

There is a real need for tumor markers in the early detection of PA, and their additional benefits may include early detection of recurrence, monitoring of therapy, avoidance of unnecessary surgery, improvement of staging and critical prognostication (Malfertheiner et al., 2008). Unfortunately, there have been no well-defined specific ones for the early detection of PA (Antonio and Manuel, 2006).

A wide array of tumor-associated antigens has been evaluated as the markers for screening and diagnosing PA (Malfertheiner et al., 2008). The detection of serum levels of Carbohydrate antigen, such as CA 19-9, CA 50, CA 242 and CA 494 has been evaluated for their utility in diagnosing PA. The level of CA 19-9 is the most useful in clinical practice, especially when combined with the level for CA 242, which improves overall specificity (Lokshin et al., 2011). However, CA 19-9 has not been proven to be of clinical use in confirming the diagnosis of PA for its low sensitivity and cross reactivity with other tumors (Habermann et al., 2011).

KRAS, a member of the *RAS* oncogene family, plays a key role in *RAS*/*MAPK* signaling, which is involved in multiple cellular processes, including proliferation, differentiation and apoptosis (Karnoub et al., 2008; Huang C et al., 2013). The human tumors harbor an activating mutation in *KRAS* by over 30%, mainly of lung, colon, pancreatic cancers, and hematopoietic neoplasms (Eijk et

*[1]Department of Laboratory Medicine, [2]Surgical Department, Zhongshan Hospital, Fudan University, Shanghai, China [&]Equal contributors *For correspondence: guo.wei@zs-hospital.sh.cn*

al., 2010). In the era of targeted therapy, *KRAS* mutations can be highly predictive of the patient's response to antiepidermal growth factor receptor (EGFR) antibody such as cetuximab and panitumumab (Massarelli et al., 2007; Di Fiore et al., 2007). These therapies are exclusively effective in tumors with wild-type KRAS. Therefore, screening tumors for possible *KRAS* mutations has been gaining increasing attention because of its multiple clinical applications.

In our study, we developed a new approach to detecting *KRAS* mutations and in the meanwhile, combined CA 19-9 detection with *KRAS* mutations, as an early diagnosis PA for better treatment and prognosis.

Traditionally, the detection of *KRAS* mutations is performed on DNA extracted from tumors tissues. However, the sampling of these tissues and the methods used are inaccurate, time consuming, and cannot be used for easy routine screening to determine disease recurrence and response to treatment.

Recent reports have demonstrated that it can be detected in plasma-circulating DNA of patients with advanced tumors, such as PA and NSCLC, and it is originated from either the primary tumor or metastatic sites gaining access to the peripheral circulation through normal neighboring vessels, or via newly formed capillaries by tumor-induced angiogenesis (Laurent-Puig et al., 2002). Further data indicate that it may function as biomarkers of diagnosis, prognosis and as surrogate markers of therapeutic efficacy in general as well as specifically for molecularly targeted agents (Dawood et al., 2010). Most of the growing evidence indicates that detecting *KRAS* mutations in the plasma-circulating DNA may provide useful information to the clinician in diagnosing and treating PA, NSCLC and colorectal carcinomas (Maheswaran et al., 2008; Shyamala et al., 2008; Dawood et al., 2010).

Various methods have been described for the detection of *KRAS* gene mutations, such as Restriction Fragment Length Polymorphism polymerase chain reaction (PCR-RELF) (CHEN et al., 2004), mutagenic PCR analysis (Boldrini et al., 2004), pyrosequencing (Ogino et al., 2004), real-time PCR (Amicarelli et al., 2007) and Sanger sequencing. Currently, the gold standard for *KRAS* mutations detection is conventional PCR amplification followed by direct sequencing (Sanger sequencing) on the primer of downstream. However, Sanger sequencing can only detect mutations whose ratio is as low as 20% thanks to its limited sensitivity (Wittwer et al., 2002); moreover, it is expensive and time-consuming and therefore considered impractical in the standard clinical setting.

In the current study, co-amplification-at-lower denaturation-temperature PCR (COLD-PCR) was employed as a novel modification of the conventional PCR method so as to go beyond the limitations currently encountered in detecting mutations (Li et al., 2008). COLD-PCR, combined with unlabeled-probe HRM, an integration of our previous approaches, was thus developed for detecting *KRAS* codon 12 and 13 mutations in plasma-circulating DNA of PA, thereby expanding the scope of the modular diagnosis application in the clinic (Wei et al., 2012).

Materials and Methods

Cell lines and specimens

KRAS wild-type cell lines (G2 Hepatocellular carcinoma cells) and *KRAS* mutant cell lines (AsPC-1 metastatic pancreatic carcinoma cells) were obtained from the Cell Bank of the Chinese Academy of Sciences.

Thirty-six pancreatic adenocarcinoma tissues and plasma specimens were obtained as the disease group, and 24 gastrointestinal tissues and plasma, as the disease control group from January to October 2009 at Zhongshan Hospital of Fudan University, during which their plasma specimens were collected, fractionated into several vials and stored at -70°C. And as the healthy control group, 25 healthy plasma specimens were collected via the same procedures in accordance with the approval of internal Ethics Review Board.

Extraction of DNA

The genomic DNA was extracted from the tissues via DNA Mini Kit (Qiagen, Germany) based on the instructions (Qiagen Corp, Hilden, Germany). The plasma-circulating DNA was extracted from the plasma specimens via QIAamp Circulating Nucleic Acid Kit (Qiagen, Germany).

Detection of CA-199

Commercially available kits (Roche, Germany) were employed to test serum specimens for CA-199 by electrochemiluminescence irnmunoassay (ECLIA) on the E170 P-Modular (Roche, Germany).

Unlabled-probe High-resolution Melting analysis

The general approach for the detection of *KRAS* mutation in our previous research was to use unlabled-probe PCR followed by HRM. The sequences for *KRAS* amplimer were as follows: forward 5`-GCCTGCTGAAAATGACTGAA-3 and reserve 5`-GAATGG TCCTGCACCAGTAA-3` (166bp); the sequences for wild-type probe to span the *KRAS* codons 12 and 13 as follows: 5`-AACTTGTGGTAGTTGGA GCTGGT GGCGTAGGCAAGAG-3` (37bp,3`-blocked).

The PCR was performed in a 20-μl reaction volumes containing 2×Premix Taq [1.25U/25μl TaKaRa Taq HS, 0.4mM dNTP mixture, 3mM Mg2+ (TaKaRa, China)], 10μM forward primer (1:15 dilutions), reverse primers and unlabeled probe (sangon, China) 0.5μl, fluorescent dye SYTO-9 (Invitrogen, CA) 0.6μl on the Mastercycler Gradient (Eppendorf, Germany).

The unlabled-probe PCR and HRM was run as follows: one cycle of 95°C for 10 min (pre-denaturation), 55 cycles of 95°C for 10 sec, 56°C for 15 sec, 72°C for 25 sec (amplification), one cycle of 95°C for 2 min, 40°C for 2 min and a melt from 65 to 85°C rising at 0.2°C per sec. Their analysis was performed on the Rotor-Gene Q PCR Amplification Instrument (Qiagen Corp. Hilden, Germany).

COLD-PCR analysis

COLD-PCR is based on the observations for critical denaturation temperature (Tc) of each DNA sequence. Tc,

Table 1. Conventional PCR and COLD-PCR Based on Unlabeled-probe HRM Examining DNA of 36 Pancreatic Carcinoma Tissues

Specimen Number	Conventional PCR	COLD-PCR	TA-DNA clone and Sequence
9, 18, 20, 23, 27, 29, 35	wild	mutation	35G>A
10, 13, 22, 25, 33	wild	mutation	38G>A
7, 15, 21, 28	wild	mutation	35G>T
24	wild	mutation	37G>A
19	wild	mutation	34G>C
2, 4, 5, 6, 11, 26, 34, 36	mutation	mutation	-

*A total of 36 pancreatic adenocarcinoma tissue samples were examined via both conventional PCR and COLD-PCR based on unlabled-probe HRM, t-A DNA cloning and sequencing used to verify the different results of the two methods

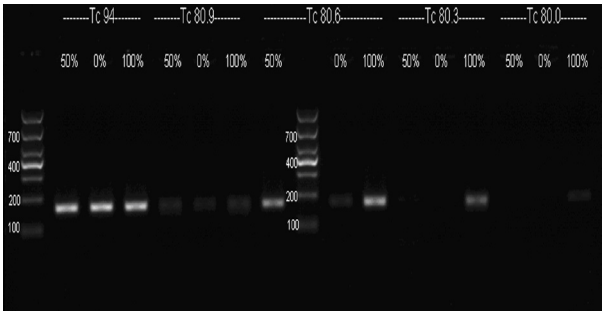

Figure 1. Determination of Critical Tc

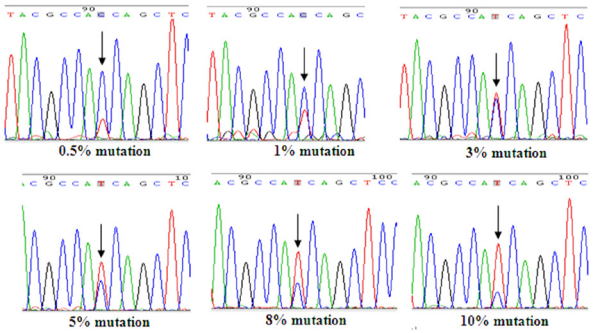

Figure 2. Sensitivity of Sanger Sequencing Improved by COLD-PCR HRM

dependent on the DNA sequence, is a temperature 0.5-1°C below Tm of the target sequences and below which PCR efficiency drops abruptly.

A series of denaturation temperatures (Tc) were applied to the running of the PCR, starting at Tm and at 0.3°C intervals downward, until it was too low to amplify any specific PCR products. For verification, the PCR products were visualized on a 2% agrose gel with the appropriate size markers with a UV imager (Tanon, China). The optimal Tc was identified as the lowest temperature that yielded substantial PCR products with reliable reproducibility.

COLD-PCR-based unlabeled-probe HRM analysis

Two consecutive runs of PCR were performed prior to the unlabeled-probe HRM analysis, Tc of 80.6°C as the first-run of COLD-PCR, and the conventional thermocycling conditions as the second-run of enhancing-PCR. The procedures of COLD-PCR were identical to those of the conventional PCR, and the two consecutive PCR cycling was modified as follows: 95°C for 10 min, followed by 20 cycles of 80.6°C for 30s, 56°C for 30s, 72°C for 30s and the other 40cycles of 95°C for 10s, 56°C

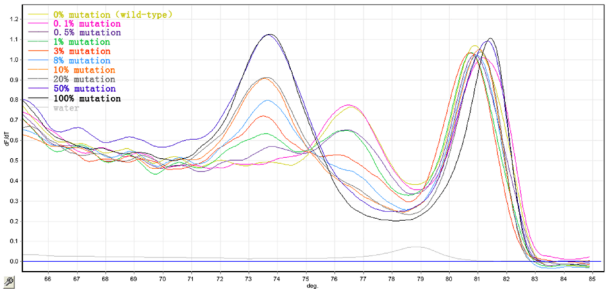

Figure 3. Sensitivity of COLD-PCR Based on Unlabeled Probe HRM Analysis

for 15s, 72°C for 25s constantly. And the unlabeled-probe HRM condition was a melt at a rise of 0.2°C per second from 65° to 85°C.

Sensitivity of COLD-PCR-based unlabeled-probe HRM and Cold-PCR-based Sanger Sequencing

KRAS wild-type cell lines were mixed in various ratios with the *KRAS* mutant ones to obtain dilutions of 0%, 0.1%, 0.5%, 1%, 3%, 8%, 10%, 20%, 50% and 100%, respectively, all of which were tested following DNA extraction. The entire DNA mixtures were simultaneously subjected to COLD-PCR-based unlabeled-probe HRM followed by Sanger Sequencing.

Sample detection

To detect *KRAS* mutations in the plasma-circulating DNA of PA, both conventional PCR and unlabeled-probe COLD-PCR were performed followed by HRM analysis. For the samples with mutations, COLD-PCR-based unlabeled-probe HRM was used to detect mutations in the matched tissues. For further verification, T-A DNA cloning and sequencing were applied to the differences.

Statistical analysis

All statistical analyses were performed using SPSS 15.0 software and Microsoft Excel. The receiver operating characteristic curve (ROC) and respective area under the curve (AUC) were calculated for *KRAS* mutation and/or CA 19-9 to provide more accurate information to distinguish patients with PA from control subjects using Binary logistic regression analysis followed by ROC Curve analysis. The sensitivity, specificity, and accuracy of COLD-PCR-based unlabeled-probe HRM were calculated according to the standard definitions.

Results

Determination of Critical Tc

It was found that 80.6°C was the appropriate Tc for COLD-PCR, below which all the wild-type, mutant and mismatched sequences were equally amplified (Figure1).

Sensitivity of COLD-PCR based-HRM and COLD-PCR-based sanger sequencing

With COLD-PCR, the sensitivity of Sanger sequencing reached 0.5% (Figure 2), whereas that obtained after conventional PCR did only 20%; that of COLD-PCR based on unlabeled-probe HRM analysis, 0.1% (Figure 3).

Detection of KRAS mutations

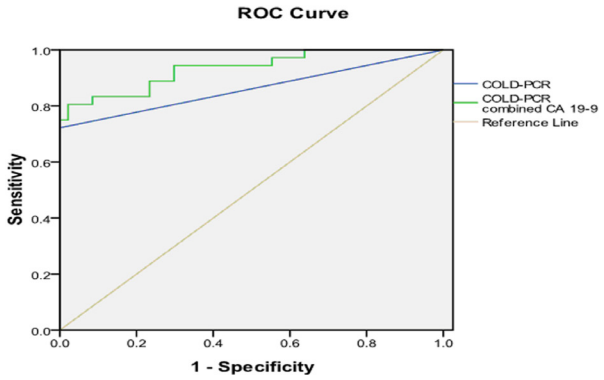

Figure 4. ROC Curve

KRAS mutations were identified in 26 of 36 plasma-circulating DNA Samples (72.2%), none detected in the disease control and/or healthy control group (Table 1).

KRAS mutations were identified both in 26 PA tissues samples and in 26 plasma-circulating ones, the results consistent with each other.

Specificity and sensitivity of KRAS mutations in PA diagnosis

To discriminate those with PA from the disease control and/or healthy control group, the group area under the ROC curves (AUC) of COLD-PCR based unlabeled probe HRM analysis turned out to be 0.861. When combined with CA 19-9, AUC increased to 0.934 (Figure 4).

The sensitivity, specificity, and accuracy of COLD-PCR-based on unlabeled probe melting analysis for *KRAS* mutations in plasma-circulating DNA samples were tested to 80.6%, 87.5% and 83.3%, respectively.

Discussion

The *RAS* family genes, originally identified as oncogenes in acutely transforming retroviruses (Vossen et al., 2009) have three highly homologous *RAS* proteins encoded by KRAS, HRAS and NRAS genes. A high frequency of *RAS* mutations has been found in many types of tumor; approximately 30% of all human cancers develop 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 (Krypuy et al., 2006); and there is an association between *KRAS* gene status and the therapeutic effect of epidermal growth factor receptor (EGFR) inhibitors (Deramaudt et al., 2005). Thus, its detection could be of clinical importance to the clinician in diagnosing and treating PA (Paez et al., 2004; Wu et al., 2008).

The approaches to mutation detection include Sanger sequencing, pyrosequencing, matrix-assisted laser desorption/ionization time-of flight (MALDI-TOF) mass spectrometry, restriction fragment length polymorphism (RFLP) assay, high-resolution melting and others. In the past decade, many published reports have indicated that the above-mentioned methods present the limitation of a low sensitivity, even though there is a slight increase.

At the initial step of mutation detection, however, these approaches are PCR-based genetic tests, which amplify both variant and non-variant alleles with approximately equal efficiency. Thus, the focus falls on the downstream assays to identify the minor variant alleles in the majority of wild-type alleles; therefore, researchers are dedicated to their improvement. Although the screening mutations in well microdissection isolated tumor tissues are reliable, these approaches have limitation in the samples with low-level mutations at the initial DNA amplification.

COLD-PCR is a recently developed new form of PCR that enriches the minor variant alleles in the wild-type allele background (Arvanitakis et al., 2004). A single nucleotide mismatch in the double-stranded DNA sequence will lead to a subtle difference in the melting temperature (Tm) of DNA sequence (Lipsky et al., 2001). Depending on the sequence context and position of the mismatch, Tm changes of 0.2-1.5°C are common for sequences up to 200 bp or higher. Thus, it is educible that there exists a critical denaturation temperature (Tc) that only variant alleles and heteroduplexes of the mutant are selectively amplified. With no specific instruments and pricy commercial kits, in the current study, the replacement of the conventional PCR with COLD-PCR can increase mutation-detection sensitivity by 5- to 100-fold with different downstream assays in various sample types.

Since the first use of COLD-PCR, much literature has indicated its application to molecular diagnostics assays. In the set-up of the PCR assay, however, the Tc plays an important role in its sensitivity, stability and reproducibility. To identify the optimal Tc in the current study, we carried out a set of PCR procedures at graded denaturation temperatures below the Tm (80.0°C, 80.3°C, 80.6°C and 80.9°C) to amplify a wild-type, homogenous and heterogenous-mutated cell line DNA. With the comparison of the PCR products amplified along the conventional Tm (94°C), the ones amplified below the temperature 80.9°C showed a selective amplification of mutated sequences; nonetheless, the efficiency of PCR assays took on a descending trend. We chose 80.6°C to amplify specifically the mutated sequence with reliable reproducibility as the Tc (Figure 1). To verify the impact of the mutation enrichment, the serial dilutions of homogenous-mutated cell line DNA were subjected to COLD-PCR followed by Sanger sequencing. The sensitivity of Sanger sequencing was increased to 0.5% after COLD-PCR, when compared with its well-known limited one by 20%, suggesting that replacing the conventional PCR with COLD-PCR could increase the sensitivity of mutation-detection by approximately 40-fold.

In practice, the downstream assay in the current study was characterized by being simpler, higher performance, shorter turn around-time and lower cost. Furthermore, unlabeled-probe melting with saturated fluorescent dye was chosen to increase the sensitivity of detection. The unlabeled-probes were designed to be complementary to the wild-type sequences so that all the potential mutations were identified. To ensure the upstream enrichment step, the proportion of the primers was adjusted to perform COLD-PCR followed by asymmetric PCR consecutively. Upon amplification, high-resolution melting was conducted on the HRM Platform in the form of a closed-

tube test. To validate the performance of the novel KRAS, the sensitivity of the COLD-PCR-based on unlabeled probe melting analysis was determined through a dilution; consequently, mutation as low as 0.1% was detected, when compared with that as low as 3% out of the conventional-PCR-based unlabeled probe melting analysis, which suggested that COLD-PCR-based unlabeled probe HRM analysis could be of a preferable approach to *KRAS* mutation detection.

Plasma-circulating DNA released by tumor has been reported to be capable of carrying somatic oncogene or tumor suppressor gene mutations. A number of studies have viewed the mutation analysis of candidate genes in circulating free DNA as a genomic biomarker for certain types of tumors. Early in 1999, Castells A et al. detected *KRAS* codon 12 mutations in plasma-circulating DNA to make a diagnosis of those with PA, achieving the highly specific, low-sensitive results through RFLP and SSCP. In addition, COLD-PCR based unlabeled probe HRM combined CA 19-9 were not only of high diagnostic value, but also easier to be accepted as a diagnostic basis by clinicians rather than COLD-PCR based unlabeled probe HRM only.

In the current study, the establishment of COLD-PCR-based unlabeled probe HRM analysis could enable the sensitivity and specificity of *KRAS* mutations detection in circulating free DNA. To demonstrate the diagnostic utility of *KRAS* mutations detection in the circulating free DNA of patients with PA mingling with non-pancreatic carcinoma and healthy individuals by COLD-PCR-based unlabeled probe melting analysis, we set up a disease group of 36 PA subjects, a disease control group of 24 gastrointestinal disease patients and a healthy control group of 25 healthy individuals, isolated plasma-circulating DNA, and obtained nucleic acids of guaranteed concentration and purity for *KRAS* mutation detection. Then we applied the COLD-PCR-based unlabeled probe HRM analysis to screen the potential *KRAS* mutations in the plasma-circulating DNA sample. As a clinical widely accepted tumor marker, CA19-9 was also detected to provide an adjunctive diagnostic aid in PA.

KRAS mutations eventuated in 26 (72.2%) PA cases and none in the healthy and/or disease control group (Table 1). For discriminating patients with PA from the healthy and disease control group area under the ROC curves (AUC) was 0.861. When combined with CA 19-9, the AUC increased to 0.934 (Figure 4). COLD-PCR combined CA 19-9 were not only of high diagnostic value, but also easier to be accepted as a diagnostic basis by clinicians rather than COLD-PCR only.

The sensitivity, specificity, and accuracy of COLD-PCR based unlabeled probe melting analysis for *KRAS* mutation in circulating free DNA samples were 80.6%, 87.5% and 83.3%, respectively.

The limitation of the current study was the number of the specimens. Next, the unlabeled probe designed complementary to the wild-type sequence could detect the potential existence rather than the types of *KRAS* mutations. In addition, the most common types of *KRAS* mutations in PA were found to be 35G>A (46%) and 35G>T (32%) with a decreased Tm (Liew et al., 2004), while some type of G>C mutation could be missed in spite of the use of the fast COLD-PCR step.

In conclusion, the data of the current study showed that COLD-PCR-based unlabeled probe melting analysis in plasma-circulating DNA could be an easy, noninva¬sive, and highly sensitive approach to the clinical mutation detection of *KRAS* codons 12 and 13, and that the *KRAS* mutations detection in circulating free DNA combined with CA 19-9 could represent a promising candidate biomarker for the early screening and diagnosis of PA.

Acknowledgements

This research is supported by the by grants from the State Key Laboratory of Clinical College Construction Project (/); the National Science & Technology Pillar Program during the 12th Five-year Plan Period (2012BAI37B01); the Research Fund of Tumorigenesis and Invasion Principle Key Laboratory of Ministry of Education (KLCCI2014-3).

References

Amicarelli G, Shehi E, Makrigiorgos GM, et al (2007). FLAG assay as a novel method for real-time signal generation during PCR: application to detection and genotyping of *KRAS* codon 12 mutations. *Nucleic Acids Res*, **35**, 131.

Antonio Jimeno, Manuel Hidalgo (2006). Molecular biomarkers: their increasing role in the diagnosis, characterization, and therapy guidance in pancreatic cancer. *Mol Cancer Ther*, **5**, 787-96.

Arvanitakis M, Van Laethem JL, Parma J, et al (2004). Predictive factors for pancreatic cancer in patients with chronic pancreatitis in association with *KRAS* gene mutation. *Endoscopy*, **36**, 535-42.

Boldrini L, Gisfredi S, Ursino S, et al (2007). Mutational analysis in cytological specimens of advanced lung adenocarcinoma: a sensitive method for molecular diagnosis. *J Thorac Oncol*, **2**, 1086-90.

Chen CY, Shiesh SC, Wu SJ (2004). Rapid detection of *KRAS* mutations in bile by peptide nucleic acid-mediated PCR clamping and melting curve analysis: comparison with restriction fragment length polymorphism analysis. *Clin Chem*, **50**, 481-9.

Dawood S (2010). Novel biomarkers of metastatic cancer. expert review of molecular diagnostics. *Expert Rev Mol Diagn*. **10**, 581-90.

Deramaudt T, Rustgi AK. (2005). Mutant *KRAS* in the initiation of pancreatic cancer. *Biochim Biophys Acta*, **1756**, 97-101.

Di Fiore F, Blanchard F, Charbonnier F, et al (2007). Clinical relevance of *KRAS* mutation detection in metastatic colorectal cancer treated by Cetuximab plus chemotherapy. *Br J Cancer*, **96**, 1166-9.

Eijk RV, Puijenbroek MV, Amiet R, et al (2010). Chhatta sensitive and specific *KRAS* somatic mutation analysis on whole-genome amplified DNA from archival tissues. *J Molecular Diagnostics*, **12**, 27-34.

Gary Y, Timothy D, Wagner, et al (2005). Multimodality approaches for pancreatic cancer. *Cancer J Clin*, **55**, 352-67.

Guo W, Zhang C, Wu J, et al (2012). Unlabeled-probe high-resolution melting to detect *KRAS* codon 12 and 13 mutations in pancreatic adenocarcinoma tissues. *Clin Chem Lab Med*, **50**, 1035-40.

Habermann J, Bunger K, Bunger S, et al (2011). Serum biomarkers for improved diagnostic of pancreatic cancer:

a current overview. *J Cancer Res Clin Oncol*, **137**, 375-89.
Huang C, Wang WM, Gong JP, et al (2013). Oncogenesis and the clinical significance of K-ras in pancreatic adenocarcinoma. *Asian Pac J Cancer Prev*, **14**, 2699-701.
Karnoub AE, Weinberg RA (2008). *RAS* oncogenes: split personalities. *Nat Rev Mol Cell Biol*, **9**, 517-31.
Krypuy M, Newnham GM , Thomas DM, et al (2006). High resolution melting analysis for the rapid and sensitive detection of mutations in clinical samples: *KRAS* codon 12 and 13 mutations in non-small cell lung cancer. *BMC Cancer*, **6**, 295-306.
Laurent-Puig P, Lecomte T, Berger A, et al (2002). Detection of free-circulating tumor-associated DNA in plasma of colorectal cancer patients and its association with prognosis. *Intl J Cancer*, **100**, 542-8.
Liew M, Pryor R, Palais R, et al (2004). Genotyping of single-nucleotide polymorphisms by high-resolution melting of small amplicons. *Clin Chem*, **50**, 1156-64.
Li J, Wang L, Mamon H, et al (2008). Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. *Nature Med*, **14**, 579-84.
Lipsky RH, Mazzanti CM, Rudolph JG, et al (2001). DNA melting analysis for detection of single nucleotide polymorphisms. *Clin Chem*, **47**, 635-44.
Lokshin A, Brand E, Nolen RE, et al (2011). Serum biomarker panels for the detection of pancreatic cancer. *Clin Cancer Res*, **17**, 805-16.
Maheswaran S, Sequist LV, Nagrath S, et al (2008). Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med*, **359**, 366-77.
Malfertheiner P, Fry L, Monkemuller CK, et al (2008). Molecular markers of pancreatic cancer: development and clinical relevance. *Langenbecks Arch Surg*, **393**, 883-90.
Massarelli E, Varella-Garcia M, Tang X, et al (2007). *KRAS* mutation is an important predictor of resistance to therapy with epidermal growth factor receptor tyrosine kinase inhibitors in non-small-cell lung cancer. *Clin Cancer Res*, **13**, 2890-6.
Ogino S, Kawasaki T, Brahmandam M, et al (2005). Sensitive sequencing method for *KRAS* mutation detection by pyrosequencing. *J Mol Diagn*, **7**, 413-21.
Paez JG, Janne PA, Lee JC, et al (2004). EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science*, **304**, 1497-500.
Pitman MB, Lewandrowski K, Shen J, et al (2010). Fernandez-del Castillo, C. Pancreatic cysts preoperative diagnosis and clinical management. *Cancer Cytopathology*, **118**, 1-13.
Robert M, Beazley, lsidore Cohn, Jr (1981). Pancreatic cancer. *CA Cancer J Clin*, **31**, 346-58.
Maheswaran S, Sequist LV, Nagrath S, et al (2008). Detection of mutations in EGFR in circulating lung-cancer cells. *N Engl J Med*, **359**, 366-77.
Vossen RH, Aten E, Roos A, et al (2009). High-resolution melting analysis (HRMA)-more than just sequence variant screening. *Human Mutation*, **30**, 860-6.
Wittwer CT, Reed GH, Gundry CN, et al (2003). High-resolution genotyping by amplicon melting analysis using LCGreen. *Clin Chem*, **49**, 853-60.
Wu CC, Hsu HY, Liu HP, et al (2008). Reversed mutation rates of *KRAS* and EGFR genes in adenocarcinoma of the lung in Taiwan and their implications. *Cancer*, **113**, 3199-208.