Clinica Chimica Acta 413 (2012) 1532–1535

Contents lists available at SciVerse ScienceDirect

Clinica Chimica Acta

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

Application of pyrosequencing technique for improved detection of K-Ras mutation in formalin-fixed and paraffin-embedded prostate carcinoma tissues in Chinese patients

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ARTICLE INFO

Article history:
Received 31 May 2012
Accepted 3 June 2012
Available online 23 June 2012

Keywords:
Pyrosequencing
K-Ras mutation
Formalin-fixed and paraffin-embedded
Prostate carcinomas

ABSTRACT

Background: The purpose of this study was to comparatively evaluate the efficacy of pyrosequencing (PS) and Sanger sequencing (SS) methods for detecting K-Ras codon 12 and 13 mutations in formalin-fixed paraffin-embedded (FFPE) prostate cancer (PCa) samples from Chinese patients.
Methods: The cancer cell lines, including the LS174T G12D mutant (GGT to GAT) and the COLO320 wild-type, were tested to determine the limitation of detection and reproducibility of the PS method. In addition, 101 PCa patient samples, 13 benign prostatic hyperplasia (BPH) and 12 normal adjacent tissue samples, were assayed by PS and SS to evaluate their detection abilities for K-Ras mutations in codons 12 and 13.
Results: The PS assay was able to reproducibly detect 5% mutant alleles and had an intra-assay variability of 4.21% and inter-assay variability of 11.37%. The PS assay detected a higher number of K-Ras mutations in PCa samples than the SS assay (8.91% vs. 3.96%). Correlation and stratification analyses of the PCa samples and K-Ras mutation status revealed no associations between age, serum prostate specific antigen (PSA), depth of invasion (pT category), or Gleason score.
Conclusions: We demonstrated that the PS method detected more K-Ras mutations in codons 12 and 13 of FFPE prostate cancer samples from Chinese patients than the traditional SS method. In addition, the K-Ras mutation was more frequent in Chinese population than in Western populations but was similar to that of other Eastern populations, suggesting that these K-Ras mutations may contribute to the pathogenesis of prostate carcinoma in Asian patients.

1. Introduction

Prostate cancer (PCa) is recognized as one of the principal medical problems currently faced by the male population. PCa prevalence has steadily and remarkably increased over the past two decades in China. In Shanghai alone, the estimated incidence of PCa increased from 1.8–2.4 per 100,000 in 1990 to 4.5–7.7 per 100,000 in 2000, and then to ~10.0 per 100,000 in 2004 [1]. Furthermore, most newly diagnosed Chinese PCa patients are symptomatic and have reached a metastatic stage. This relatively late stage diagnosis is accompanied by poorer prognosis and is believed to be due to the lack of routine PCa screening methods, including prostate specific antigen (PSA) assay and digital rectal examination [2].

The RAS/RAF/ERK signaling pathway mediates cellular responses to extracellular signals that regulate cell proliferation, differentiation, and apoptosis. An important Ras family member, the K-Ras small GTPase, is particularly sensitive to mutation and harbors so-called mutation 'hotspots' in codons 12 and 13 [3]. K-Ras mutations are frequently detected in human cancers, including prostate cancer [4]. Studies of K-Ras mutation status in PCa patients have identified ethnic-specific prevalence patterns; for example, 7.3% of Korean PCa patients [5] and ~10% of Japanese PCa patients [6,7] are reported to carry K-Ras mutations, while <3% of patients from the U.S. were reported as positive for K-Ras mutations [7]. Such K-Ras data have rarely been reported in Chinese PCa patients [4].

The most frequently used strategies for assessing K-Ras mutation status in tumor samples are direct sequencing methods. Sanger

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doi:10.1016/j.cca.2012.06.008

sequencing (SS) is considered by many to be the gold standard for mutation testing in clinical samples. However, SS has a modest limit of detection, and accumulating evidence has suggested that its sensitivity may be insufficient compared to the more recently developed pyrosequencing (PS) method. The accuracy of SS, however, is known to be highly variable according to the target sequence and laboratory conditions [8]. PS is based on the sequencing-by-synthesis approach that uses real-time, nonelectrophoretic nucleotide extension. This simple, robust, and sensitive methodology has led to PS being successfully applied in clinical and experimental settings to diagnose mutations in tumors, perform single nucleotide polymorphism genotyping, and evaluate pro-/anti-oncogenic alterations in methylation status [9–12]. The detection limit of PS has been reported as 5% of mutant alleles, making it particularly attractive for use with tumor samples that contain abundant non-neoplastic cells [8,13–15]. In addition, PS is readily applicable to whole-genome DNA samples, making it useful in large-scale studies. In fact, previous studies have investigated the detection limit of PS for K-Ras mutations, and demonstrated that the detection limit nearly reached the 5% level [8,13,16–19].

Here, we performed a systematic comparative analysis of the PS and SS methods for detecting K-Ras mutations in codons 12 and 13 from formalin-fixed paraffin-embedded (FFPE) PCa samples of Chinese patients. Prevalence of the K-Ras mutation in these patients and their clinicopathological characteristics (age, serum PSA, pT category, and Gleason score) were also analyzed to uncover any correlation that may be exploited in future diagnostic or therapeutic strategies.

2. Materials and methods

2.1. Formalin-fixed paraffin-embedded prostate samples

Tissue samples from 101 prostate adenocarcinomas and 13 cases of benign prostatic hyperplasia (BPH) were obtained from Renji Hospital, School of Medicine, Shanghai Jiaotong University and Huashan Hospital, Fudan University. In addition, non-neoplastic adjacent prostate tissue samples were obtained from 12 of the 101 PCa patients for use as controls. Hematoxylin and eosin (H&E) stained sequential sections from this cohort were evaluated by two pathologists to estimate the percentage of tumor cells for each sample and patient. All of the tumor samples were confirmed to contain >80% tumor cells. All patients provided informed consent for experimental use of their stored tissues.

2.2. Cell lines

To determine the limitation of detection and reproducibility of the PS method for K-Ras mutations, two human colorectal carcinoma cell lines were tested: LS174T, which harbored a K-Ras G12D mutant (GGT to GAT); and COLO320, which was a confirmed non-mutated, wild-type line. Both cell lines were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (GibcoBRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), and incubated at 37 °C in an atmosphere of 5% CO_2.

2.3. DNA extraction

For each sample, five sequential, unstained, 10 μm-thick sections were processed by the QIAamp DNA FFPE Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA was extracted from the cell lines by using the QIAamp DNA Mini Kit (Qiagen) and following the manufacturer's protocol. All DNA samples were quantified by spectrophotometric measurement on a NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA).

2.4. DNA Amplification and mutation analysis by direct DNA sequencing

DNA from case samples was analyzed for two common K-Ras mutations (in codons 12 and 13) by using nested-PCR and the primers are shown in Table 1. DNA samples (10 ng) were PCR amplified with reagents from the HotStarTaq Plus Master Mix (Qiagen) and the gene-specific primers listed in Table 1 (forward and reverse, 10 pmol each) in a 25 μL final volume reaction mix. Cycling conditions were as follows: one cycle of 95 °C for 5 min; 40 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s; one cycle of 72 °C for 5 min; indefinite hold at 4 °C. The PCR amplicons were purified and subjected to DNA sequencing by the ABI 3500 Genetic Analyzer (Applied Biosystems Inc., Foster City, CA, USA) with the same primers used for PCR amplification. The sequences were compared with the reference sequences.

2.5. DNA amplification and mutation analysis by pyrosequencing

Samples were biotin-labeled by PCR amplification in the reaction mix described above, except at a 50 μL final volume. Cycling conditions were as follows: one cycle of 95 °C for 3 min; 45 cycles of 95 °C for 10 s, 56 °C for 20 s, and 72 °C for 30 s; one cycle of 72 °C for 5 min; indefinite hold at 4 °C. Following amplification, 10 μL of the biotinylated PCR product was immobilized on streptavidin-coated sepharose beads and washed in 70% ethanol. The purified biotinylated PCR product was released into the PyroMark Q96 sequencer (Biotage, Uppsala, Sweden) along with PyroMark Gold reagents (Qiagen) and 0.3 μmol/L sequencing primer (Table 1).

Negative control DNA was extracted from the COLO320 cell line, and positive control DNA was extracted from the LS174T cell line. Both control DNA samples were confirmed by direct DNA sequencing. Negative control, positive control, and blank (no DNA template) samples were included in each run.

2.6. Statistical analysis

Fisher's exact test was used to comparatively analyze the correlation of K-Ras mutations with clinicopathologic features of PCa samples/patients. *P*-values <0.05 were considered to be statistically significant.

3. Results

3.1. Limitation of detection and reproducibility of PS and SS assays for K-Ras mutations in FFPE PCa samples

Serial dilutions of mutant K-Ras cells (LS174T) and wild-type cells (COLO320) were subjected to direct sequencing by PS and SS methods. The undiluted mutant sample (100%) was expected to contain 50% mutant alleles, if each cell contained one wild-type and one mutant allele. The PS assay was able to reproducibly detect 5% of mutant alleles in the wild type DNA background (COLO320); however, the limit of detection for the SS assay was 20%. Mutation levels of <5% could not be detected by the PS assay. To test the intra- and inter-assay variabilities (presented as the coefficient of variation (%)) of PS, respectively, we

Table 1
PCR and sequencing primers used for K-Ras mutation analysis.

K-Ras primer — 12, 13	Sequence (5′–3′)	PCR product size, bp
External-F	GTTCTAATATAGTCACATTT	245
External-R	ACTCATGAAAATGGTCAGAGAAACCTTTAT	
Internal-F	TGTAAAACGACGGCCAGTCACATTTTCATTATTTTTATTATAAGGC	234
Internal-R	AGAAACCTTTATCTGTATCAAAGAATG	
Pyro-F	TAAGGCCTGCTGAAAATGACT	118
Pyro-R	Biotin-TTGGATCATATTCGTCCACAA	
Pyro-S	TTGTGGTAGTTGGAGCT	