

KRAS Mutations: A Possible Biomarker for Advanced Prostate Cancer

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ABSTRACT

Prostate cancer is an important type of cancer in males with the highest mortality rate after the lung cancer, especially in industrialized countries. RAS oncogenes, originating from proto-oncogenes with point mutations, play an important role in cancer cell proliferation. The RAS proto-oncogene mutations occur in 25% of human cancers. The aim of the study was to investigate the potential mutation of KRAS gene at exon 2 (codons 12/13), exon 3 (codon 61) and exon 4 (codon 117/146) in Turkish patients with prostate cancer. The case group was comprised of 45 paraffin-embedded prostate cancer tissues. The control group was comprised of 20 healthy samples. The mutation analysis was performed by using PCR-based High Resolution Melting (HRM) analysis. The DNA samples of case and control groups were isolated and evaluated by HRM. Chi-square and Fisher's exact tests were used to evaluate the relationship between mutations in KRAS gene and prostate cancer: $p < 0.05$ was considered as statistically significant. There were mutations in exon 2 (1 patient) and exon 3 (3 patients) of KRAS. In exon 4, no mutations were determined. The results of this study suggest that exon 2 and exon 3 mutations in KRAS gene may be effective in the development of prostate cancer. In addition, the results of this study may provide insight into the efficacy of anti-EGFR treatment on cases without KRAS mutation.

Keywords

HRM, KRAS, Prostate Cancer.

Introduction

Cancer is a multifactorial disease affected by both genetic and environmental factors. The mutations in the proto-oncogenes which stimulate the growth of cells and the tumor suppressor genes may cause the transformation of normal cells [1,2]. Three mechanisms are defined for proto-oncogenes transform to oncogenes which are point mutations, chromosomal rearrangements and gene amplifications. It is known that especially point mutations cause this transformation in cancer development [3].

In human tumors, transformation to oncogenes from proto-oncogenes is mostly encountered in RAS genes. 25% of human cancers showed oncogenic RAS mutations [4]. RAS proto-oncogenes encode p21 protein which has guanosine triphosphate activity and has role in cell proliferation [5]. In mammals, 3 different RAS genes were identified as H(arvey)-RAS, K(irsten)-

RAS and N(euroblastoma)-RAS. KRAS and NRAS gene mutations are more common in human cancers [6]. KRAS gene encodes a 21 KDa GTP-binding protein in the Epidermal Growth Factor Receptor (EGFR) [7]. RAS proteins are placed on the cytoplasmic side of the cell membrane. They are active in GTP-bound conformation and inactive in GDP-bound conformation (Figure 1). GAP and GEF regulate the change between these conformations. RAS proteins transmit growth stimulating signals from tyrosine kinase membrane receptors to different molecules starting signal transduction pathways [8,9].

Some mutations in RAS genes inhibit the GTP-GDP change resulting the accumulation of active Ras proteins in cells. This situation cause constitutive activation of Ras proteins and the induction of uncontrolled cell proliferation [10,11]. The most common aminoacid changes in KRAS oncogene are present in exon 2 (codons 12 and 13), exon 3 (codon 61) and exon 4 (codon 117 and 146) [12]. The mutations at especially codons 12 and 13 cause Ras protein to bind to GTP longer resulting in more proliferation

signal. In some tissues such as colon, pancreas, prostate and lung, many other mutations with RAS gene mutations cause cancer cell formation [13]. In pancreas and colon carcinomas, KRAS mutations are more common often in codons 12 and 13. In various populations, KRAS mutations were encountered in prostate cancer patients [14].

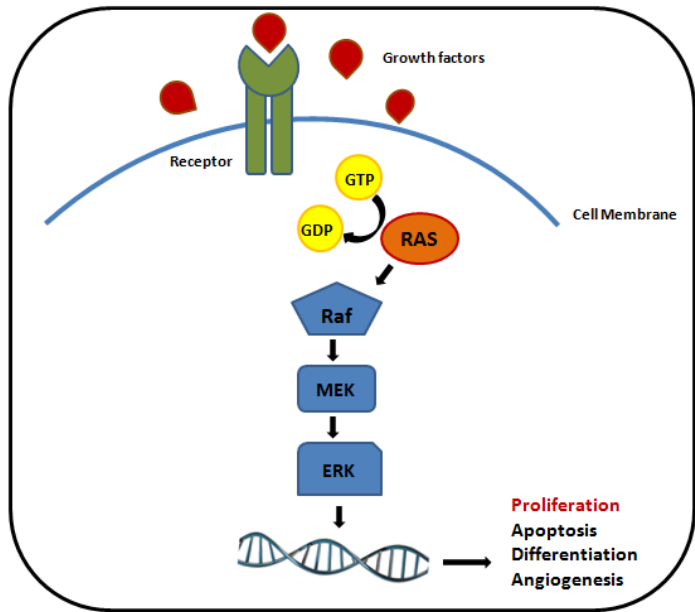


Figure 1: The biological role of the Ras/Raf/MEK/ERK signaling pathway in the occurrence and development of Prostate Cancer.

Prostate cancer is the fourth most common cancer worldwide and the second most commonly diagnosed cancer worldwide in men [15]. The diagnosis and staging of this cancer are of great medical interest. For several decades, prostate specific antigen (PSA) has been used as a blood-based biomarker. PSA is a protein mainly secreted by prostate cells making it suitable to be biomarker. However, PSA does not have sufficient specificity for diagnosis [16].

KRAS mutations are considered as prognostic biomarkers for clinical outcome of patients and anti-EGFR targeted monoclonal antibody therapies [7]. However enough response could not be obtained for the patients who have KRAS and NRAS mutant cells. For the choice of these drugs, molecular genetic tests should be performed for RAS genes in order to understand whether they are mutant or not. In this study, the aim is to determinate probable mutations in exon 2 (codons 12 and 13), exon 3 (codon 61) and exon 4 (codons 117 and 146) of KRAS genes in prostate cancer patients.

Subjects and Methods

Study Subjects

In this study, 65 individuals comprised 45 subjects with prostate cancer and 20 healthy subjects. In case group, the material selected for mutation analysis was formalin-fixed and paraffin-embedded (FFPE) section. The control group is composed of healthy individuals without any metabolic disorders including cancer. DNA samples of control group were isolated from the peripheral

blood. The study was approved by the Ethical Committee.

DNA Isolation from FFPE Tissue

Firstly, deparaffinization was performed for DNA isolation from FEPE samples. 10 µm thick sections of FFPE were used and the samples were treated with xylol, absolute alcohol and 75% alcohol at appropriate temperature and times. For DNA isolation, lysis buffer containing proteinase K was added in deparaffinized tissues and samples were incubated 37°C overnight. After incubation, DNA was extracted with phenol/chloroform and precipitated with ethanol.

Mutation Analysis

Mutation analysis of KRAS gene (exons 2, 3 and 4) was performed using HRM technique based on real-time PCR. The reaction was carried out using primers (Table 1) covering the target site on each exon (BioRad Precision Melt Supermix). HRM amplification was carried out in 20 µl volume containing 50-100 ng genomic DNA, 1X Precision Melt Supermix, 200 nM of each primers and DNase-free water. PCR reactions were performed as follows: an initial denaturation at 94 °C for 2 min was followed by 40 cycles of denaturation at 95°C for 10 s, annealing at 60°C for 30 s, elongation at 72°C for 30 s. Following PCR amplification, high-resolution melting was performed: heated to 95°C for 30 s and then cooled to 60°C for 1 min and performed from 65°C to 95°C, rising at 0.2°C/s.

Locus	Forward Primer	Reverse Primer
KRAS Exon 2	TTATAAGGCCTGCTGAAAT-GACTGAA	TGAATTAGCTGTATCGT-CAAGGCACT
KRAS Exon 3	CCAGACTGTGTTTCTCCCTT	CACAAAGAAAGCCCTC-CCCA
KRAS Exon 4	ACAGGCTCAGGACTTAG-CAAGAAGT	AGCATAATTGA-GAGAAAACTGA

Table 1: Sequence of primers used in the study [17,18].

Statistical Analysis

Chi-square and Fisher's exact tests were used to evaluate the relationship between mutations in KRAS gene and prostate cancer. p<0.05 was considered as statistically significant.

Results

The mutations in exons 2, 3 and 4 of the KRAS gene were detected by HRM analysis. After evaluating of the HRM products according to the agarose gel electrophoresis and melting curves analysis (Figure 2), mutation analysis of the following regions was performed using the Bio-Rad Precision Melt Analysis-Software.

According to the results of HRM analysis, mutation in exon 2 of KRAS was detected at 1 case, and mutation in exon 3 of KRAS was detected at 3 cases. Mutation was not found in the exon 4 of KRAS gene (Figure 3). The statistical analysis results indicate that the mutation of the exon 3 is three times more risky than the mutation of the exon 2 in the prostate cancer patients. However, this risk was not statistically significant (p = 0,616 [OR: 3,143; 95% CI: 0,314 - 31,420]).

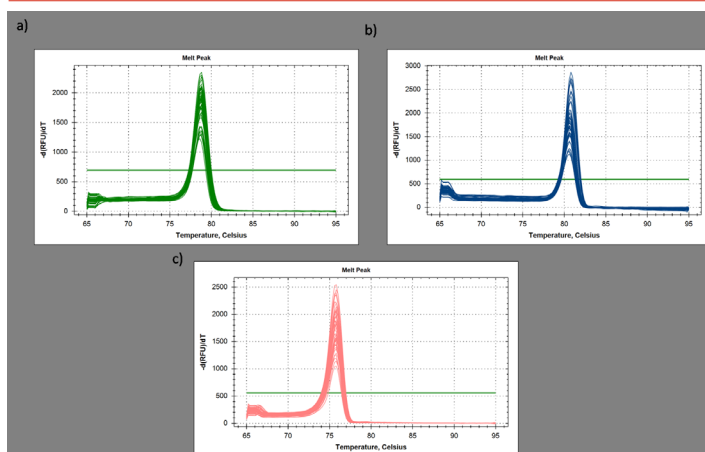


Figure 2: Melting curves of exon 2 (a), 3 (b) and 4 (c).

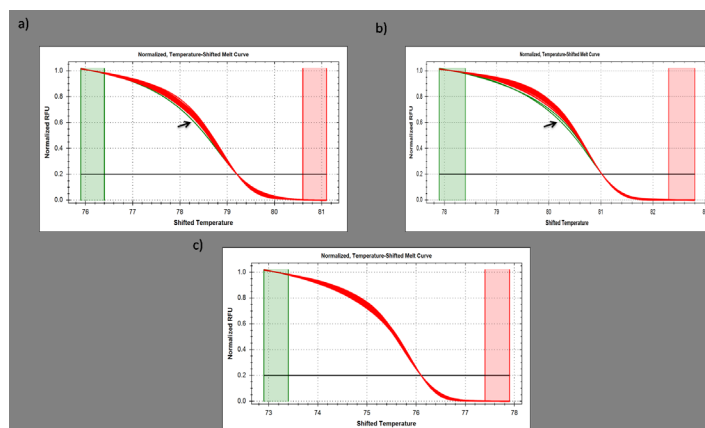


Figure 3: Results of HRM analysis: exon 2 (a), 3 (b) and 4 (c). Arrows indicate the mutant genotype.

Discussion

Early diagnosis of prostate cancer is achieved by measuring prostate-specific antigen (PSA) level and Digital rectal examination (DRE). Typical treatment methods for prostate cancer which is curable at early stage are androgen deprivation therapy (ADT), surgery and radiation therapies [19]. However, there is no effective method for treating the advanced prostate cancer. For this reason, the elucidation of the molecular mechanism of the disease will greatly contribute to the development of new treatment strategies and possible disease markers.

Cancer is a complex disease in which cells exhibit altered gene expression, uncontrolled proliferation, invasion and loss of function. The accumulation of critical mutations in important genes that involved in proliferation and cell death is effective in this process [20]. In particular, the continuous activation of signaling pathways, which stimulate cell proliferation, due to mutations is associated with cancer development. RAS/RAF/MEK/MAPK pathway mediates cellular responses to growth signals, differentiation and programmed cell death [21]. Constitutive activation of this pathway because of mutation of upstream targets such as KRAS and BRAF is common in various human cancers, including prostate carcinoma in human [22]. The incidence of KRAS gene mutations in prostate cancer cases varies

among populations. For example, KRAS mutations were found in 7.3% cases in Korea [23] and 9.1% cases in China [24].

The response to therapeutic agents may vary due to the different genomic characteristics of cancer cells [25,26]. The use of drugs which stop in stages prior to the Ras protein does not provide adequate response in cells carrying the mutated KRAS gene. This situation prevents patients from using such medicines in the clinic. For example, it is known that metastatic colorectal cancer patients with mutations in the KRAS gene codons 12, 13 (exon 2), 61 (exon 3), 117 and 146 (exon 4) do not respond to Cetuximab or Panitumumab therapy targeting EGFR [27,28]. Therefore, before using chemotherapeutic drugs targeting the EGFR cell receptor, molecular genetic tests should be conducted to determine whether the KRAS gene is mutated.

The aim of the study was to investigate the potential mutation of KRAS gene at exon 2 (codons 12/13), exon 3 (codon 61) and exon 4 (codon 117/146) in Turkish patients with prostate cancer by using HRM technique based on PCR. According to the results, mutation in exon 2 of KRAS was detected in 1 case (2.2%), and mutation in exon 3 of KRAS was detected in 3 cases (6.6%). No mutation was found in the exon 4 of KRAS gene. The case whom was exon 2 mutation was detected is characterized as M0 (no distant metastasis) and N1 (spread to the one or more nearby lymph nodes) according to the TNM staging system. When looking at the overall profile of the patients diagnosed with mutation in the exon 3 of KRAS gene, a patient's Gleason score is 7 as well as N3 (metastasis in a lymph node or nodes, >5 cm) for TNM staging. Gleason score of another patient is 7, and TNM stage is N2 (a lymph node or multiple nodes involvement, 2-5 cm). Last patient's Gleason score is 6 and TNM stage is N1 (single lymph node involvement).

In summary, it has been determined that the above-described cases with KRAS gene mutations are locally advanced prostate cancer cases, rather than organ limited prostate cancer cases. It is known that RAS gene family mutations produce a dominant signal in the direction of cell proliferation which supports cancer development and tumor transformation.

In this study, it was seen that the disease progressed locally in cases where RAS gene mutation was observed. It is necessary that evaluation should include all parameters such as PSA values, Gleason grade and clinic phase. Furthermore, molecular approaches are critically important in determining the pathological stage and the form of targeted therapies in these cases. The results of this study, which can be seen as a preliminary study in the identification of KRAS gene mutations in Turkish patients who have been diagnosed with prostate cancer, should be supported by further studies in which the number of samples is increased.

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