RESEARCH ARTICLE

Mutation Analysis of KRAS and BRAF Genes in Metastatic Colorectal Cancer: a First Large Scale Study from Iran

Aghigh Koochak1, Nasser Rakhshani1*, Mohammad Hadi Karbalaie Niya2, Fahimeh Safarnezhad Tameshkel1, Masoud Reza Sohrabi1, Mohammad Reza Babaee1, Hamid Rezvani3, Babak Bahar1, Farid Imanzade4, Farhad Zaman1, Mohammad Reza Khonsari1, Hossein Ajdarkosh1, Gholamreza Hemmasi1

Abstract

Background: The investigation of mutation patterns in oncogenes potentially can make available a reliable mechanism for management and treatment decisions for patients with colorectal cancer (CRC). This study concerns the rate of KRAS and BRAF genes mutations in Iranian metastatic colorectal cancer (mCRC) patients, as well as associations of genotypes with clinicopathological features. Materials and Methods: A total of 1,000 mCRC specimens collected from 2008 to 2012 that referred to the Mehr Hospital and Partolab center, Tehran, Iran enrolled in this cross sectional study. Using HRM, Dxs Therascreen and Pyrosequencing methods, we analyzed the mutational status of KRAS and BRAF genes in these. Results: KRAS mutations were present in 33.6% cases (n=336). Of KRAS mutation positive cases, 85.1% were in codon 12 and 14.9% were in codon 13. The most frequent mutation at KRAS codon 12 was Gly12Asp; BRAF mutations were not found in any mCRC patients (n=242). In addition, we observed a strong correlation of KRAS mutations with some clinicopathological characteristics. Conclusions: KRAS mutations are frequent in mCRCs while presence of BRAF mutations in these patients is rare. Moreover, associations of KRAS genotypes with non-mucinous adenocarcinoma and depth of invasion (pT3) were remarkable.

Keywords: Mutation analysis - KRAS - BRAF - metastatic colorectal cancer, pyrosequencing - high resolution melting

Introduction

Colorectal cancer (CRC) is one of the most frequent cancer types worldwide, with more than 70,000 new affected per year in the United States (Fransen et al., 2004, Jemal et al., 2011). CRC progresses through a multi-step carcinogenic process with a gathering of epigenetic and genetic alterations, including KRAS and BRAF mutation (Imamura et al., 2012). The KRAS proto-oncogene encodes a GTPase which trigger signal transduction cascade of Influence of epidermal growth factor receptor/mitogen-activated protein kinases (EGFR/MAPK pathway), resulting in recruits and activates BRAF (Campbell et al., 1998, Fransen et al., 2004, Guedes et al., 2013). BRAF, another factor of the EGFR/MAPK signaling pathway, produces a serine-threonine protein kinase that is a downstream molecule of actuated KRAS (Chen et al., 2014). BRAF mutant induces a signaling cascade involving factors in the MAPKs, resulting in cell propagation (Allegra et al., 2009, Guedes et al., 2013). It has been demonstrated that mutation of KRAS and BRAF genes frequently observed in the early stages of CRC (Roth et al., 2010). Approximately 30% to 40% of CRC tissues harbor a mutated KRAS gene (Andreyev et al., 1998; Andreyev et al., 2001), and 90% of those mutations found in exon 2 of the KRAS gene (at codons 12/13) (Janakiraman et al., 2010, De Roock et al., 2011). This mutant gene create a constitutively active ras factor that leads to EGFR-independent activation of the MAPK signaling pathway (Bos et al., 1987; Bamford et al., 2004) and subsequent induces cell growth and resistance to apoptosis (Rajagopalan et al., 2002; Benvenuti et al., 2007; Schubbert et al., 2007). Recent studies have shown that Almost 15% of CRC harbor the BRAF mutation (Deng et al., 2004; Roth et al., 2010) and approximately all BRAF-mutated CRC are existing within the KRAS wild-type cancers (Imamura et al., 2012). Mutations of the KRAS gene have been broadly studied as prognostic biomarkers for CRC (Hsieh et al., 2012). On the other hand the BRAF mutation has been correlated with lesser...
prognosis in several studies (French et al., 2008; Farina-Sarasqueta et al., 2010; Roth et al., 2010). However, mutation analysis of KRAS and BRAF genes, as members of EGFR/MAPK pathway, before the administration of anti-EGFR-targeted therapies for patients with CRC has become important (Rizzo et al., 2010). In CRCs, population-based investigations have proposed that the mutations might be correlated with some tumor features (Hsieh et al., 2012). However, the mutation rate and clinicopathological characteristics of KRAS/BRAF genes in Iranian CRC patients stay has been unclear. The aim of our study was to investigate the mutational activation of two members of the MAPK pathway, the KRAS gene in 1000 mCRC and BRAF gene in 242 mCRC tissue at stage IV in Iranian population by using HRM and Pyrosequencing techniques.

Materials and Methods

Study population

The biopsy specimens were obtained from 1000 Iranian mCRC patients including 427 (42.7%) female (median age 55 yrs.) and 573 (57.3%) male (median age 57 yrs.); their pathological data are listed in Table 1. These patients between 2008 and 2012 were referred to the Mehr Hospital and molecular diagnostic laboratory of Partolab, the main referral center for cancerous patients in Iran that located in Tehran, Iran. Written informed consent was obtained from all patients before testing. The study was approved by the ethical committee of Iran University of Medical sciences, Tehran, Iran.

Hematoxylin and Eosin (H&E)-stained slides from all CRC cases were reviewed by a sophisticated pathologist and at least 50% neoplastic tissues selected for mutation analysis. Unstained slides were immersed in xylene for 5 minutes and twice in ethanol 100% for 5 minutes. Tumor areas were then delimited, by comparison with correspondent H&E stained slides, and macro dissected.

DNA extraction

Genomic DNA was extracted from dissected tissue using the methods described by the QIAamp® DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany). Concentration (ng/ul) and purity (OD 260/280nm) of DNA was quantified by spectrophotometry with NanoDrop ND-1000® (Thermo Fisher Scientific Inc., Waltham, MA, USA). Extracted DNA was stored at - 20°C before use.

High resolution melting (HRM)

To distinguish KRAS and or BRAF mutant from wild-type specimens, High resolution melting (HRM) was performed as a screening method. PCR amplification and HRM were performed on a LightCycler® 480 II Real-Time System (Roche Diagnostics, Basel, Switzerland). Analysis provided with the software LightCycler® 480 Gene Scanning Software Version 1.5 (Roche diagnostics) (Figure 1). PCR reaction mixtures with a final volume of 10 μl contained: The 0.5 µM forward primer, 0.5 µM reverse primer, 0.5 µM unlabelled probe with a 3’-conjugated C3 spacer, 0.01 µg genomic DNA in 1× LightScanner Master mix (Idaho Technologies, Salt Lake City, UT, USA). Assay for KRAS performed by following program: one cycle of initial denaturation at 95°C 2 min; 50 cycles of 30 s 95°C (denaturation); 30 s 64°C (annealing); 30 s 72°C (extension); and one cycle of 60 s 72°C, 20 s 95°C, 20 s 55°C; final melting in two steps follows: Melt 1 (55-78oC at 0.06oC/s) followed by Melt 2 (78- 95°C, 0.06°C/s) and recorded of the fluorescent level. The termocycler conditions for BRAF HRM assay were: 95°C 10 min; 40 cycles of 20 sec 90°C, 20 sec 67°C, 20 sec 72°C; a final extension at 72°C 10 min. The heteroduplex cycle performed at 95°C 5 min and 40°C 1 min, then, melting at 70°C to 90°C with 25 acquisitions/°C and a 1 minute cooling to 40°C with a ramp rate of 2.2°C/second.

The LightCycler software plotted the melting peak of the fluorescent signal corresponding to the temperature

Figure 1. HRMA Melting Profiles Obtained from Tissues Carrying Mutant KRAS. The figure shows the results of Gene Scanning analyses. A) Normalized and B) difference graph, containing wild-type samples (green) and mutated samples (blue and red).
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Pyrosequencing

Extracted DNA samples were amplified for codons 12 and 13 of KRAS gene using primers from the PyroMark KRAS v.2.0 Q96 kit using Veriti 96 well Applied BioSystem thermal cycler. PCR reactions for KRAS codons 12 and 13 were performed under the following condition: the mixture was heated at 95°C 5 min, 45 cycles of 95°C 15 s, 57°C 30 s, 72°C 15 s, then, held at 72°C 5 min. PCR components final concentrations were: 1x PCR buffer, 2 mM MgCl₂, 0.125 mM dNTPs, 0.2 μM Forward primer and 0.2 μM Reverse biotinylated primer, 1U of AmpliTaq polymerase (Perkin Elmer, Waltham, USA) and 2 ng/μl DNA template.

BRAF was accomplished in those samples considered positive by HRM (like KRAS), in order to confirmation of HRM analysis results. After HRM analysis profile, Pyrosequencing was carried out using the Qiagen PyroMark BRAF kit according to the manufacturer protocol. Sequencing primers for BRAF codon 600 were obtained from the PyroMarkBraf v.2.0 kit. Sequence analysis was conducted using the PyroMark Q96 software.

Running of 12 μl PCR product on 1.5% agarose gel (Sigma-Aldrich, St. Louis, USA) used to confirm successful amplification. PyroMark Q96 analysis Software used to analysis of sequenced PCR products by EpigenDX Company (Worcester, USA). Purification of PCR products performed by illumira GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Little Chalfont, UK), according to manufacturer instructions.

Sequencing PCR products were run by 1 μL of purified PCR amplification products on an PyroMark ID Pyrosequencing machine (QIAGEN, Germany) according to kit protocol, and the respective electropherograms were analyzed with PyroMark Q96 analysis Software (QIAGEN, Germany) (Figure 2). All electropherograms were read manually.

Dxs Therascreen

TheraScreen Dxs KRAS Mutation Kits KR-21 and KR-22 (Qiagen, Hilden, Germany) that were designed to detect six mutations in codon 12 (Gly > Ala, Asp, Arg, Cys, Ser, and Val) and one in codon 13 (Gly > Asp) of the KRAS oncogene. Internal reaction control and a synthetic control template were into commercial test kit. Calculation of the KRAS mutation degree were the difference between the control reaction and the allele-specific reaction. Two characteristics of its primers: 3´ ends were sequence-specific to detect mutations (comprise the PCR-Amplification Refractory Mutation System, PCR-ARMSW), and Real-time PCR-Scorpion W primer tags, that into double-stranded DNA were fluoresce emission.

LightCyclerW480 II (Roche Applied Science, Penzberg, Germany) used for PCR reactions corresponding to manufacturer protocol (TheraScreen K-RAS Mutation

### Table 1. Demographical and Pathological Characteristics of CRC Patients

| Clinical and pathological characteristics | N   | %   |
|------------------------------------------|-----|-----|
| Sex                                      |     |     |
| Male                                     | 573 | 57.3|
| Female                                   | 427 | 42.7|
| Age (y)                                  |     |     |
| ≤50                                      | 325 | 32.5|
| >50                                      | 675 | 67.5|
| Tumor type                               |     |     |
| Non Mucinous adenocarcinoma              | 898 | 89.8|
| Mucinous adenocarcinoma                  | 86  | 8.6 |
| Signet ring cell adenocarcinoma          | 16  | 1.6 |
| Tumor differentiation                    |     |     |
| Well Differentiated                      | 439 | 43.9|
| Moderate Differentiated                  | 384 | 38.4|
| Poor differentiated                      | 164 | 16.4|
| Undifferentiated                         | 13  | 1.3 |
| pT (Depth of invasion)                   |     |     |
| p Tis                                    | 14  | 1.4 |
| p T1                                     | 14  | 1.4 |
| p T2                                     | 53  | 5.3 |
| p T3                                     | 812 | 81.2|
| p T4                                     | 107 | 10.7|
| Lymph nodes involvement                  |     |     |
| Involved                                 | 667 | 66.7|
| Uninvolved                               | 333 | 33.3|
| Mutant                                   | 0   | 0   |
| Pathological Grade                       |     |     |
| Low grade                                | 823 | 82.3|
| High grade                               | 177 | 17.7|

Figure 3. Frequency Distribution of the Different Mutation types Found in KRAS
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A total of 1000 Iranian mCRC patients tested for KRAS mutations and 242 subsets (mCRC tissue at stage IV) of them analyzed for BRAF mutations using HRM, Pyrosequencing and TheraScreen DxS methods, respectively. Table 2 characterizes the results by more details.

Table 2. Frequency of KRAS Mutations According to Clinical and Pathological Features

| Clinical and pathological characteristics | KRAS wild type | KRAS mutant, N (%) |
|------------------------------------------|----------------|--------------------|
| N (%) | Total | Codon 12 | Codon 13 |
|-------------------------------|----------------|------------------|
| Sex                           |                |                  |
| Male                          | 402 (60.5%)    | 171 (50.9%)      | 143 (83.6%) |
| Female                       | 262 (39.4%)    | 165 (49.1%)      | 143 (86.7%) |
| Age (y)                       |                |                  |
| ≤50                           | 235 (35.4%)    | 90 (26.8%)       | 74 (28.3%)  |
| >50                           | 429 (64.6%)    | 246 (73.2%)      | 212 (61.7%) |
| Tumor type                    |                |                  |
| Non-Mucinous adenocarcinoma   | 602 (90.7%)    | 298 (88.7%)      | 250 (83.9%) |
| Mucinous adenocarcinoma       | 46 (6.9%)      | 38 (11.3%)       | 34 (10.5%)  |
| Signet ring cell adenocarcinoma | 16 (2.4%)   | 0 (0.0%)         | 0 (0.0%)   |
| Tumor differentiation         |                |                  |
| Well Differentiated           | 265 (39.9%)    | 173 (51.5%)      | 77 (44.5%)  |
| Moderate Differentiated       | 279 (42.0%)    | 108 (32.1%)      | 86 (43.9%)  |
| Poor differentiated           | 109 (16.4%)    | 54 (16.1%)       | 39 (22.6%)  |
| Undifferentiated              | 11 (1.7%)      | 1 (0.3%)         | 1 (0.3%)   |
| pT (Depth of invasion)        |                |                  |
| p Tis                         | 0 (0.0%)       | 14 (4.2%)        | 0 (0.0%)   |
| p T1                          | 14 (2.1%)      | 0 (0.0%)         | 0 (0.0%)   |
| p T2                          | 33 (5.0%)      | 20 (5.9%)        | 13 (8.3%)  |
| p T3                          | 547 (82.4%)    | 265 (78.9%)      | 201 (75.8%)|
| p T4                          | 70 (10.5%)     | 37 (11.0%)       | 33 (20.6%) |
| Lymph nodes involvement       |                |                  |
| Involved                      | 399 (60.1%)    | 268 (79.7%)      | 239 (87.9%)|
| Uninvolved                    | 265 (39.9%)    | 68 (20.3%)       | 58 (14.7%) |
| Total mCRC                    | 1000 patients  | 664 (66.4%)      | 336 (33.6%)|

Statistical analysis

SPSS version 20 software (SPSS Inc., Chicago, IL, USA) was used for statistical analyses and the basic descriptive and frequency features were used. After the calculation of arithmetic and standard mean, double-sided chi square/fisher-exact tests and t tests were used to compare genotype frequency and incidence between the various groups. P values less than 0.05 were considered to be statistically significant.

Results

Patient characteristics

A total of 1000 Iranian mCRC patients tested for KRAS mutations and 242 subsets (mCRC tissue at stage IV) of them analyzed for BRAF mutations using HRM, Pyrosequencing and TheraScreen DxS methods, respectively. Table 2 characterizes the results by more details.

Frequency of KRAS mutations

We found a mutation of KRAS (codon 12 or 13) in 33.6% cases (n=336). Of KRAS mutation positive cases 85.1% were codon 12 mutant and 14.9% were codon 13 mutant (Table 1). The most common mutation at KRAS codon 12 was Gly12Asp, as shown in Figure 3. Other mutations founded in codon 12 were Gly12Val, Gly12Ser, Gly12Ala, Gly12Cys and Gly12Arg. Also the most frequent mutation at codon 13 was Gly12Asp. All detected mutations of KRAS in our study have been previously described as oncogenic (table3). None of the KRAS mutation positive cases had BRAF mutation, respectively.

Frequency of BRAF mutations

We analyzed the mutational status of BRAF gene, using HRM and Pyrosequencing methods. Among 242 patients of our mCRC specimens, BRAF mutations were not detected. According to our results the BRAF mutation occurs at very low frequency in mCRC.

Associations of Clinicopathological feature with KRAS mutations

We analyzed whether KRAS genotypes correlated with any distinguishing clinicopathological and morphological features, including sex, age of patients, histological findings, depth of invasion (pT) and Lymph nodes involvement. Our results, as shown in table 2, indicate that KRAS mutations occurred at a statistically higher frequency in older patients (>50) than in younger patients (≤50) (P=0.0001). Further statistical tests revealed that KRAS mutations have a tendency to occur in Non-mucinous adenocarcinoma than other tumor types (P=0.0001). In addition, there was a significant association of KRAS genotype with depth of invasion, so that KRAS mutations occur at a high rate in pT3 than pTis, pT1, pT2 and pT4 (P=0.0001). It is also worth mentioning that KRAS mutation tended to occur at a more frequency in male cases than in female cases, according to results of
The overall BRAF mutational frequency ranges from 3.3% to 13% in the different ethnic groups (Hsieh et al., 2012). But no such rate could be observed in our study and we determine all of Iranian mCRC grade IV patients contain wild type BRAF gene. This finding of the current study is consistent with those of Naghibalhossaini and zamani who found no V600E mutation of BRAF gene in Iranian population (Naghibalhossaini et al., 2011). It is challenging to explain this result, but it might be related to geographical variation and different ethnic groups (Yokota et al., 2011). Although the sample size in our study is reliable, however, it is suggested that further research be undertaken. In accordance to reported results in the previous literature (Rajagopalan et al., 2002; Ahlquist et al., 2008; Amado et al., 2008), KRAS and BRAF mutations are mutually exclusive in CRCs. Similarly, our results support this hypothesis and shown that none of the 336 CRCs with KRAS mutations were not coexistent with the mutation in BRAF.

In order to association investigation of clinico-pathological characteristics with KRAS genotypes, we also collected and analyzed the information on patient’s sex and age, tumor type, tumor differentiation, lymph nodes involvement and depth of invasion. There are a statistically significant association between KRAS mutations and older patients (more than 50 years) (P=0.0001). These observations suggested that KRAS gene is more disposed to the mutation with increasing of age. We also found that KRAS mutations occurred at a statistically higher frequency in non-mucinous adenocarcinoma (P=0.0011), and pT3 (P=0.0001). These data proposed that KRAS mutations might related to crucial event favoring the tumor type of non-mucinous adenocarcinoma and pT3. However, the molecular mechanisms of this tendency are unclear and more research on this topic needs to be undertaken. No statistically significant association in other selected clinicopathological features were found according to KRAS genotypes. In conclusion, the current study indicated that 33.6% of 1000 colorectal cancer sample in Iranian population showed KRAS mutations. Also mutational status of KRAS was associated with high age of patients, tumor type of non-mucinous adenocarcinoma and pT3 (P=0.0001). These data proposed that KRAS mutations might related to crucial event favoring the tumor type of non-mucinous adenocarcinoma and pT3. However, the molecular mechanisms of this tendency are unclear and more research on this topic needs to be undertaken. No statistically significant association in other selected clinicopathological features were found according to KRAS genotypes. In conclusion, the current study indicated that 33.6% of 1000 colorectal cancer sample in Iranian population showed KRAS mutations. Also mutational status of KRAS was associated with high age of patients, tumor type of non-mucinous adenocarcinoma and pT3, this is an important issue for future research.

Acknowledgements

The authors widely acknowledged the patients and their families for participation in this project. This work was supported by Iran University of Medical Sciences.

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DOI:http://dx.doi.org/10.7314/APJCP.2016.17.2.603

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