symposium article

Patterns of K-*ras* mutation in colorectal carcinomas from Iran and Italy (a Gruppo Oncologico dell'Italia Meridionale study): influence of microsatellite instability status and country of origin

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Background: K-*ras* mutations are a key step in colorectal cancer progression. Such mutations have been widely studied in case series from Western countries but there are few data on the rate and spectrum of mutations in tumors from countries where the epidemiological features of the disease are different.

Patients and methods: Tumor samples from 182 Iranian colorectal cancer patients (170 sporadic cases and 12 HNPCC cases) were screened for K-*ras* mutations at codons 12, 13 and 61 by sequencing analysis. The cases were also characterized for microsatellite instability at mononucleotide repeats by PCR and fragment analysis, and classified according to microsatellite instability status. The frequency and the spectrum of K-*ras* mutations were compared with those observed in a series of colorectal cancer patients from Italy.

Results: K-*ras* mutations were observed in 68/182 (37.4%) cases. Mutation frequencies were similar in HNPCC-associated, sporadic MSI-H and sporadic microsatellite-stable (MSS) tumors. However, the G13D substitution was more frequent in HNPCC (3/4, 75%) and sporadic MSI-H (7/11, 63.6%) tumors compared to sporadic MSS tumors (11/53, 20.4%) ($P < 0.01$). Comparison of mutations in the two series from Iran and Italy showed a significantly higher frequency of G13D among Italian patients.

Conclusions: While the frequency of K-*ras* mutations could be similar, the mutational spectrum could be differentially influenced by genetic and environmental factors.

Key words: K-*ras* mutations, colorectal carcinoma, HNPCC, MSI, Iran, Italy, gene-environment interaction

introduction

Cancer development and progression is a multi-step process based on the accumulation and clonal selection of somatic mutations in key cancer-related genes [1]. Colorectal carcinoma (CRC) provides a classic model for the study of such mutations [2]. Several epidemiological studies strongly suggest that the differences in CRC incidence observed among world populations could be at least partly attributable to differences in environmental factors, with particular regard to dietary components [3, 4]. Thus studying and comparing the molecular characteristics of CRCs from populations differing in ethnicity and environmental exposures could advance our understanding of the gene-environment interactions that influence colorectal carcinogenesis.

Mutations activating the K-*ras* proto-oncogene are considered a key step in the progression from normal colorectal epithelium to carcinoma [2]. K-*ras* is activated by point mutations resulting in single amino acid substitutions. Mutations in codons 12, 13 and 61 have been found in approximately 25%–50% of all CRCs [5–7]. Different dietary carcinogens have been shown to induce characteristic K-*ras* point mutations [8, 9].

High-level microsatellite instability (MSI-H) is a form of genomic instability that occurs in hereditary non-polyposis colorectal cancer (HNPCC) and in about one-fifth of all sporadic CRCs [10]. MSI-H is attributed to defects in DNA

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mismatch repair (MMR). Although genes with repetitive sequences are clear targets of defective MMR, mutations in non-repetitive sequences are also found in MSI-H tumors. MSI-H CRCs, whether inherited or sporadic, tend to be right-sided and to show poorly differentiated mucinous phenotype. It has been a matter of controversy whether CRCs with deficient versus competent MMR share the same pattern of somatic alterations in K-ras.

A number of studies analyzed the rate and spectrum of K-ras mutations in relation to MSI status. Most authors reported similar frequencies of K-ras mutations in HNPCC-related and sporadic CRCs [11–14] as well as in MSI-H and microsatellite-stable (MSS) CRCs, although this was not confirmed in other studies [15, 16]. Overall the vast majority of the studies were based on patients from Western countries, and there are few data available on the mutational characteristics of tumors from developing countries. The incidence rates of colorectal cancer in developing countries have been shown to be lower compared with the figures observed in the West. However a marked increase in CRC incidence has been observed during the last decades in various countries, including Iran [17–19]. A possible explanation for the reported CRC increase in Iran might be related to changes in dietary habits and lifestyle.

We have analyzed K-ras mutation rate and spectrum in a hospital-based Iranian CRC series presenting a relatively high frequency of clinically-diagnosed HNPCC [20]. The spectrum of K-ras mutations detected in this case series has been correlated with the presence or absence of clinical HNPCC criteria and with tumor MSI status. Furthermore, the frequency and pattern of K-ras mutations found in the Iranian series have been compared with those observed in a well-characterized CRC series from southern Italy [21].

patients and methods

cases and DNA extraction

One hundred and ninety formalin-fixed, paraffin-embedded CRC cases from patients who underwent surgical resection from February 1998 to September 2003 in two major hospitals in Tehran (Atieh and Mehr) were selected for this study. Patients with CRC associated with inflammatory bowel disease, familial adenomatous polyposis and patients who had undergone preoperative chemotherapy or radiotherapy were not included. Out of 182 patients whose samples could be successfully analyzed, 79 (43.4%) were females and 103 (56.6%) males. Fifty-eight (32%) tumors were located in the right side of the colon, 65 (35.7%) in the left colon and 53 (29%) in the rectum, the location of six (3.3%) tumors was not specified. The study was reviewed and approved by the ethics committee of the Digestive Disease Research Center of Tehran University of Medical Sciences. Archived histological sections were reviewed by the collaborating pathologists in Tehran. Colorectal adenocarcinoma diagnosis was confirmed after review at the Pathology Section of the Center of Excellence on Ageing (CeSI), Chieti, Italy.

For each case an area with at least 50% neoplastic cells and an area including normal *muscularis propria* and/or CRC-unaffected mucosa was identified on H&E-stained slides and used to guide manual microdissection for DNA extraction. Serial sections of 15-µm thickness were prepared for DNA extraction. Selected areas were dissected from de-waxed step-sections by gentle scraping. Scraped tissue was digested by incubation for 1–2 days at 56°C in 100 µl of buffer containing TRIS (50 mM pH 8,5), EDTA (1 mM), TWEEN 20 (0,5%) and proteinase K (20 mg/ml). The extracted DNA was

purified with the QIAamp DNA minikit (Qiagen, Hilden, Germany) following manufacturer's instructions.

K-ras analysis

De-waxing, microdissection, DNA extraction and PCR set-up were all performed in a dedicated laboratory free of contamination from PCR products. Exons 1 and 2 of K-ras were individually amplified by 40-cycle PCRs using primers specific for the K-ras gene. Primers for exon 1 were: K-ras -F: 5′-TTTTATTATAAGGCCTGCT-3′ and K-ras -R: 5′-GTCCTGCACCAGTAATATGC-3′. PCRs were in 30 µl volumes with 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 200 µM dNTPs, 1.5 units of AmpliTaq Gold (Applied Biosystems, Foster City, CA), 0.5 µM of each primer and 1–3 µl of DNA extract. PCR conditions were 40 cycles of 50°C for 45 s, 72°C for 45 s and 94°C for 30 s using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA). Primers and PCR condition for exon 2 were as described by Semczuk et al. [22]. PCR products were visualized by ethidium bromide staining on 2% agarose gel electrophoresis and purified with Montage PCR microfilters (Millipore Corporation, Bedford, MA). PCR products were directly sequenced using the ABI PRISM Big Dye™ Terminator v3.1 Cycle Sequencing Ready Reaction Kit and visualized by capillary electrophoresis with an ABI 310 Genetic Analyzer according to the supplier's instructions (Applied Biosystems, Foster City, CA, USA). All samples with mutations were verified by two independent cycle sequencing PCR reactions and analysis of both DNA strands.

MSI analysis

DNA from microdissected normal and tumor areas was purified as described above. MSI analysis was based on two mononucleotide repeats, BAT25 and BAT26, known to be the most sensitive markers of MSI-H status and widely regarded as sufficient for the identification of CRCs with MMR defect [23, 24]. PCRs for repeats were performed using 6-FAM-labelled forward primers. The sequences of the oligonucleotides were as follows: BAT25-F: 5′-CTAAAGAGTTTTGTGTTTTG-3′ and BAT25-R: 5′-GGCTCTAAAATGCTCTG-3′; BAT26-F: 5′-TACTTTTGACTTCAGCCAG-3′ and BAT26-R: 5′-CCAATCAACATTTTTAACCC-3′. PCR for both markers were carried out in 20 µl volumes containing PCR buffer, 1.5 mM MgCl₂, 0.4 µM of primers, 0.2 mM each dNTP and 0.5 units AmpliTaq Gold™. PCRs consisted of an initial denaturation step at 94°C for 10 min, followed by 35 cycles at 94°C for 10 s, 55°C for 30 s and 72°C for 2 min, and a final extension step at 72°C for 10 min. An aliquot (1.5 µl) of the product was mixed with 0.05 µl of ROX size standard, 9 µl formamide and 0.45 of ddH₂O. The samples were then denatured and loaded onto an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The data were processed using GeneScan software. A difference in the electrophoretogram pattern between normal and tumor DNA was regarded as evidence of MSI for that marker. The tumors were classified as MSI-H when at least one of the two markers showed tumor-associated allele shifts.

comparisons of K-ras mutation frequencies

Patients belonging to families fulfilling the Amsterdam II criteria (i.e. at least three members with an HNPCC-associated cancer—colorectal, endometrial, small bowel, ureter, renal pelvis—in at least two successive generations, one being a first-degree relative of the other two and at least one diagnosed before the age of 50 years), were classified as HNPCC [25]. The remaining CRC cases were classified according to MSI status in MSS and MSI-H. The spectra of K-ras mutations (codon distribution and type of mutation) were compared in these subgroups.

The overall observed frequency and spectrum of K-ras mutations were also compared with the relevant data from 160 CRC patients from southern

Italy previously described by Bazan et al. [21]. Statistical comparisons were performed by χ^2 and Fisher's exact tests using SPSS for IBM PC (Chicago, IL). P values <0.05 were considered significant.

results

Of the 182 Iranian CRCs analyzed in this study 68 (37.4%) showed K-ras mutations. The frequency of K-ras gene mutations observed in women (34/79, 43%) was slightly higher than that observed in men (34/103, 33%), but the difference was not significant ($P = 0.12$).

Of all CRCs, 12 cases were classified as HNPCC according to Amsterdam II criteria. MSI analysis was done in all 182 tumors; all HNPCC cases were MSI-H, of the remaining 170 sporadic cases 33 (19.4%) were MSI-H and 137 (80.6%) did not show microsatellite instability at the markers tested and were classified as MSS.

In the 68 samples with K-ras mutations, codon 12 was the most frequently mutated (45/68 cases, 66%), followed by codon 13 (22/68 cases, 32.5%), while codon 61 was the least mutated (1/68 cases, 1.5%). K-ras mutations were then compared in the three CRC subgroups (MSI-H HNPCC-associated, 12 cases; sporadic MSI-H, 33 cases; sporadic MSS, 137 cases). The overall prevalence of K-ras mutations was not different between these groups [HNPCC-associated: 4/12 (33.3%), sporadic MSI-H: 11/33 (33.3%) and sporadic MSS: 53/137 (38.7%), Table 1]. However the frequencies of K-ras mutations at codons 12 and 13 differed according to MSI status (Table 1). Sporadic MSS CRCs harbored a significantly lower frequency of mutations at codon 13 compared with sporadic MSI-H or HNPCC-associated CRCs (23% versus 63.5% and 75% respectively; Table 1).

K-ras mutation types also tended to differ in these subgroups. G to A transitions and G to T transversions were observed with similar frequencies (47% and 49%, respectively) in sporadic CRCs with MSS phenotype. However, G to A transitions were the most frequent mutation in HNPCC-associated CRCs

(100%, 4/4 with K-ras mutation) and in MSI-H CRCs (82%, 9/11 with K-ras mutation; Table 1).

The type of K-ras amino acid change also varied among the three CRC subgroups. The frequency of glycine to aspartate (G13D) substitutions at codon 13 was significantly higher in HNPCC-associated and sporadic MSI-H cases compared with sporadic MSS cases (Table 1). There was also a trend ($P = 0.099$) towards a higher frequency of G12V, resulting from a G to T substitution, in sporadic MSS cases compared with the other two subgroups (Table 1).

The frequency and spectrum of K-ras mutations found in the Iranian case series were then compared with those observed in the CRC patients from southern Italy (Table 2). In the Italian series the frequency of K-ras mutations (74/160, 46.3%) was higher than that observed in the Iranian CRCs (68/182, 37.4%), but the difference did not reach statistical significance ($P = 0.09$). We also compared the spectrum and codon distribution of the K-ras mutations detected in the two series. G to A transitions were more frequent in the series from Italy (Table 2). Codon 13 was more frequently affected in the Italian CRCs, whereas codon 12 was equally affected in both series (Table 2). Comparison of the two series with regard to K-ras amino acid change showed that G13D was significantly more frequent in the Italian CRCs. Other K-ras amino acid changes did not differ between the two series (Table 2).

discussion

It is widely accepted that mutations that activate K-ras are among the critical transforming genetic alterations occurring during human colorectal tumorigenesis [2]. Although K-ras mutations have been widely studied in CRCs from Western countries there are few data on K-ras mutation rate and spectrum in CRCs from developing countries, such as Iran. Iran has an age-adjusted CRC incidence rate of 6–7.9 per 100 000 persons/year [17, 26], which is remarkably lower than the rates reported in Western countries, ranging from 20 to 40 per

Table 1. Frequency of K-ras mutations, nucleotide substitutions and amino acid changes in three clinical settings of 182 colorectal cancers

	HNPCC (n = 12)	MSI-H (n = 33)	MSS (n = 137)	P value
K-ras mutation				
Present	4 (33.3%)	11 (33.3%)	53 (38.7%)	0.8
Mutated codon[a]				
Codon 12	1 (25%)	4 (36.5%)	40 (77%)	<0.01
Codon 13	3 (75%)	7 (63.5%)	12 (23%)	
Total nucleotide substitutions[b]				
G → A	4 (100%)	9 (82%)	25 (47%)	<0.01
G → T	0 (0)	2 (18%)	26 (49%)	
Amino acid changes[c]				
G12D transition at 2nd pos. codon 12 (Asp)	1 (25%)	2 (18.2%)	13 (24.1%)	0.9
G12C transversion at 1st pos. codon 12 (Cys)	0 (0)	1 (9.1%)	8 (14.8%)	0.6
G12V transversion at 2nd pos. codon 12 (Val)	0 (0)	1 (9.1%)	17 (31.5%)	0.1
G13D Transition at 2nd pos. codon 13 (Asp)	3 (75%)	7 (63.6%)	11 (20.4%)	<0.01

[a]Codon 61 was only mutated in one tumor and was not considered for statistical analysis.
[b]A → C (codon 61) and G → C each were observed in only one tumor and were not considered for statistical analysis.
[c]G12S, G12A, G13C and Q61H were each observed in one tumor and were not considered for statistical analysis.

Table 2. Frequency of K-*ras* mutations, types of nucleotide substitution and amino acid changes in 182 CRCs from Iran and 160 CRCs from Italy

	Iran (n = 182)	Italy (n = 160)	P value
Cases with K-*ras* mutation	68 (37.4%)	74[a] (46.3%)	0.09
Mutated codon			
Codon 12	45 (24.7%)	46 (28.8%)	0.5
Codon 13	22 (12.1%)	34 (21.3%)	<0.05
Total nucleotide substitutions			
G → A	38 (21%)	51 (32%)	<0.05
G → T	28 (15.4%)	21 (13%)	0.6
G → C	1 (0.5%)	2 (1.3%)	0.6
Amino acid changes[b]			
G12D transition at 2nd pos. codon 12 (Asp)	16 (8.8%)	17 (10.6%)	0.6
G12C transversion at 1st pos. codon 12 (Cys)	9 (4.9%)	9 (5.6%)	0.8
G12V transversion at 2nd pos. codon 12 (Val)	18 (9.9%)	13 (8.1%)	0.6
G13D transition at 2nd pos. codon 13 (Asp)	21 (11.5%)	32 (20%)	<0.05

[a]In CRCs from Italy 80 K-*ras* mutations were observed in 74 patients.
[b]G12S, G12A, G13C and Q61H were not considered for statistical analysis due to very low frequency in both series.

100 000 persons/year [27]. Nevertheless, the frequency of familial forms of CRC such as HNPCC seems to be relatively high in Iran (4.7%) [20]. This is consistent with the results of our study showing a relatively high percentage of MSI-H CRCs meeting HNPCC criteria in the presently analyzed series. Overall, we found 45/182 (24.5%) patients with MSI-H phenotype (including HNPCC patients), a rate higher than reported in studies from Western countries (12%–17%) [10, 28]. This may also be related to the relatively high number of right-sided CRCs in the series (58/182, 32%), which is consistent with results from a population-based study in Iran indicating a relatively high rate of proximal colon cancer [17].

In this study, conducted on 182 Iranian CRCs from Tehran, we found an overall K-*ras* mutation rate of 37.4%. This rate is within the range of 25%–50% reported in studies of other CRC series [5–7]. The frequency of K-*ras* mutations did not differ between HNPCC-associated, sporadic MSS and sporadic MSI-H CRCs. In line with this observation there is some evidence [11–14] that HNPCC-associated CRC may progress through molecular pathways similar to those of sporadic CRC with regard to K-*ras* mutations. It has also been reported that the subset of sporadic MSI-H CRC with hypermethylation of the hMLH1 promoter is less frequently mutated in K-*ras* compared with sporadic MSI-H CRC negative for hMLH1 methylation and to both HNPCC-associated and MSS CRC [14].

Mutations of K-*ras* result in specific amino acid substitutions that lead to permanent activation of the encoded p21 ras protein [29]. It is well known that activating K-*ras* mutations cluster at codons 12 and 13 (GTP-binding domain). In the current Iranian series, 98.5% of the detected mutations occurred at these codons and only one CRC carried a mutation at codon 61. This confirms that codons 12 and 13 are preferentially involved in CRC progression also in the Iranian population. Looking at codons 12 and 13, we found significant differences between HNPCC-associated, sporadic MSI-H and sporadic MSS CRC. The frequency of K-*ras* mutations at codon 12 was higher in sporadic MSS cases whereas mutations at codon 13 were much more common in HNPCC-associated and sporadic MSI-H CRCs.

The type of K-*ras* mutation also differed between CRC subgroups. The frequency of G to A transitions was significantly higher in HNPCC-associated and sporadic MSI-H CRC than in MSS CRC. Thus hereditary and sporadic tumors with MSI-H seemed to preferably harbor G to A transitions, suggesting a link between this specific type of mutation and MMR defect. In this respect it could be noteworthy that defects in other DNA repair systems, such as O6-methylguanine-DNA methyltransferase (MGMT) activity, are linked to inability to protect from G to A transition in K-*ras* induced by alkylating agents [30–32]. It has been suggested that other repair systems could also contribute to this repair mechanism [31]. Further grouping of K-*ras* mutations with regard to type and nucleotide position showed that G13D, resulting from a G to A transition at the second nucleotide of codon 13, was significantly less frequent in sporadic MSS CRC while it had the highest frequency in HNPCC-associated CRC. This is in concordance with other reports, which showed that G13D is the most common type of K-*ras* mutation in HNPCC-associated CRC [13, 14]. In addition, consistently with other studies [14, 33] the frequency of G12V was considerably high (31.5%) in sporadic MSS CRCs (Table 1).

Apart from genetic factors, accumulating evidence suggests that environmental factors such as diet could be involved in CRC progression and could induce specific K-*ras* mutations. Diet-related carcinogens can induce K-*ras* mutations [34] and dietary components were shown to influence the rate and spectrum of K-*ras* mutations in CRC [8]. Dietary factors may also affect clonal selection by modifying the growth of tumors harboring specific K-*ras* mutations [35, 36]. This could link specific types of K-*ras* mutations with specific nutritional patterns, deficient or excessive in particular dietary component(s).

We compared the rate and spectrum of CRC-associated K-*ras* mutations from Iran and Italy as representatives of countries with differences in CRC incidence [26, 27]. We found an overall rate of K-*ras* mutations of 37.4% in the Iranian CRC series and of 46% in the Italian series. Although there is a considerable

difference between these two rates, this did not reach statically significance. Moreover, G to A transitions were the most common mutation type observed both in Iranian (57.6%) and in Italian (70.8%) CRCs, a finding in agreement with reports concerning other populations [5, 7]. There was a higher frequency of G to A transitions in CRC from Italy compared to Iran. Although mutations in codon 12 were similar in both series, codon 13 was more frequently affected in CRC from Italy. Comparison of the specific types of K-*ras* mutation between the two series revealed that there was a significantly higher frequency of G to A transition mutations in the second base of codon 13 (G13D) among Italian CRCs, which mainly contributed to the higher G to A transition rate and higher codon 13 mutation frequency. This type of mutation has been linked with high consumption of refined grain [8], a dietary pattern directly associated with CRC risk [37, 38].

The Italian diet, particularly in southern Italy, is characterized by a high intake of refined grain such as pasta and white bread as the top two sources of energy [39, 40]. In contrast, the Iranian diet is wheat-based with a variety of unrefined, unleavened, whole-wheat breads comprising the main staple. Wholegrain foods may reduce the risk of several types of neoplasms, particularly of the digestive tract, including CRC [41].

In conclusion, in the currently studied Iranian series HNPCC-associated CRCs, sporadic MSI-H and sporadic MSS shared similar K-*ras* mutation frequencies; however, the spectrum of K-*ras* mutations differed in the above mentioned CRC subsets. Specifically, G13D mutations in K-*ras* seemed to be associated with CRC characterized by defect in MMR, as indicated by MSI-H phenotype. Furthermore, we found differences in the amino acid changes in the series from Iran compared with that from Italy. These could be attributed to differences in dietary habits. Overall our data suggest that the spectrum of K-*ras* mutations could be differentially influenced by genetic and environmental factors.

acknowledgements

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