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ÖZGÜN ARAŞTIRMA/ORIGINAL ARTICLE

Kolon Adenokarsinomlarında KRAS Mutasyonlarının Sıklığı ve Lenf Nodu Metastazı ile İlişkisi

Incidence of KRAS Mutations in Colon Adenocarcinomas and its Association With Lymph Node Metastases

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ÖZ

Amaç: Gastrointestinal sistemin en yaygın malignitesi olan kolon kanserleri mortalite ve morbiditenin ana nedenlerinden biridir. Kolorektal kanserlerin %30-40'ında KRAS gen mutasyonu izlendiğinden tedaviye başlamadan önce mutasyon taraması önemlidir. Bu çalışmadaki amacımız orta derece diferansiye kolon adenokarsinomlarında KRAS kodon 12, 13, 61 mutasyon durumunu belirlemek ve bu mutasyonların lenf nodu metastazı ile ilişkisini araştırmaktır.

Gereç ve Yöntem: Fırat Üniversitesi Tıp Fakültesi Tıbbi Patoloji Anabilim Dalı arşivinden seçilen 60 adet lenf nodu metastazlı ve 60 adet lenf nodu metastazı olmayan toplam 120 adet orta derecede diferansiye kolon adenokarsinomu örneğinden uygun primer çiftleri PZR ile çoğaltılıp sekans analizi ile KRAS mutasyon durumu belirlenmiştir.

Bulgular: Çalışmamıza alınan 120 olguda 40 tanesi kodon 12 de ve 5 tanesi kodon 13 de olmak üzere %37.5 (45/120) oranında KRAS mutasyonu izlendi. KRAS kodon 61 de mutasyona rastlanmadı.

Sonuç: Çalışmamızda KRAS mutasyon durumu ile lenf nodu metastazı arasında anlamlı bir ilişki bulunmadı. Bu çalışma küçük bir alanda öncesinde tanı almış sınırlı sayıda olguda yapıldı; ancak çok merkezli ve daha geniş çalışmalar için bir ön izlenim taşıma özelliğindedir.

ABSTRACT

Objective: Colon adenocarcinoma, one of the most frequent malignant tumors of the gastrointestinal system, is a major cause of morbidity and mortality. As KRAS mutations are encountered in 30-40% of colorectal carcinomas, a mutation screening is required before therapy commences. In this study, we aim to determine KRAS codon 12, 13 and 61 mutations in moderately differentiated colon adenocarcinomas and assess whether these mutations are associated with lymph node metastases.

Material and Method: A total of 120 moderately differentiated colorectal carcinomas, 60 with lymph node metastases and 60 without, were included. Samples underwent PCR with appropriate primers and Sanger sequencing was carried out to determine their KRAS mutation status.

Results: Out of the 120 cases included in our study, 40 carried codon 12, 5 carried codon 13 mutations. In total, 37.5% of cases had a KRAS mutation (45/120). No mutation was detected in codon 61.

Conclusion: Our study has not shown a significant association between the presence of KRAS mutations and lymph node metastasis. This study was conducted in a limited number of patients and in a pre-defined, small area; but can be used as a preliminary step for multicenter and larger studies.

Introduction

Colorectal adenocarcinoma is the most prevalent malignant tumor of the gastrointestinal system and is a leading cause of morbidity and mortality (1). Colorectal cancers are the 3rd most frequent cancers worldwide and comprise the 4th most common cause of mortality (2). In the United States of America, 150000 people get diagnosed with colorectal cancer and 50000 die of the disease every year (3).

98% of colorectal carcinomas are adenocarcinomas. The majority develop from adenomatous polyps and are amenable to treatment with successful outcome when diagnosed early (4). Colorectal carcinomas arise following a sequence of molecular events and this event is characterized by a progression from normal mucosa to changes in a single crypt, adenomatous polyps and adenocarcinoma (5). One of the important actors in colorectal adenocarcinoma pathogenesis is the EGFR (Epidermal Growth Factor Receptor) activation and resultant RAS-MAPK (Rat Sarcoma-Mitogen Activated Protein Kinase) pathway stimulation. RAS gene mutations are thought to be an initiating factor of colorectal tumors, with RAS-mutated adenomas progressing to cancer faster than those without (6). Targeted therapy modalities developed in recent years depend on targeting this pathway. Codons 12, 13 and 61 of KRAS are mutation hotspots (7, 8). These mutations result in activation of RAS protooncogene-regulated pathways, autonomous cell growth and proliferation (6, 9).

Information on KRAS mutation status is used to determine potential therapeutical efficiency of EGFR-targeting agents. Lack of response to EGFR-targeting agents on patients with KRAS mutations has been reported repeatedly (10-12). Because 17-25% of all human tumors and 30-40% of colorectal carcinomas harbor KRAS mutations, screening for mutations is important before the onset of therapy (10).

In the present study, we aim to evaluate KRAS codon 12, 13, 61 mutations and assess the relationship between these mutations and lymph node status of tumors.

Material and Method

Patient Selection

Sixty cases of node-positive moderately differentiated colorectal adenocarcinomas and 60 node-negative cases of moderately differentiated colorectal adenocarcinomas, all of which were diagnosed and archived between 1996 and 2014 in Firat University School of Medicine Department of Pathology were included in the study. Cases were reviewed for diagnostic confirmation. The study was conducted with Firat University Ethics Committee for Non-Interventional Studies' approval number 06 dating 01.28.2014. Patients were informed about the study, their consent was obtained from themselves and legal representatives. The study was conducted in accordance with Helsinki Declaration Ethical Principles for Medical Research Involving Human Projects.

Mutation Detection

Isolation of DNA from Paraffin Blocks

Five 20 µm-thick sections were prepared per block and placed in Eppendorf tubes. Deparaffinization was achieved after treatment with heat, xylene and alcohol. For lysis, samples were kept at a 37˚C water bath overnight after addition of Proteinase K, Tris-HCl solution (1M), EDTA (0.5M) and SDS (10%). DNA extraction was done using the phenol-chloroform method. DNA was purified through ethanol precipitation. Samples were assessed for DNA concentration and purity, those that were considered sufficient in amount and quality were kept in +4˚C for further use. Cases that did not yield DNA of adequate quality underwent another round of DNA extraction.

Sanger Sequencing

A polymerase chain reaction was carried out before Sanger sequencing to amplify the target sequence. For this end, two primer pairs were designed, one to cover K-RAS gene exon 2 (245 bp) and the other for exon 3 (197 bp). Two different sets of PCR were run using these primer sets (One cycle of the following; 5 min at 94 °C, 1 min at 55 °C, 1 min at 72 °C. Then 35 cycles of the following; denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, elongation at 72 °C for 1 min. Then 1 cycle of the following; 5 min at 94 °C, 1 min at 55 °C, 7 min at 72 °C). PCR products were run on agarose gels and PCR was repeated for the samples that did not yield bands of sufficient quality. All samples were run at the ABI 3100 Genetic Analyzer with the forward primer for sequencing and results were analyzed using Sequencing Analysis 5.1 software.

Statistical Analyses

Pearson correlation analysis was used to prepare the statistics and to assess the relationship between parameters. The distribution of genotypes and allele frequencies among patients was done by chi-square analysis. A non-parametric test, Mann-Whitney U was used to evaluate the differences among groups. $p<0.05$ was considered statistically significant.

Results

Clinicopathological Distribution of Cases

Seventy-one (59.2%) of the 120 cases included in the study were men, 49 (40.8%) were women. Of the 60 cases without lymph node metastases (no LNM), 31 (51.6%) were men and 29 (48.3%) were women; of those that had lymph node metastases (with LNM), 40 (66.6%) were men and 20 (33.3%) were women. There was no statistically significant difference among groups with regard to sex ($p>0.05$). Average age was 61.76±12.67 (30-89). Cases without LNM had an average age of 63.35±13.07, cases with LNM had an average age of 60.18±12.17. No statistically significant difference was found between groups on average ages ($p>0.05$). The smallest tumor diameter was 1.5 cm and the biggest tumor was 13 cm; average tumor diameter was 5,37±2.31. No significant difference was noted between the tumor sizes of the two groups ages ($p>0.05$).

Tumors' localization distribution was as follows; 11 (9%) in caecum, 33 (27.5%) in the right colon, 6 (5%) in the transverse colon, 29 (24.1%) in the left colon and 41 (34.1%) in the sigmoid colon. In our study, it was noted that tumors of the transverse colon and left colon tended to have a higher rate of lymph node metastases; this difference was found to be statistically significant ($p<0.05$). Nine cases without LNM (15%) were located in the caecum while only two of cases without LNM (3.3%) were located in the caecum. Only one of the cases without LNM (1.6%) was located in the transverse colon and 10 (16.6%) were in the left colon; those with LNM had 5 cases (3.3%) located in the transverse colon and 19 (31.6%) in the left colon.

KRAS Mutation Status

Forty-five of all the cases included in the study displayed KRAS mutations (35%); 75 (62.5%) did not. All mutated cases showed alterations in the second exon. In 40 cases (88.9%) the mutation was in codon 12, in 5 cases (11.1%) in codon 13. No mutations were detected in codon 61.

In cases without LNM, 21 (35%) had KRAS mutations and 39 (65%) had none. Twenty-four (40%) cases with LNM displayed KRAS mutations, 36 (60%) did not. Cases with LNM tended to have a higher frequency of KRAS mutations but this difference was not statistically significant ($p>0.05$). Forty (88.9%) of mutated cases had alterations in codon 12, 5 (11.1%) in codon 13. In the 21 cases without LNM that harbored KRAS mutations, 18 (85.7%) had them in codon 12, 3 (14.3%) in codon 13. Twenty-two (91.6%) of the 24 cases with LNM had codon 12 mutations, 2 (8.4%) had them in codon 13. There was no statistically significant difference between the groups in terms of the mutated codon ($p>0.05$).

Associations of Clinicopathological Variables with KRAS Mutations

Twenty-seven of the 45 mutated cases (60%) were men and 18 (40%) were women. Of the 75 non-mutated cases, 44 (58.6%) were men and 31 (41.3%) were women. Mutated cases had a mean age of 59.95±11.2, mean age of non-mutated cases was 62.85±13.4. sex and age were not statistically associated with the mutation status ($p>0.05$ for both). Mutated cases had a mean tumor diameter of 5.70 2.46 cm, wild-type tumors had a mean diameter of 5.18 2.21 cm; tumor size and mutation status were not statistically related ($p>0.05$). In the 45 mutated cases, 6 tumors were located in the caecum, 13 in the right colon, 2 in the transverse colon, 10 in the left colon and 14 in the sigmoid colon. Among the 75 non-mutated cases, 5 primary tumors were located in the caecum, 20 in the right colon, 4 in the transverse colon, 19 in the left colon and 27 in the sigmoid colon. There was no statistically significant difference between KRAS mutation status and tumor localization ($p>0.05$).

Associations of variables of age, gender, tumor localization, tumor diameter and mutation status were assessed separately in patient groups that did and did not have lymph node metastases; no statistically significant association was detected.

Distribution of KRAS Point Mutations

In the total of 45 cases that harbored KRAS mutations; in codon 12, 16 point mutations (35.5%) were Gly12Asp (Glycine-Aspartate), 11 (24.4%) were Gly12Val (Glycine-Valine), 5 (11.1%) were Gly12Cys (Glycine-Cysteine), 5 (11.1%) were Gly12Ala (Glycine- Alanine), 3 (6.6%) were Gly13Ser (Glycine-Serine). Five (11.1%) of the codon 13 mutations were Gly13Asp. The point mutation distribution of the 21 mutated cases that had no LNM was as follows: 7 (33.3%) Gly12Asp, 5 (23.8%) Gly12Val, 3 (14.3%) Gly12Cys, 2 (9.5%) Gly12Ala, 1 (4.7%) Gly13Ser. Three cases (14.3%) that had codon 13 mutations had Gly13Asp alterations. The point mutation distribution of the 24 mutated cases that had LNM was as follows: 9 (37.5%) Gly12Asp, 6 (25%) Gly12Val, 2 (8.3%) Gly12Cys, 3 (12.5%) Gly12Ala, 2 (8.3%) Gly13Ser. Both codon 13 mutations (8.3%) were Gly13Asp alterations.

Discussion

Rapid advances in molecular biology enable better portrayal of physiopathological properties of diseases. One of the best explained cancers is the colon carcinoma. Delineation of pathogenetic mechanisms underlying colon cancer development has increasing value in early diagnosis, assess prognosis and implement additional suitable therapy options. KRAS gene mutations are detected in 17-25% of all human tumors and 30-40% of colorectal carcinomas; newly-developed EGFR blockers do not show effects on KRAS-mutated cases. Therefore, both primary and metastatic lesions undergo mutation analysis, where cases with wild-type KRAS mutation can receive targeted therapy (EGFR blocker) (10, 12).

The presence of KRAS mutations are used as a harbinger of resistance to anti-EGFR antibody treatment (13). Cetuximab and panitumumab, two anti-EGFR agents, show efficacy in 10-20% of patients with colorectal cancer (14, 15). American Society of Clinical Oncology recommend all cases of colorectal carcinoma to be assessed for KRAS mutations before the induction of anti-EGFR antibody treatment and the cancellation of this therapy if a KRAS mutation is detected (15, 16).

KRAS mutation frequency of colorectal carcinomas in different ethnic groups (such as Italy, Sweden, China, Germany, Japan) range between 20% and 50% (17-21). Our study is a retrospective study that assessed the KRAS mutation status in a limited set of colorectal adenocarcinoma; the locality of the study precludes representation of the whole population. However, tumor and patient characteristics of the cases chosen for the study is congruent with that of the general population. In the present study, 37.5% of the total 120 patients displayed KRAS mutations; this ratio is similar to those reported in the literature.

The facts that KRAS mutation testing provides for patient-based treatment options and prevents loss of resources and time spent on unnecessary treatment modalities have driven researchers to discover new genetic markers to provide both prognostic information and allow prediction of therapy outcomes (12, 22, 23). For this purpose, in addition to BRAF that has already taken its place in routine testing, it will efforts are made to biomarker panels expanded to include PTEN, NRAS and PIK3CA (24). However, because the roles of the latter two genes are not fully elucidated, molecules other than KRAS and BRAF are not in routine use (12, 25).

Phase 3 studies have shown that KRAS-mutated colorectal tumors do not benefit from agents targeting EGFR (10-12, 26). The most frequent mutations in colorectal cancers are on KRAS codons 12 and 13, rarely on codon 61 (7, 8, 16). The present study displayed that 37.5% of cases had KRAS mutations and mutations were found on codons 12 or 13, with no alterations in codon 61.

Several different techniques can be implemented to determine KRAS mutations; with real-time PCR with specially designed primers, pyrosequencing and Sanger sequencing among them (27-29). In addition to its high sensitivity and specificity, Sanger sequencing has an advantage of detecting all and any base changes. The present study implemented Sanger sequencing to detect both hot-spot mutations and other mutations, but no base changes were observed out of the frequently mutated sites.

A literature overview has shown us that colorectal carcinomas with lymph node metastases tend to have a lower incidence of KRAS mutations than those without lymph node metastases (30, 31). Despite the lack of a statistically significant association, the present study shows a higher rate of KRAS mutations in lymph node – positive cases, which contradicts previous reports on the subject. However, larger sets of cases need to be evaluated to validate the difference in mutation rates. In addition, in parallel to the present study, many others assessing the relationship between KRAS mutation status and clinicopathologic data have found no statistically significant relationship (32, 33).

Conclusion

This retrospective study demonstrates the associations between major clinicopathological variables and KRAS mutation status in a limited set of colon adenocarcinomas encountered in and around Elazig province of Turkey. Despite the lack of a statistically significant association, there was a tendency for a higher frequency of KRAS mutations among colorectal carcinomas with lymph node involvement. This finding needs re-testing in larger series with a more diverse patient participation.

Yazarlık katkısı: Fikir/Hipotez: GV, İHÖ, EÖ Tasarım: GV, İHÖ, EÖ Veri toplama/Veri işleme: GV, İHÖ, EÖ Veri analizi/Makalenin hazırlanması: GV, EÖ Makalenin kontrolü: GV

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