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Different types of K-Ras mutations are conversely associated with overall survival in patients with colorectal cancer

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Abstract. A glycine to valine substitution at codon 12 (G12V) in Kirsten-Ras (K-Ras) gene has been associated with reduced overall survival in colorectal cancer patients; however, the effect of other K-Ras mutations than G12V still remains unclear. Therefore, we investigated the role of different K-Ras mutations on overall survival in a homogeneous, large patient cohort with standardized therapy and uniform analysis of K-Ras mutation status. The study included 342 patients with histopathologically proven colorectal cancer. Survival data were provided by the federal agency for statistics in Austria. Occurrence of K-Ras mutations at codons 12, 13 and 61 were determined by capillary sequencing. The overall K-Ras mutation frequency in carcinoma tissue was 28%. Carriers of the G12V mutation at the K-Ras gene showed a significantly decreased overall survival compared to carriers of the wild-type [HR=2.56 (1.15-5.69)]. Other mutations than G12V were associated with better overall survival compared to wild-type [HR=0.44 (0.2-0.99)]. In conclusion, for the first time, our study showed clearly that different types of K-Ras mutations are conversely associated with overall survival in patients with colorectal cancer.

Introduction

Colorectal cancer (CRC) is the second most common incidental form of cancer, with 376,400 new cases diagnosed and 203,700 related deaths annually in Europe (1). Genetics play a key role in predisposition, initiation and progression of CRC. The multistep progression from normal colon epithelium to adenomatous polyp and finally to invasive cancer is characterized by two classes of genes, tumor-suppressor genes and proto-oncogenes (2,3). In this context, mutations in the proto-oncogene Kirsten-Ras (K-Ras) are involved in 22-46% of colorectal carcinogenesis (4-9).

Mutations in the K-Ras gene, especially a glycine to valine substitution at codon 12 (G12V), has been proposed to be an independent risk factor for reduced overall survival in colorectal cancer patients (5). Moreover, recent data have shown that mutations activating the Ras signalling pathway are also predictive for response to anti epidermal growth factor receptor (EGFR) monoclonal antibodies. The presence of mutations in the K-Ras gene has been associated with the lack of response to anti-EGFR monoclonal antibody treatment (10-14). Thus, there is increasing clinical relevance in the detection of mutations in the K-Ras gene with sensitive molecular methods to improve risk stratification and therapeutic strategies.

However, experimental *in vitro* data suggest that codon 12 K-Ras mutations differ in carcinogenic potential and prognostic significance (15,16). Previous studies merely showed G12V mutation to be an independent risk factor for reduced overall survival. Therefore, we aimed to further elucidate the role of different K-Ras mutations in overall survival in a large, homogeneous patient cohort with standardized therapy and uniform analysis of K-Ras mutation status. We investigated the association between G12V and other K-Ras mutations, considering the fact that different mutations may show different effects on overall survival.

Materials and methods

Patients. We considered 342 patients with histologically proven colorectal cancer at the Department of Pathology at the Academic Teaching Hospital Feldkirch from January 2003 to October 2006. Inclusion criteria were histologically confirmed colorectal cancer and patients undergoing radical surgery. After appropriate investigational review board

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Table I. Patient characteristics.

	Total n=342
Gender (m/f), n	195/147
Age (years), mean ± SD	69 ± 11.5
Staging (UICC), n	
Stage I	72
Stage II	140
Stage III	101
Stage IV	29
Grading, n	
G 1	104
G 2	205
G 3	28
G 4	1
Unknown	3
Causes of death, n	
Tumor	54
Non-tumor related	3
Tumor localisation, n	
Colon	245
Rectal	97

Table II. Non-synonymous K-Ras mutation status of patients with colorectal carcinoma.

Amino acid change	Total n=96/342 (%)
Codon 12	
G12S (Serine)	3.5
G12D (Aspartate)	31.2
G12C (Cysteine)	11.5
G12V (Valine)	18.8
G12R (Arginine)	3.1
G12A (Alanine)	7.3
Codon 13	
G13D (Aspartate)	20.8
Codon 61	
Q61H (Histidine)	2.1

approval, these 342 formalin fixed, paraffin embedded tissue blocks were recovered. All tumors were graded by an experienced pathologist using 6th edition of UICC classification (17). Sections were cut from regions of the tumor containing as high a proportion of tumor cells as possible (typically ≥50%). All materials were unlinked from their identifiers before being subjected to DNA extraction and genetic analysis. Clinicopathological variables of the patients were gender, age, tumor localisation, UICC stage, and K-Ras status. Follow-up survival data were provided by the Federal Agency for Statistics in Austria.

Analysis of K-Ras mutations. Genomic DNA was extracted from deparaffinized tumor tissue samples using the peqGOLD® Tissue DNA mini kit (PEQLAB Biotechnologie Ltd., Erlangen, Germany) according to the manufacturer's instructions. Mutation analysis of K-Ras codons 12, 13 and 61 was carried out by direct sequencing of amplified PCR-products spanning respective codons. The mutations at codons 12 and 13 were determined by using the following PCR sense primer: 5'AAAGGTACTGGTGGAGTATTTGATAG and antisense primer: 5'ACAAGATTTACCTCTATTGTTGGATC. For determination of mutations at codon 61 PCR was performed using the sense primer: 5'GGAAGCAAGTAGTAATTGATGGAGA and antisense primer: 5'GCATGGCATTAGCAAAGACTCA. Primers were purchased from VBC-Biotech Service GmbH, Vienna, Austria. PCR was performed in a 15 μl volume mix, comprising 5-10 ng genomic DNA, 0.3 Units AmpliTaq Gold® DNA Polymerase (Applied Biosystems, Forster City, CA), 1X AmpliTaq Gold DNA Buffer I (Applied Biosystems), 0.5 μM of each primer and 0.2 mM deoxynucleotide triphosphate under the following amplification conditions: 15 min at 95˚C and 50 cycles at 94˚C for 30 sec, 55˚C for 30 sec and 72˚C for 40 sec, followed by a last step at 72˚C for 5 min. Purified PCR products were sequenced on an ABI Prism 3130 (Applied Biosystems), automated sequencer.

Statistical analysis. Overall survival was defined as the time from histopathological diagnosis to death from any cause. Univariate and multivariate analysis was performed using Cox's model for proportional hazards survival analysis. Hazard ratios (HR) and 95% confidence intervals (CI) of the hazard ratios were calculated from the individual Cox multivariate analysis. Survival curves were generated using the Kaplan-Meier method. The survival curves were compared using the log-rank Mantel-Cox test. $p<0.05$ were considered to indicate statistical significance. Statistical analyses were performed with the software package SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL, USA).

Results

Patient characteristics. We enrolled 342 patients from an Austrian cohort with colorectal cancer diagnosed during January 2003 and October 2006. Over a mean follow-up period of 26 months (±13), 57 deaths were recorded; 54 of them were tumor related. Follow-up data were not available for 17 patients (5%). The baseline patient characteristics are presented in Table I.

Genotyping revealed an overall mutation frequency of 28% (n=96) in K-Ras gene codons 12, 13 and 61; 21.7% of K-Ras mutations occurred at codon 12, 5.8% at codon 13 and 0.6% at codon 61. The mutation status is specified in Table II.

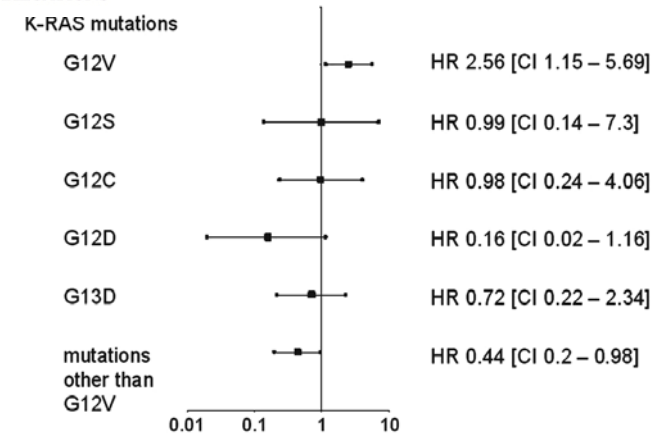

Figure 1. Results from Cox regression analysis of each individual mutation compared to the wild-type. The G12R, G12A and Q61H mutations were excluded from individual Cox regression analysis because no fatalities occurred. HR, hazard ratio; CI, confidence interval (95%). Hazard ratios were calculated by Cox regression analysis.

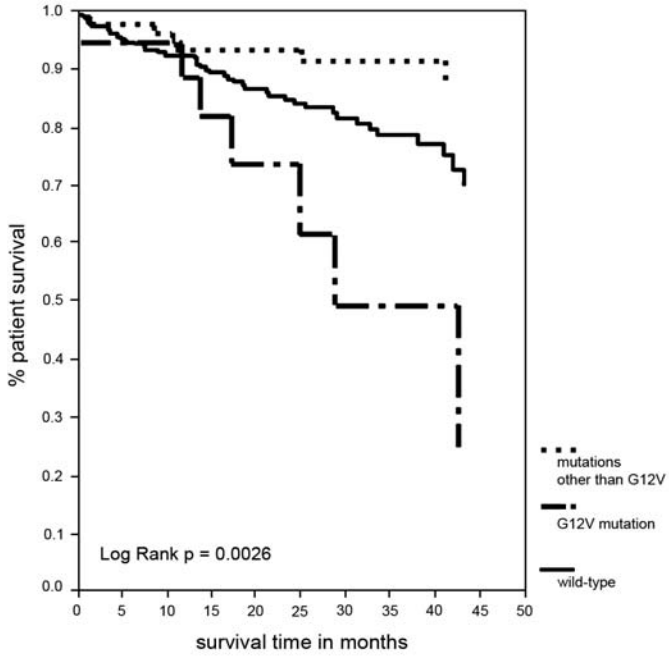

Figure 2. Kaplan-Meier survival curves according to three risk models. K-Ras mutations other than G12V, wild-type, and G12V were compared. Overall survival decreased significantly for carriers of K-Ras mutations other than G12V over carriers for the wild-type to those who carried the G12V mutation. p-value was calculated by log-rank test.

The most common mutation was a G to A change at codon 12 resulting in a glycine to aspartate substitution (G12D).

Association of determined genotypes with overall survival. We analyzed the effect of each individual mutation compared to the wild-type. Results from Cox regression analysis are shown in Fig. 1. The G12V mutation showed a significant risk for decreased overall survival (p=0.022). Mutations other than G12V (n=72) showed only a moderate, non-significant effect on overall survival. However, by combining other mutations than G12V a significant protective effect on overall survival could be observed (p=0.046). Concordantly, due to the contrary effect of K-Ras mutations, the G12V mutation compared to the wild-type together with other mutations showed worst overall survival [HR=2.95 (1.34-6.53); p=0.007]. In the same way, best overall survival was observed by merged remaining mutations compared to the wild-type together with the G12V mutation [HR=0.4 (0.18-0.89); p=0.024]. Adjustment for age, gender and UICC tumor stage in multivariate Cox regression analysis confirmed these results [HR=2.42 (1.07-5.46); p=0.033 and HR= 0.31 (0.14-0.7); p=0.005, respectively].

Additionally, Kaplan-Meier survival curves were graphically displayed according to the three risk models: K-Ras mutations excepting G12V, wild-type, and G12V. Overall survival decreased significantly for carriers of K-Ras mutations other than G12V over carriers for the wild-type to those who carried the G12V mutation (Fig. 2).

Discussion

Our study provides new insights into the influence of K-Ras mutations on overall survival in patients with colorectal cancer. In contrast to G12V mutation, combined remaining mutations at codons 12, 13 and 61 showed a benefit on overall survival.

Genotype frequencies in the presented study are concordant with literature reports showing K-Ras was mutated in 22-46% of sporadic colorectal cancers (4-9). However, reported prognostic impact of the K-Ras mutations provided inconsistent results (18-22). A large meta-analysis suggested that the G12V mutation at codon 12 in the K-Ras gene increases the risks of recurrence or death in patients with colorectal cancer (5). These data are supported by our findings that G12V mutation affects overall survival. Earlier studies could not demonstrate a significant impact on overall survival of other K-Ras mutations. However, in our study, K-Ras mutations other than G12V, showed a moderate, but non-significant effect on overall survival. By combining these mutations a clear survival benefit was observed. This is in concordance with a previous report suggesting the K-Ras-dependen tumor development results in the formation of less aggressive neoplasms (23).

In vitro studies showed that different Ras mutations may influence the oncogenesis unequally because of their different effects on signal transduction mechanisms (15,16,24). Normal Ras cycle occurs between a GDP bound inactive and a GTP bound active form of Ras. Mutant Ras remain longer in the GTP-bound state, resulting in a more persistent growth signal (24,26). It is proposed that lower affinity of GTP to G12D allows G12D an escape from the oncogenic GTP-bound state, whereas GTP tightly bound to G12V generates a more persistent, potentially oncogenic signal. Furthermore, differences in the effector region of G12D und G12V could modify interactions with downstream signalling molecules (15). Due to these effects, the influence of different K-Ras

mutations on overall survival might be explained by the fact that heterogeneity of K-Ras mutations in colorectal cancer may differ in carcinogenic potential (15,27).

Additionally, K-Ras is a potential biomarker for anti-epidermal growth factor (EGFR) targeted treatment of metastatic colorectal cancer (mCRC) patients (10-13). Prospective randomized studies are ongoing to elucidate the impact of different K-Ras mutations on antibody treatment in combination with chemotherapy. Findings of these studies offer the possibility to adapt therapy to each patient's individual K-Ras mutational status. Consequently, potential adverse effects of treatment can be avoided and leads to cost reduction of increasingly limited financial funds.

Considering that tumor aggressiveness *in vivo* can be influenced by several genetic and epigenetic factors the study requires cautious interpretation. Nevertheless, *in vitro* data showed consistent differences in carcinogenic potential of different K-Ras mutations. Due to the rare incidence of individual K-Ras mutation in colorectal cancer tumor cells, our study may appear underpowered for detecting significant associations of individual mutations with overall survival. However, our investigation clearly demonstrates an association between combined K-Ras mutations and overall survival.

To the best of our knowledge this is the first study with the largest sample size showing antidromic effects of non-synonymous mutations of the K-Ras gene on overall survival. In contrast to other studies we provide several advantages for the investigation of the role of K-Ras mutations on overall survival in colorectal cancer patients: All patients were diagnosed centrally; the patient cohort was homogeneous and regionally restricted; standardized therapy was offered to all patients with colorectal cancer; there was uniform analysis of K-Ras mutations on codon 12, 13 and 61. Using these methods, the relationship between the genetic profile of colorectal cancer and overall survival can be reliably assessed.

In conclusion, our results suggest that different mutations on the K-Ras gene show different effects on overall survival in patients with colorectal cancer. G12V mutation affects overall survival adverse, whereas other mutations influence overall survival favourable as we could show for the first time. Further studies are in progress to elucidate the impact of each individual K-Ras mutational status with respect to treatment.

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