Clinicopathologic characteristics and gene expression analyses of non-\textit{KRAS} 12/13, \textit{RAS}-mutated metastatic colorectal cancer

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Background: \textit{KRAS} mutations in codons 12 and 13 are present in \sim 40\% of all colorectal cancers (CRC). Activating mutations in codons 61 and 146 of \textit{KRAS} and in codons 12, 13, and 61 of \textit{NRAS} also occur but are less frequent. The clinicopathologic features and gene expression profiles of this latter subpopulation of \textit{RAS}-mutant colorectal tumors have not yet been clearly defined but in general are treated similarly to those with \textit{KRAS} 12 or 13 mutations.

Patients and methods: Records of patients with metastatic CRC (mCRC) treated at MD Anderson Cancer Center between December 2000 and August 2012 were reviewed for \textit{RAS} (\textit{KRAS} or \textit{NRAS}) and \textit{BRAF} mutation status, clinical characteristics, and survival outcomes. To study further with an independent cohort, data from The Cancer Genome Atlas were analyzed to define a gene expression signature for patients whose tumors feature these atypical \textit{RAS} mutations and explore differences with \textit{KRAS} 12/13-mutated colorectal tumors.

Results: Among the 484 patients reviewed, \textit{KRAS} 12/13, \textit{KRAS} 61/146, \textit{NRAS}, and \textit{BRAF} mutations were detected in 47.7\%, 3.0\%, 4.1\%, and 7.4\%, respectively, of patients who were tested for each of these aberrations. Lung metastases were more common in both the \textit{KRAS} 12/13-mutated and atypical \textit{RAS}-mutated cohorts relative to patients with \textit{RAS}/\textit{BRAF} wild-type tumors. Gene expression analyses revealed similar patterns regardless of the site of \textit{RAS} mutation, and \textit{in silico} functional algorithms predicted that \textit{KRAS} and \textit{NRAS} mutations in codons 12, 13, 61, and 146 alter the protein function and drive tumorgenesis.

Conclusions: Clinicopathologic characteristics, survival outcomes, functional impact, and gene expression profiling were similar between patients with \textit{KRAS} 12/13 and those with \textit{NRAS} or \textit{KRAS} 61/146-mutated mCRC. These clinical and bioinformatic findings support the notion that colorectal tumors driven by these \textit{RAS} mutations are phenotypically similar.

Key words: colorectal cancer, \textit{KRAS}, next-generation sequencing, gene expression, survival

introduction

Estimated to be responsible for more than 50 000 deaths in 2014, colorectal cancer (CRC) remains the second leading cause of cancer-related mortality for men and women combined in the United States [1]. Mutations in codons 12 or 13 of \textit{KRAS} occur in \sim 40\% of all metastatic colorectal cancers (mCRC) [2, 3] and generate constitutive activation of the mitogen-activated protein kinase (MAPK) pathway [4, 5]. The presence of these mutations is predictive for a lack of response to treatments with monoclonal antibodies against the epidermal growth factor receptor (EGFR) like cetuximab and panitumumab in patients with mCRC [6–8].

Recent data have shown that mutations in codons 61 and 146 of \textit{KRAS}, or in codons 12, 13, and 61 of \textit{NRAS}, occur in \sim 18\% of mCRC patients [9, 10] and can induce malignant transformation \textit{in vitro} of colorectal cells [11]. Previously classified as \textit{KRAS} ‘wild-type’ tumors based on practices testing only for mutations in codons 12 or 13 of \textit{KRAS}, these less common atypical \textit{RAS} mutations have been shown to negate the presumed benefit of anti-EGFR therapies for patients with \textit{RAS} wild-type tumors [9, 10].
Scant information exists regarding the clinicopathologic features and gene expression patterns of these non-traditional activating RAS mutations in patients with CRC. To that end, we reviewed the records of 484 patients with mCRC evaluated and treated at our institution and characterized their clinical phenotypes according to demographic information, anatomical locations of tumor metastases, and survival outcomes. Using an independent cohort of patients with CRC from The Cancer Genome Atlas (TCGA) dataset, gene expression patterns were analyzed in patients with RAS-mutated tumors to explore differences according to the site of RAS mutation.

**materials and methods**

**patients and samples**

The MD Anderson Cancer Center institutional database was searched and reviewed under an Institutional Review Board protocol for clinical characteristics of patients treated, including demographic information, mutation status, and sites of tumor involvement. Tumor DNA was isolated and sequenced for particular mutations using Ion Torrent next-generation sequencing (Life Technologies, Germany). Results were analyzed to assess differences according to mutational status of each patient.

**gene expression analyses**

Because we did not have enough tissue to perform gene expression profiling from the tumors of patients treated at MD Anderson, we analyzed the clinical, mutation, and RNA sequencing data for CRC from the TCGA dataset in order to evaluate differences between various NRAS and KRAS mutations using an independent dataset. Tumor samples were categorized according to KRAS and NRAS mutation status. Analyses were limited to the set of patients for which both gene mutation and RNA sequencing data were available. In order to assess the molecular similarity between the KRAS 12/13 tumors with the non-traditional RAS-mutated tumors, we carried out differential expression analyses using RNA sequencing data. Gene expression signatures were identified for both the KRAS 12/13 and the non-traditional RAS-mutated tumors by comparing each group with the RAS/BRAF wild-type tumors.

**in silico functional impact analyses**

To assess the biological consequence of the KRAS and NRAS mutations identified in the TCGA project and studied in the MD Anderson cohort, we annotated each mutation with a ‘driver versus passenger’ status and predicted in silico their functional impacts. CHASM was used to predict driver statuses. To obtain functional impact predictions, we used VariantTools [12] with dbNSFP v2.0 which provides predictions from SIFT, LRT, and MutationTaster tools. A complete description of the Materials and Methods section is provided in the supplementary Materials and Methods section, available at *Annals of Oncology* online.

**results**

**patient demographics**

Among the 484 patients with mCRC evaluated at our institution between August 2000 and December 2012, 301 (62.2%) had stage IV disease at the time of diagnosis, with a median follow-up period of 3.0 years. The remaining 183 patients initially underwent resection for stage I–III CRC but developed metastatic disease at a later point in time. Comparing patients with these mutation profiles, there were no appreciable differences in age at initial diagnosis, gender, ethnicity, or tobacco history (supplementary Table S1, available at *Annals of Oncology* online).

**clinicopathologic features**

The majority of patients (97.7%) were tested for mutations in codons 12, 13, 61, and 146 of the KRAS oncogene upon diagnosis of metastatic disease. Within this group, 178 (37.6%) and 48 (10.1%) demonstrated KRAS 12 and 13 mutations, respectively (supplementary Table S2, available at *Annals of Oncology* online). Tumors of 14 patients (3.0%) harbored KRAS 61 or 146 mutations. NRAS mutations were present in 10 patients (4.1% of tumors analyzed). BRAF mutations were detected in 32 of the 435 (7.4%) tumors tested.

The clinicopathologic features of patients stratified by the presence of activating RAS or BRAF mutations were investigated. Patients with KRAS 12/13 mutations and those with BRAF mutations (mutually exclusive of one another) were both more likely than their wild-type counterparts to have right-sided primary tumors when compared with those with KRAS/ NRAS/BRAF wild-type tumors (Table 1). Relative to the wild-type subgroup, mucinous tumors were more common in the KRAS 12/13-mutated and BRAF-mutated cohorts (P < 0.001). However, no significant differences in distribution of primary tumor sites, histopathological subtype, or degree of differentiation were detected between patients with KRAS 12/13 mutations and those with atypical RAS mutations. Of the four subgroups, BRAF mutations were associated with the most poorly differentiated tumors (P = 0.006). Individuals with KRAS 12/13 mutations had similar patterns of metastatic spread as those with atypical RAS mutations, and both of these groups were more likely than their wild-type counterparts to develop lung metastases (Figure 1).

Overall survival from the time of metastasis diagnosis was computed and assessed with the Kaplan–Meier method (Figure 2). Differences between subgroups according to mutation status were noted with a global P-value of <0.001. Overall survival was significantly shorter for the KRAS 12/13-mutated cohort relative to the RAS/BRAF wild-type cohort [38.7 months versus 57.6 months, respectively; hazard ratio (HR) for death 1.49, 95% confidence interval (CI) 1.13–1.97; P = 0.006]. For the cohort of patients with atypical RAS-mutated mCRC, the median overall survival was 47.4 months. No significant difference in survival was identified between these patients and patients with KRAS mutations in codons 12 or 13 (HR 1.77, 95% CI 0.93–3.39; P = 0.08). Additionally, the presence of BRAF mutation was found to be the worst prognostic marker of the four subgroups with a median survival was 24.3 months, representing significantly shortened survival relative to the RAS/BRAF wild-type patients (HR 4.71, 95% CI 2.27–9.81; P < 0.001).

**TCGA analysis of clinical variables**

Of 227 patients with colon (n = 154) and rectal (n = 73) primary tumors sequenced in the respective TCGA projects, 34.3% of tumors were mutated in KRAS on codons 12 and 13 (n = 78) and 4.8% were mutated in KRAS on codons 61 and 146 (n = 11, based on merged colon and rectal project data). Activating
mutations in NRAS on codons 12 and 13 were present in 3.5% of tumors (n = 8) with an additional 4.8% being mutated in NRAS on codon 61 (n = 11).

We carried out the following mutation-based comparisons: (i) KRAS 12/13-mutated versus wild-type RAS/BRAF wild-type; (ii) atypical RAS-mutated versus RAS/BRAF wild-type; and (iii) KRAS 12/13-mutated versus atypical RAS-mutated. Between patients with the KRAS 12/13-mutated tumors and those with the atypical RAS mutations, no unique associations with clinicopathologic features were identified according to mutation status when evaluating association in age at initial diagnosis, gender, ethnicity, and stage at initial diagnosis. These results are consistent with those from the MD Anderson patients examined in our institutional study (data not shown). Sites of distant spread in those patients with metastatic disease were unavailable in the TCGA dataset and therefore were not considered.

functional impacts of KRAS and NRAS mutations

All three in silico prediction algorithms [12] utilized in this study showed similar results and suggested that any mutation within codons 12, 13, 61, and 146 of KRAS or NRAS is damaging (supplementary Table S3, available at Annals of Oncology online). In particular, MutationTaster and SIFT were concordant and predicted that each of these mutations were damaging. LRT showed consistency across mutations within each gene, identifying all of the KRAS 12/13/61/146 mutations as damaging but produced an unknown status for all NRAS mutations. Finally, CHASM classified each of the KRAS and NRAS mutations as drivers, predicting them to induce a malignant effect. These algorithms were unable to predict functional effects of compound substitutions; thus, predictions of the KRAS Q61L mutation are unavailable.

TCGA gene expression profiling

Differential expression analyses of the TCGA RNA sequencing data when comparing KRAS 12/13-mutated tumors with RAS/BRAF wild-type tumors resulted in a KRAS 12/13 gene expression signature with 88 up- and 54 down-regulated genes. BRAF-mutated tumors were associated with microsatellite instability and clustered with the RAS-mutated tumors, even though there was no overlap between BRAF mutations and RAS mutations for any single tumor. The unsupervised clustering suggests that the KRAS 12/13 and the atypical RAS-mutated groups of tumors have similar gene expression profiles (Figure 3). To characterize this similarity, we identified a gene expression signature for the atypical RAS-mutated tumors compared with RAS/BRAF wild-type tumors that included 38 up- and 18 down-regulated genes. Then, an enrichment analysis to detect pathways putatively associated with the RAS-mutated gene expression signature in both groups identified four up-regulated and two down-regulated pathways that included pathways critical to VEGF signaling, tumor cell extravasation, and integrin signaling (supplementary Table S4, available at Annals of Oncology online).

discussion

Using both retrospective clinical data and bioinformatic analyses, we conclude that the clinicopathologic features of patients with mCRC featuring atypical RAS-mutated tumors are similar to those of their KRAS 12/13-mutated counterparts. Recent data reported in the PRIME and FIRE-3 trials assessed the impact
of NRAS mutations and ‘atypical’ KRAS mutations in patients with mCRC [9, 10, 13]. These patients demonstrated similar outcomes when compared with patients harboring KRAS 12/13-mutated mCRC when treated with anti-EGFR therapies. In our study, patient characteristics and tumor pathologic characteristics were overall similar between KRAS 12/13-mutated and atypical RAS-mutated CRC and hence provide further evidence for integrating these different KRAS mutations and NRAS mutations into a ‘RAS-mutated’ profile when characterizing subpopulations of patients with mCRC.

We found no differences in the age of diagnosis, gender, ethnicity, or tobacco history with the development of a RAS-mutated tumor relative to those who have RAS wild-type disease. However, patients with KRAS 12 or 13 mutations were more likely to have right-sided primary tumors when compared with patients whose tumors were RAS/BRAF wild-type. This pattern was also validated when we analyzed an independent cohort of patients with CRC from the TCGA dataset. While right-sided primary tumors have traditionally been associated with BRAF mutations and not KRAS mutations, other series have also reported that KRAS 12/13 mutations may be more common in the cecum than their wild-type counterparts [14].

KRAS mutation status has been previously implicated with a specific distributional pattern of metastatic disease. For example, in one prior study [15], KRAS 12 or 13 mutations were associated with an increased propensity for lung metastases relative to patients with KRAS wild-type mCRC. Our results corroborate these findings, as patients with KRAS 12/13 mutations and atypical RAS mutations were both more likely to harbor lung metastases relative to their RAS wild-type counterparts. However, no differences were noted in our study when comparing the distribution of other locations for metastases between the KRAS 12/13 and the atypical RAS cohort. No differences in tumor grade or histological subtype were detected either between these two subgroups. Taken together, these findings highlight the concept that the clinical and pathologic presentations of these two subpopulations of mCRC are similar.

When survival between patients with various RAS mutations and patients with RAS type-disease was compared, patients with KRAS 12/13-mutated CRC were found to have a shortened survival in an era with availability of EGFR monoclonal antibodies. However, of particular interest were the outcomes for patients with RAS mutations outside the more commonly studied codons 12 and 13 of KRAS. In these patients, the median overall survival was closer to that of the KRAS 12/13-mutated patients relative to the RAS wild-type cohort. However, our small sample of patients with atypical RAS mutations likely resulted in an underpowering of the survival analysis for moderate difference. Four patients with these atypical RAS mutations had been treated with either cetuximab (n = 2) and panitumumab (n = 2) before evaluation at our institution, and all progressed at the time of first restaging. These findings corroborate the idea that patients with RAS mutations may not respond to therapies against the EGFR and behave akin to patients with KRAS 12/13-mutated mCRC exposed to anti-EGFR agents.

We recognize several limitations to our study. Due to the overall small number of patients with KRAS 61 or 146 or NRAS mutations, we chose to pool all patients with these variants into one group. In doing so, we assume that all mutations behave similarly to one another, despite the fact that previous studies have suggested that KRAS G13D mutations may respond better to cetuximab in mCRC relative to other KRAS exon 2 mutations [16] and the particular codon mutated may influence the
predictive behavior of a given biomarker. Delineating the specific phenotypes of these individual aberrancies will require large efforts of cooperative groups in the future.

Another limitation is that all patients were evaluated at a single institution during a time in which testing for these mutations were not within the standard-of-care in the management of mCRC, thus potentially introducing ascertainment bias into our analysis. To address this bias further, we interrogated a completely independent dataset from the TCGA to compare various clinical and molecular characteristics of patients with RAS-mutated CRC. Specifically, when comparing the age at initial diagnosis of cancer, gender, ethnicity, site of primary tumor, and stage at diagnosis, we confirmed that there were no differences between patients with KRAS 12/13 mutations and those with non-KRAS 12/13 RAS-mutated tumors (data not shown). Moreover, analysis of mutation and gene expression data from the TCGA provided molecular evidence to support our clinical findings. We annotated KRAS and NRAS mutations identified in the TCGA CRC dataset using multiple computational prediction algorithms. Our analysis has shown that non-traditional RAS mutations are predicted to be deleterious as mutations, and to drive tumor progression. This suggests that the non-traditional RAS mutations are functionally relevant, akin to KRAS codon 12 and 13 mutations. Additionally, through differential expression analysis, our results suggest that tumors harboring non-traditional RAS mutations have similar gene expression profiles compared with tumors with KRAS mutations on codons 12 and 13. Furthermore, the differentially expressed genes in both groups compared with RAS/BRAF wild-type tumors are representative of pathways related to metastasis, providing additional support that these two groups of tumors behave similarly. It is interesting that the BRAF-mutated tumors clustered in gene expression analyses with the RAS-mutated tumors, a finding which may be explained by common sets of genes involved in the RAS/RAF/MAPK signaling pathway.

We conclude here that the clinicopathologic features and tumor cell gene expression patterns in patients with KRAS or NRAS-mutated mCRC appear to be similar. These findings are important because patients with mCRC harboring these RAS mutations are managed similarly. The similarities across the various RAS mutations analyzed in the MD Anderson institutional data and in the bioinformatic analyses lend further credence to the notion that patients with atypical RAS-mutated CRC are
phenotypically and molecularly similar to those whose tumors are driven by mutations in codons 12 or 13 of KRAS.

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disclosures

The authors have declared no conflicts of interest.

references

A simple technique to estimate best- and worst-case survival in patients with metastatic colorectal cancer treated with chemotherapy

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Background: Patients with incurable cancer usually want specific information about prognosis, and clinicians’ estimates are often inaccurate. Studies in breast and lung cancer have suggested that simple multiples of the median overall survival (OS) can accurately estimate the time at which 90%, 75%, 25% and 10% of patients are alive.

Patients and methods: We identified 46 phase III randomised clinical trials of chemotherapy in metastatic colorectal cancer, representing data from 29,011 patients. We extracted data on demographics, treatment and survival from 96 patient cohorts and assessed agreement with the estimated survival time points, calculated as 0.25, 0.5, 2 and 3 times the median OS.

Results: Median OS was 16.8 months in the trials. There were 342 assessable time points. For 301 of these, the estimated survival time was within 0.75–1.33 of the actual survival time (88% agreement). The worst agreement (76%) was at the earliest (90%) level of survival.

Conclusions: Simple multiples of the median OS give reasonable estimates of the times at which different survival levels are reached in patients with metastatic colorectal cancer. Taken with previous studies, these findings are likely to be valid across a large range of patients. We would encourage clinicians to think of prognosis as a trajectory, and to consider quoting survival ranges instead of point estimates, in discussions with patients.

Key words: survival, prognosis, metastatic colorectal cancer, review

introduction

Estimating survival in patients with incurable cancer is difficult, and predictions made by medical staff are often inaccurate [1, 2]. Such predictions are important for patients, and may influence their decisions on treatment; they are also important in planning services [3].

When asked for prognosis, many clinicians quote the median overall survival (OS). However, the median represents the time by which half the patients will have died and the relevance of this figure is not clear to patients [4, 5]. Ideally, patients should have access to better prognostic information without the need for complex formulae or electronic aids. There has been substantial work on estimating survival in the last few weeks of life, often in the hospice environment (see [6] for an overview). However, there is much less work on estimating survival in those patients who cannot be cured but may have life expectancies measured in months and years. Such patients have high