RAS Mutations Affect Pattern of Metastatic Spread and Increase Propensity for Brain Metastasis in Colorectal Cancer

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Abstract

**Background**—RAS and PIK3CA mutations in metastatic colorectal cancer (mCRC) have been associated with worse survival. We sought to evaluate the impact of RAS and PIK3CA mutations on cumulative incidence of metastasis to potentially curable sites of liver and lung and other sites such as bone and brain.

**Methods**—We performed a computerized search of the electronic medical record for mCRC cases genotyped for RAS or PIK3CA mutations in our institution from 2008 to 2012. Cases were reviewed for patient characteristics, survival, and site-specific metastasis.

**Results**—Among the 918 patients identified, 477 cases were RAS wild-type and 441 cases had a RAS mutation (394 at KRAS exon 2, 29 at KRAS exon 3 or 4, and 18 in NRAS). RAS mutation was significantly associated with shorter median overall survival (OS) and on multivariate analysis independently predicted worse OS (HR 1.6, p<0.01). RAS mutant mCRC exhibited a significantly higher cumulative incidence of lung, bone, and brain metastasis and on multivariate analysis was an independent predictor of involvement of these sites (HR 1.5, 1.6, and 3.7, respectively). PIK3CA mutations occurred in 10% of the 786 cases genotyped, did not predict for worse survival, and did not exhibit a site-specific pattern of metastatic spread.

**Conclusion**—The metastatic potential of CRC varies with the presence of RAS mutation. RAS mutation is associated with worse OS and increased incidence of lung, bone, and brain metastasis. An understanding of this site-specific pattern of spread may help inform physicians’ assessment of symptoms in patients with mCRC.
INTRODUCTION

Mutations in the RAS and PIK3CA oncogenes are early events in colorectal cancer (CRC) development, arising in the adenoma stage and adenoma-carcinoma transition of the classic adenoma-carcinoma sequence, respectively. RAS proteins are small GTP-binding proteins that regulate cell proliferation, survival, and differentiation. Phosphatidylinositol 3-kinase (PI3K), a lipid kinase that regulates cell growth and survival, is a heterodimer composed of a catalytic subunit, encoded by the gene PIK3CA, and a regulatory subunit. PIK3CA mutations can lead to constitutive activation of PI3K signaling, most commonly through activating mutations in the kinase domain (e.g., H1047R) or mutations in the helical domain that relieve an inhibitory interaction with the regulatory subunit (e.g., E542K, E545K). Some studies have reported an association between RAS and PIK3CA mutations and increased recurrence and worse survival in CRC. The etiology of this worse prognosis is not known.

The site-specific pattern of metastatic spread of CRC impacts patient outcomes. CRC with limited metastatic involvement of the liver or lung can be resected, achieving curative outcomes in up to 50% of patients. Thus, tumors with limited involvement of these sites exhibit improved outcomes, while tumors with a predilection to spread to sites, such as the peritoneum or brain, are associated with worse outcomes. Recent data suggest that tumor mutation profile may influence sites of relapse or sites of metastatic involvement. For example, we and others have found that BRAF mutation is associated with peritoneal spread, a pattern of disease progression that may be the basis of the poor prognosis of these tumors. Additionally, among patients with metastatic CRC (mCRC) undergoing resection of liver metastasis, those with KRAS mutations were more likely to develop lung metastases. We have also recently reported that KRAS mutation affects recurrence risk and sites of recurrence after hepatectomy for CRC, with lung, bone, and brain metastases occurring more often in patients with KRAS mutations. In this study, we assembled a large series of patients with mCRC to define the correlation between mutations in the RAS and PIK3CA oncogenes and patients’ survival and pattern of metastatic spread.

PATIENTS AND METHODS

Patient Population

Cases were derived from patients seen at Memorial Sloan Kettering Cancer Center (MSKCC) with mCRC who had their tumors submitted for KRAS genotyping between 2008 and 2012. Beginning in 2008, genotyping was performed in patients with mCRC as part of standard-of-care to guide the use of epidermal growth factor receptor (EGFR) targeting antibodies. Tumor sequencing was performed in all patients following in-house resection of metastatic disease and by physician request for all other patients. The number of cases...
sequenced in our molecular pathology laboratory, therefore, closely approximates the population of mCRC patients at MSKCC.

We performed a computerized search of electronic medical records to identify all cases that were sequenced during this period. We identified 1095 unique patients with mCRC whose tumors were genotyped for \textit{KRAS} exon 2 mutations between 2008 and 2012, including 786 cases genotyped for extended \textit{RAS} and \textit{PIK3CA} mutations. Cases with a \textit{KRAS} exon 2 mutation or an extended \textit{KRAS} or \textit{NRAS} mutation were analyzed together in the \textit{RAS} mutant group for a total of 441 \textit{RAS} mutant cases and 477 \textit{RAS} wild-type cases.

\textbf{Sequence Analysis}

Genomic DNA was extracted from formalin fixed paraffin embedded (FFPE) tissue obtained from biopsies or surgical resections. Sequencing was performed on a metastatic specimen in cases where tissue was available from metastasectomy or diagnostic biopsy, and on the primary tumor in all other cases. Prior to October 2010, testing was performed by Sanger sequencing of \textit{KRAS} exon 2. Subsequently, a mass-spectrometry based assay (Sequenom, San Diego, CA) was used to detect mutations in \textit{KRAS} and \textit{NRAS} (codons 12, 13, 61, 117, and 146) and \textit{PIK3CA} (codons 88, 345, 420, 542, 545, 1043, and 1047) as previously described\textsuperscript{12}. Mutations were confirmed either by a separate Sequenom assay or by Sanger sequencing. Patients with a \textit{KRAS} exon 2 mutation identified by Sanger sequencing and patients with a hotspot mutation in \textit{KRAS} or \textit{NRAS} identified by Sequenom testing were analyzed together in the \textit{RAS} mutant group.

\textbf{Data Collection}

Cases were analyzed for specific clinical characteristics, including age, gender, primary tumor site, stage at diagnosis, sites of metastatic disease, metastasectomy, previous treatment, and survival. Tumors arising from the cecum to distal transverse colon were classified as right-sided, and tumors arising from the splenic flexure to rectosigmoid junction were classified as left-sided. Metastatic sites were identified by review of medical records and/or imaging. All research was conducted under appropriate Institutional Review Board/Privacy Board protocols and waivers.

\textbf{Statistical Analysis}

Associations between clinicopathologic characteristics and tumor mutation status were analyzed using the Chi-square test for categorical variables and the Wilcoxon Rank-Sum test for continuous variables. Overall survival (OS) was examined from date of metastatic disease to date of death or last available follow up. Log-rank test was used to examine whether OS differed by mutation status. Cox proportional hazards model was used to evaluate the independent effect of mutation status on OS after adjusting for the following known confounders: age at diagnosis, gender, tumor location, synchronous tumor, and surgery. Surgery was treated as a time-dependent covariate in the multivariate model.

Cumulative incidence function was used to estimate the probability of liver, lung, bone, or brain metastasis from the date of initial metastasis in the subset of patients who did not have these sites involved at the time of diagnosis of metastatic disease. Patients who died or were
alive but developed subsequent disease at other sites were treated as competing events. Competing regression model was used to further evaluate the independent association between RAS mutation and the probability of lung, bone, and brain metastasis, separately. The models were adjusted for variables that were significantly associated at p ≤0.1 level with time to lung, bone, and brain metastasis on univariate analyses.

Cumulative incidence function was also used to estimate the probability of metastasectomy from the time of mCRC diagnosis, considering death as competing event. Gray’s test was used to compare the cumulative incidence functions by mutation status.

All p-values were based on 2-tailed statistical analysis, and p<0.05 was considered to indicate statistical significance. All analyses were performed with SAS Version 9.2 (SAS Institute, Cary, North Carolina) and R (2.10.1).

RESULTS

Mutation Frequency

We identified 1095 unique patients with mCRC whose tumors were genotyped between 2008 and 2012 (Fig. 1). All cases were genotyped for KRAS exon 2 mutations, and 786 cases were also genotyped for extended RAS and PIK3CA mutations. We identified 701 KRAS exon 2 wild-type cases, and, of these, 524 cases underwent extended RAS mutation testing. We excluded 177 KRAS wild-type cases that were not sequenced for extended RAS mutations from analysis. Four hundred seventy seven cases were confirmed to be wild-type at KRAS exons 3 and 4 and NRAS. Extended RAS mutation analysis identified 29 non-exon 2 KRAS mutant cases, with mutations involving exon 3 in 10 cases and exon 4 in 19 cases. NRAS mutations occurred in 18 cases, in exon 2 in 8 cases and in exon 3 in 10 cases. One tumor had both KRAS G12D and NRAS G13V mutations. Cases with a KRAS exon 2 mutation or an extended KRAS or NRAS mutation were analyzed together in the RAS mutant group for a total of 441 RAS mutant cases.

PIK3CA activating mutations were identified in 76 (9.7%) cases. Mutations were identified at positions E542 or E545 in 56 tumors, H1047 in 14 tumors, N345 in five tumors, and R88 in one tumor. RAS mutations were significantly more common in the PIK3CA mutant tumors than in PIK3CA wild-type tumors (71% v 37%, p<0.01).

Patient Characteristics

The characteristics of the 918 patients with known tumor RAS mutation status are shown in Table 1. RAS mutation was significantly associated with right-sided primary tumor (41% v 24%, p<0.01) and regional lymph node involvement (61% v 54%, p=0.02). Median age at diagnosis and timing of metastasis (synchronous v metachronous) did not vary by RAS mutation status.

Supplemental table 1 lists characteristics for the 786 patients whose tumors were genotyped for PIK3CA mutations. There were no statistically significant differences in the characteristics of the patients with PIK3CA wild-type and PIK3CA mutant mCRC.
Patients in our series commonly underwent metastasectomy and hepatic arterial infusion (HAI) therapy. At 12 months after the diagnosis of metastatic disease, 48% of RAS wild-type patients and 44% of RAS mutant patients underwent surgery to remove a metastatic lesion, either curative or palliative in intent (p=0.17). There was a trend for a higher frequency of hepatectomy in the RAS wild-type patients with 53% of RAS wild-type cases and 48% of RAS mutant cases undergoing hepatectomy during this time period (p=0.07). Cumulative incidence of HAI treatment at 12 months was 31.3% for RAS wild-type cases versus 24% for RAS mutant cases (p<0.01). Among the patients with liver-limited disease at the time of diagnosis of metastasis, the cumulative incidence of HAI treatment at 12 months was 41% for RAS wild-type versus 32% for RAS mutant cases (p<0.01).

Correlation of RAS or PIK3CA Mutations with Survival

In our series, the presence of an activating RAS mutation was associated with worse OS. Median OS from the time of diagnosis of metastatic disease was 81 months for patients with RAS wild-type mCRC versus 47 months for patients with RAS mutant tumors (p<0.001) (95% CI 3.3–4.4 years) (Fig. 2A). Multivariate analysis, adjusting for age at diagnosis of metastatic disease, gender, location of primary tumor, synchronous or metachronous disease, and occurrence of metastasectomy or HAI treatment treated as a time-dependent covariates, assigned the presence of an activating RAS mutation a hazard ratio of 1.6 (95% CI: 1.29–1.90, p<0.001).

In the subset of patients who did not undergo surgery for metastatic disease, RAS mutation also correlated with significantly worse OS. In this subset, median OS was 35.2 months (95% CI: 28–44 months) for RAS wild-type mCRC patients and 28 months (95% CI: 25–32 months) for RAS mutant mCRC patients (p=0.005).

The effect of an activating PIK3CA mutation on OS was evaluated in 785 patients as one patient had no follow-up data. OS did not vary by tumor PIK3CA mutation status (Fig. 2B).

Correlation of RAS Mutation with Sites of Metastatic Involvement

At the time of diagnosis of metastatic disease, RAS mutant and wild-type tumors significantly differed in the pattern of metastatic involvement. RAS mutant cases exhibited a higher incidence of lung metastasis compared to the wild-type cases. Liver, lung, bone, and brain involvement at the time of diagnosis of metastatic disease occurred in 75%, 13%, 0.6%, and 0.9% of RAS wild-type mCRC, respectively, and in 74%, 22%, 0.9%, and 0.5% of RAS mutant mCRC, respectively. Metastases were limited to the liver at the time of diagnosis in 303 (64%) RAS wild-type and 245 (56%) RAS mutant cases (p=0.015).

We determined the cumulative incidence of subsequent site-specific metastasis after the diagnosis of metastatic disease for each of these sites in patients who did not have spread to that site at the time of diagnosis of mCRC. At 2 years, RAS mutation was associated with a significantly higher cumulative incidence of lung (32.5% v 19%, p=0.001), bone (8.8% v 4.4%, p=0.024), and brain metastasis (1.4% v 0.2%, p<0.01). The cumulative incidence of liver metastasis at 2 years did not vary by RAS mutation status (12% v 14.3%, p=0.78). Figure 3 shows the cumulative incidence curves for metastasis to each of these sites over...
time. Lung metastases were detected after initial diagnosis of mCRC in 157 RAS mutant and 151 RAS wild-type mCRC patients, developing earlier in the course of metastatic disease in the RAS mutant mCRC (Fig. 3B). Bone and brain metastasis occurred more commonly in the RAS mutant cases and never reached the same cumulative incidence in RAS wild-type and mutant mCRC. More brain metastasis were seen in the RAS mutant cases. At the end of follow-up, brain metastases were identified in 28 RAS mutant cases and 9 RAS wild-type cases (Fig. 3D).

Competing regression model was used to evaluate the independent association between RAS mutation and the probability of brain, bone, and lung metastasis, separately. For brain metastasis, univariate analysis identified a significant association with RAS mutation (HR $3.3 \ [95\% \ CI: \ 1.59–7.12], p<0.01$) and with diagnosis of lung metastasis (HR $2.3 \ [95\% \ CI: \ 1.13–4.82], p=0.022$). In multivariate analysis, RAS mutation remained an independent predictor of brain metastasis (HR $3.7 \ [95\% \ CI: \ 1.7–8.1], p<0.01$) after controlling for age at diagnosis, tumor location, and previous diagnosis of lung metastasis. For bone metastasis, univariate analysis identified a significant association with RAS mutation (HR $1.55 \ [95\% \ CI: \ 1.08–2.22], p=0.018$), rectal primary tumor (HR $1.7 \ [95\% \ CI: \ 1.2–2.5], p=0.004$), synchronous tumor (HR: $0.7 \ [95\% \ CI: \ 0.52–0.99], p=0.04$), and diagnosis of lung metastasis (HR $1.8 \ [95\% \ CI: \ 1.18–2.80], p<0.01$). In multivariate analysis, RAS mutation remained an independent predictor of bone metastasis (HR $1.62 \ [95\% \ CI: \ 1.1–2.3], p=0.012$) after controlling for age, tumor location, synchronous tumor, and diagnosis of lung metastasis. For lung metastasis, RAS mutation was associated with a HR of $1.52 \ (95\% \ CI: \ 1.21–1.92, p<0.01)$ after controlling for age at diagnosis of metastatic disease, tumor location, and synchronous tumor.

**Site-Specific Prevalence of RAS Mutations**

Figure 4 shows the distribution of RAS genotypes in all cases analyzed and in cases with metastases to lung, bone, and brain anytime during the course of their disease. In our series, the dominant RAS genotype is wild-type. However, for all of these metastatic sites, the proportion of RAS mutant cases has increased, and among cases with brain metastases, the KRAS G12D genotype has now become the dominant genotype.

**Correlation of PIK3CA Mutation with Sites of Metastatic Involvement**

We also analyzed the correlation between PIK3CA mutation status and sites of metastatic involvement. At 2 years, PIK3CA mutation was associated with a significantly higher cumulative incidence of brain metastasis ($1.4\% \ v \ 0.8\%, p=0.0013$). The cumulative incidence of liver, lung, and bone metastases at 2 years did not vary by PIK3CA mutation. Figure 5 shows the cumulative incidence curves for time to metastasis to the liver, lungs, bone, and brain by PIK3CA mutation status. In total, 25 of the cases of brain metastases were genotyped for PIK3CA mutations, and seven were found to have a PIK3CA mutation. Six of these seven patients had concurrent KRAS mutations (four in exon 2 and two in exon 4).
DISCUSSION

In this report, we have sequenced a large clinical series of patients with mCRC for mutations in the RAS and PIK3CA genes. RAS alterations occurred in 48% of mCRC cases, with mutations within KRAS exon 2 in 43%, KRAS exons 3 and 4 in 3%, and NRAS in 2% of cases. These frequencies are consistent with previous reports\textsuperscript{13,14}. PIK3CA mutations occurred in about 10% of mCRC and were significantly associated with concurrent RAS mutation.

We find that the presence of an activating RAS mutation correlates with worse OS in mCRC patients with a HR of 1.6 (95% CI: 1.3–1.9, \textit{p}<0.01) in multivariate analysis. RAS mutation was associated with a median OS that was almost 3 years shorter than that seen for wild-type cases. Anti-EGFR antibody treatment has been associated with a survival advantage of several months in randomized clinical trials. The gap in OS between the RAS mutant and wild-type cases is thus too large to be solely due to uneven treatment with anti-EGFR antibodies, suggesting a different clinical course for these two groups of tumors.

The recent CALGB/SWOG 80405 study reports median OS for mCRC of about 30 months\textsuperscript{15}. Median OS in our series was high in both RAS wild-type and mutant mCRC, exceeding three years. In our series, over half of the patients initially presented with liver-limited metastatic disease and a large proportion of patients underwent metastasectomy or received HAI therapy. Our series is likely enriched for patients who undergo metastasectomy because genotyping at our institution occurs automatically for all metastasectomies performed within the institution and by physician request in other cases of metastatic disease. Additionally, there may be a referral bias to our institution for patients to undergo metastasectomy or HAI therapy. The median OS in this series is in line with other reports from our institution, including our recent report of greater than 80% OS at 3 years in patients undergoing liver resection and adjuvant HAI\textsuperscript{8,11}. Of note, an analysis of OS in the subset of patients who did not undergo surgery for metastatic disease finds median OS of 35 months and 28 months, respectively, for RAS wild-type and RAS mutant mCRC patients, a range that is in line with reported outcomes in recent treatment trials of mCRC patients.

Our data suggest that the site-specific pattern of metastatic spread may impact outcomes in mCRC. Fewer patients with RAS mutant tumors had liver-limited metastases at the time of diagnosis of metastatic disease because of an increased frequency of spread to the lungs at this time. Consequently, fewer patients with RAS mutant tumors underwent hepatectomy at 12 months from the diagnosis of metastasis and fewer patients received HAI therapy.

Metastatic spread in CRC is thought to progress sequentially in many patients, from liver to lung and then to bone and brain as late sites of involvement\textsuperscript{16–18}. Despite the shorter course of metastatic disease in RAS mutant mCRC, RAS mutation was associated with a higher cumulative incidence of CRC metastases to lung, bone, and brain. Strikingly, nearly two-thirds of the brain metastases identified occurred in RAS mutant mCRC. Consistent with these findings, Tie et al evaluated the prevalence of KRAS mutations in primary colorectal tumors and metastases from liver, lung, and brain and found that KRAS mutation was significantly more common in lung and brain metastases\textsuperscript{19}. 

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These data suggest that the metastatic potential of CRC varies by the presence of somatic mutations in the RAS oncogene and that RAS mutation may be associated with an affinity for specific organs. Schluter et al reported that colon cancer colonization of distant organs requires specific cell interactions within the microvasculature of the distant organs and that mechanical entrapment is not sufficient for colonization\textsuperscript{20}. Using intravital microscopy of the microvasculatures of rat liver, lung, intestine, skin, muscle, spleen, and kidney \textit{in vivo}, they investigated the flow of circulating cells after injection of the rats with colon cancer cells. They found that the colon cancer cells arrest primarily in the organs that are commonly targets for metastases, such as the liver or lung, and rarely arrested in the renal and other capillary systems (mesenteric, skin, muscle) despite their smaller microvasculature diameter. A recent study by Urosevic et al evaluates the mechanism by which KRAS mutant colorectal tumors form lung metastasis as a sequential step in progression from established liver metastasis\textsuperscript{21}. In a KRAS mutant CRC model, downregulation of p38 MAPK signaling led to increased lung colonization through increased expression of the cytokine parathyroid hormone-like hormone (PTHLH), which contributed to colon cancer extravasation to the lung by modulating the lung microvasculature.

RAS regulation of focal adhesion kinase (FAK) may potentially explain the increased frequency of brain metastases in RAS mutant tumors. Activated RAS leads to dephosphorylation of FAK at Y397, facilitating focal adhesion turnover at the leading edge of cells\textsuperscript{22}, and leads to ERK-mediated dephosphorylation of PRP-PEST at S571, increasing the interaction of PTP-PEST and FAK to promote migration, invasion, and metastasis\textsuperscript{23}. FAK activation has been found to promote cellular invasion through the blood-brain barrier\textsuperscript{24}.

We did not identify an association between PIK3CA mutation and baseline patient characteristics or survival. Our series was limited to patients with metastatic disease, and other series focusing on metastatic patients also do not report a correlation between PIK3CA mutation and survival\textsuperscript{25–27}. In early stage disease, PIK3CA mutation has been associated with both unchanged and worse outcomes\textsuperscript{6, 28, 29}. Our analysis of PIK3CA mutant mCRC was not powered to detect a small effect on OS. Due to smaller patient numbers, we did not specifically evaluate the impact of helical versus kinase domain PIK3CA mutations. We did find a correlation between PIK3CA mutation and brain metastases, likely resulting from the association of PIK3CA with RAS mutation and the higher frequency of brain metastases in RAS mutant mCRC.

In conclusion, our data suggest that RAS mutation is associated with worse survival and an affinity to spread to the lungs, bone, and brain. Our findings have implications for understanding the biologic effects of RAS activation in mCRC and suggest RAS mutations not only affect initiation of disease but also progression. An understanding of this pattern of spread may help inform physicians’ assessment of symptoms in patients with mCRC and alert physicians to have a lower threshold to evaluate neurologic or bony-related symptoms in patients with RAS mutant mCRC.
Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGMENTS

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Figure 1.
Flow diagram of RAS genotyping performed on cases in this series. Nine hundred eighteen patients had known RAS mutation status, consisting of 477 RAS wild-type cases and 441 RAS mutant cases.
A.

![Graph showing survival analysis with two lines representing RAS WT and RAS MUT. The graph indicates a statistically significant difference between the two groups with a p-value of <0.01.](graph_image)

**Number at Risk**

- **RAS WT**: 477
- **RAS MUT**: 441

**Years**

- 0: 345, 177, 115, 58
- 2: 305, 129, 63, 31

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Figure 2.
Kaplan-Meier estimates of overall survival (OS) from diagnosis of metastatic disease by (A) RAS and (B) PIK3CA mutation status.
Figure 3.
Cumulative incidence of site-specific metastasis to (A) liver, (B) lung, (C) bone, and (D) brain from date of first metastasis by RAS mutation status.
Figure 4.
Prevalence of RAS mutations in all cases and in cases with metastases to lung, bone, or brain.
Figure 5.
Cumulative incidence of site-specific metastasis to (A) liver, (B) lung, (C) bone, and (D) brain from date of first metastasis by PIK3CA mutation status.
Table 1

Patient characteristics by RAS mutation status

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<tr>
<th></th>
<th>RAS WT mCRC (n=477)</th>
<th>RAS Mutant mCRC (n=441)</th>
<th>P value</th>
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<td>Age (years)</td>
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<tr>
<td>Mean [SD]</td>
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<td>59 (28–87)</td>
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<td>Gender</td>
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<tr>
<td>Male</td>
<td>266 (56%)</td>
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<tr>
<td>Female</td>
<td>211 (44%)</td>
<td>214 (49%)</td>
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<td>Primary site</td>
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<td>&lt;0.01</td>
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<tr>
<td>Right colon</td>
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<td>Left colon</td>
<td>277 (58%)</td>
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<tr>
<td>Rectum</td>
<td>86 (18%)</td>
<td>72 (16%)</td>
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<tr>
<td>Timing of metastasis</td>
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<tr>
<td>Synchronous</td>
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<td>252 (57%)</td>
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<tr>
<td>Metachronous</td>
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<td>189 (43%)</td>
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<tr>
<td>Lymph nodes involved</td>
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<td>268 (61%)</td>
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<td>64 (13%)</td>
<td>56 (12%)</td>
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<td>Sites of first metastasis</td>
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<tr>
<td>Liver</td>
<td>359 (75%)</td>
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