Combined analysis of *KRAS* and *PIK3CA* mutations, MET and PTEN expression in primary tumors and corresponding metastases in colorectal cancer

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Metastasis is the main cause of mortality in patients with colorectal cancer. However, most of the targeted therapies and predictive molecular biomarkers were developed based mainly on primary tumors. The current study was conducted to determine the degree of discordance between potential predictive and/or prognostic molecular markers in primary colorectal tumors and corresponding metastases, as this could have an impact on the efficacy of targeted therapies in the advanced colorectal cancer. *KRAS*, *PIK3CA* and *BRAF* mutations were determined by Sanger sequencing and mutant-enriched polymerase chain reaction (PCR) assays in 83 paired samples, *MET* gene copy number by quantitative PCR in 59, MET expression by immunohistochemistry in 73 and nuclear and cytoplasmic expression of PTEN by immunohistochemistry in 78 and 71 pairs, respectively. A certain degree of discordance between primary tumors and corresponding metastases was demonstrated for all examined biomarkers except *BRAF* mutations. *PIK3CA* exon 9 mutations in primary tumors and loss of PTEN nuclear expression in metastases correlated with *KRAS* mutations. *KRAS* wild-type status in primary tumors was associated with loss of PTEN cytoplasmic expression and high gene copy number of *MET*. Survival and clinical data were available for 68 patients. The multiple regression analysis revealed that the right-sided tumor localization and overexpression of MET were associated with shorter overall survival.
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Colorectal cancer is the third more common tumor and the second reason of cancer-related deaths worldwide. About 25% of patients with colorectal cancer present metastases at the time of diagnosis and ∼50% of patients who undergo radical resection for primary colorectal cancer present disease dissemination during the first 5 years from diagnosis. Treatment options for patients with advanced disease have been expanded combining anti-EGFR or anti-VEGF monoclonal antibodies with chemotherapy.[1,2]

The administration of anti-EGFR monoclonal antibodies has been associated with antitumor activity in 10–20% of patients with metastatic colorectal cancer; however, recent studies suggested that the anti-EGFR-mediated antitumor activity is restricted to patients with wild-type *KRAS* tumors. Conversely, up to 65% of *KRAS* wild-type patients do not benefit from the EGFR-targeted therapy and mutations in other downstream effectors of the EGFR signaling pathway such as *BRAF, NRAS, PIK3CA* and loss of expression of PTEN seem to be responsible for this phenomenon.[3–6] However, the negative selection of the four mutant genotypes (*KRAS, BRAF, NRAS, PIK3CA*) offers modest improvements in terms of objective response rates

compared with *KRAS*, indicating that additional markers are needed in order to better predict response to EGFR-targeted therapy.[3] Therefore, taking into account the cross talk between the EGFR and MET signaling pathways, the activation of the MET receptor tyrosine kinase pathway has also been investigated as another possible mechanism of resistance to EGFR-targeted therapy.[7,8]

The majority of studies aiming to identify effective predictive molecular markers have been conducted using the tissue obtained from primary tumors. This has been mainly based on the hypothesis that metastases were fundamentally similar to primary tumors. Indeed, it has been shown that most of mutations present in metastases could also be detected in primary tumors, suggesting that migrating and metastases-generating cells already exist among the initial cell clones present in the primary tumor.[9,10] Consistently, *KRAS* and *BRAF* mutation status were concordant between primary tumors and corresponding distant metastases in ~90–100% of the cases while only few reports exist on the *PIK3CA* mutation status.[11–13] Despite these data, recent studies have demonstrated the presence of genotypic differences between the primary tumors and the metastatic lesions.[14] The genetic analyses of disseminated tumor cells and the identification of gene expression profiles that predict the metastatic potential suggested that metastasis is predetermined early during tumorigenesis.[15,16] Moreover, several reports have shown that more genes were methylated in primary colorectal tumors than in corresponding metastatic lesions and metastases can exhibit substantial differences in gene expression patterns compared with primary tumors.[17–19]

In view of these data, the heterogeneity between primary tumors and metastases emerged as an additional reason for the failure of targeted therapies in colorectal cancer; thus, the current study was conducted in order to determine the degree of discordance between potential predictive and/or prognostic molecular markers in primary tumors and corresponding metastases, and to investigate the possible associations between these biomarkers in primary tumors, as well as in metastases.

Materials and methods

Patients

The archive of the Department of Pathology of the University Hospital of Heraklion, Greece, was searched retrospectively for patients who had matched samples of colorectal primary tumors and synchronous or metachronous metastases. The search period spanned from 1998 to 2010. The samples were fixed in neutral-buffered formalin and embedded in paraffin. The hematoxylin–eosin (HE) sections from the colorectal primary tumor and metastases specimens were reviewed by a pathologist expert in gastrointestinal cancer to confirm the presence of tumor tissue.

All patients had undergone either oncologic resection or tissue biopsy of the tumors for diagnostic and therapeutic purposes.

Tumors occurring at or proximal to the splenic flexure were considered right-sided (proximal) and those in the descending and sigmoid colon were left-sided (distal). Synchronous metastases were defined as metastases detected by preoperative imaging on computed tomography or magnetic resonance imaging or during resection of the primary tumor. Metachronous metastases were detected during follow-up. Clinical data for eligible patients were obtained from the review of all available medical records regarding hospitalization and outpatient visits at the Oncology Department of the University Hospital of Heraklion. Baseline performance status was determined by the treating physician according to Eastern Cooperative Oncology Group criteria. For cancer-specific survival analysis, death attributed to colon cancer was defined as a clinical end point. Overall survival was defined as the interval between diagnosis to death or last follow-up visit.

Mutational Analysis

Tumor tissues to be genotyped were marked on standard HE-stained histologic slides; in order to achieve at least 80% tumor tissue for DNA extraction, 4 μm serial sections were manually macrodissected or microdissected using the Eppendorf Piezo-Power Microdissection system (Eppendorf, Hamburg, Germany). Genomic DNA was extracted using the QIAamp DNA Micro kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Mutational analyses of *KRAS* by Sanger sequencing were performed as previously described.[20] Exons 9 and 20 of the *PIK3CA* were sequentially amplified by two rounds of polymerase chain reaction (PCR) and subjected to by-directional automatic sequencing. The PCR primers for *PIK3CA* amplification were as follows: 99U(exon 9F)- 5'-ACAACAGTTAATTAGCAATGT AAAA-3'; 220U(exon 9F)- 5'-ACAGAGTAACAGACTAGC TAGAGAC-3'; 408L(exon 9R)- 5'-ACATGCTGAGATCAGC CAAAT-3'; 37U(exon 20F)- 5'-TCGACAGCATGCCAATCT C-3'; 45U(exon 20F)- 5'-ATGCCAATCTCTTCATAAATC-3'; 283L(exon 20R)- 5'-CAGAGTGAGCTTTCATTTTCT-3'. Sanger sequencing has a sensitivity of ~20%.

KRAS TaqMan-MGB allelic discrimination assays were designed (custom TaqMan® SNP Genotyping Assays, Applied Biosystems, Foster City, CA, USA) to detect the seven more common *KRAS* mutations; (c.35G>A (p.G12D), c.38G>A (p.G13D), c.34G>C (p.G12R), c.34G>T (p.G12C), c.34G>A (p.G12S), c.35G>T (p.G12V) and c.35G>C (p.G12A)). The amplicons were set at 80 bp. The reporter fluorophores for TaqMan-MGB probes were VIC for wild type and FAM for each *KRAS*-mutant allele. The PCR primers and probes are available upon request. The peptide nucleic acid (PNA) clamp designed to hybridize to the wild-type *KRAS* allele surrounding codons 12 and 13 was as follows:

H2N-CCTACGCCACCAGCTCC-CO-N2H (Eurogentec S.A., Seraing, Belgium). The PNA/DNA hybrid is unstable due to base pair mismatches; therefore, PNA hybridization inhibits the annealing of the wild-type *KRAS* TaqMan-MGB probes but does not inhibit the annealing of the *KRAS*-mutant TaqMan-MGB probes. The reaction was performed in a total volume of $10\,\mu l$, containing $2\,\mu l$ extracted DNA (20 ng), $5\,\mu l$ $2\times$ TaqMan® genotyping master mix (Applied Biosystems), $0.25\,\mu l$ of genotyping assay mix and 200 nM PNA; in parallel, a non-PNA-clamp reaction was performed without any PNAs. The PCR conditions were 95 °C for 10 min, followed by two-step cycling: 50 cycles of 92 °C for 15 s, and 62 °C for 90 s. In each experiment, PCR reagents without template were run in parallel as the no-template control. The sample experiment data and results were generated using an ABI 7900 real-time PCR system (Applied Biosystems) by performing an amplification run using a standard curve plate document to generate real-time PCR data and an allelic discrimination run using an allelic discrimination plate document. The fluorescence data and amplification plots were analyzed with the SDS 2.3 software (Applied Biosystems). The evaluation of *KRAS* mutation status was based on the cycle thresholds of the wild-type and mutant amplification curves as compared between the PNA and non-PNA reactions.

The assay analytical specificity was evaluated using genomic DNA (0.2, 20 ng) isolated from cell lines wild-type (HT-29) or positive (LS174, c.35G>A (p.G12D); HCT116, c.38G>A (p.G13D); HUP-T3, c.34G>C (p.G12R); KYSE410, c.34G>T (p.G12C); A549, c.34G>A (p.G12S); SW403, c.35G>T (p.G12V) and RPMI8226, c.35G>C (p.G12A)) for specific *KRAS* mutations and confirmed by sequencing. All cell lines were obtained from the A. Jung laboratory (Department of Pathology, Ludwig-Maximilians Universitat Munchen, Germany) and were cultured according to the supplier's recommendations. To assess analytical sensitivity, each of the seven *KRAS*-positive cell line genomic DNAs was serially diluted in a background of *KRAS* wild-type genomic DNA provided by the HT-29 cell line to give mutation/wild-type ratios of 100, 50, 20, 10, 5, 1, 0.2 and 0%. For example, the 1:100 mixtures contained 1 ng of mutant DNA and 100 ng of wild-type DNA. Analysis of the mean signal generated by the positive mutant probes plus PNA showed that the assay can reproducibly detect *KRAS* mutations in 0.2% genomic DNA dilution.

BRAF c.1799T>A (p.V600E) mutation detection was assessed by allelic discrimination using Taqman-MGB probes (custom TaqMan® SNP Genotyping Assays, Applied Biosystems). The amplification was performed on an ABI 7900 real-time PCR system (Applied Biosystems) in an $10\,\mu l$ mixture composed of $2\,\mu l$ of extracted DNA (20 ng), $5\,\mu l$ of $2\times$ TaqMan® genotyping master mix (Applied Biosystems) and $0.25\,\mu l$ of genotyping assay mix containing primers and probes (available on request). The reaction was performed with a preliminary step for 2 min at 50 °C, followed by denaturation for 10 min at 95 °C, then 50 cycles of two steps at 92 °C for 15 s and 62 °C for 90 s. Allelic discrimination was performed using SDS 2.3 software (Applied Biosystems).

ARMS allele-specific PCR combined with Scorpions probes for the detection of *PIK3CA* mutations (c.1633G>A (p.E545K), c.1624G>A (p.E542K), c.3140A>G (p.H1047R)) was performed as previously described.[21]

Quantitative Real-Time PCR

Relative *MET* amplification levels were determined by quantitative real-time PCR by comparison with the amplification levels of *TOP3A* residing at chromosome locus 17p11 using the following primers: MET-sense: 5'-AATTGTGTCTTTCTCTAGGCATGTCA-3'; MET-antisense: 5'-GGGAAGGAGTGGTACAACAGATTATC-3', MET probe FAM-CATCGCTCTAATTCAG-NFQ, TOP3A-sense: 5'-CCACTGCGAACTTAAGAAAACTTTG-3'; TOP3A-antisense: 5'-TTCTCTATCACAGTCAGTCCAGATCA-3', TOP3A probe VIC-AACGAGAGACTCGCCAGT-NFQ.[22] Genomic DNA from formalin-fixed paraffin embedded tissue samples, positive control (H1993 cell line for *MET* amplification), and reference (pooled PBMCs from healthy volunteers) (20 ng) was amplified for 40 cycles (15 s, 95 °C; 60 s, 60 °C) in a ABI 7900 real-time PCR system (Applied Biosystems), using the Platinum® qPCR SuperMix-UDG (Invitrogen, Carlsbad, CA, USA), 900 nM primers and 150 mM probes. Sensitivity of the assay was assessed by diluting H1993 cells with PBMCs from healthy volunteers or H2073 cells, which do not harbor *MET* amplification. Amplification of *MET* was clearly distinguished in DNA samples from 10% of H1993 cells mixed with 90% PBMCs (or 90% H2073) from those of PBMCs (or H2073 cells). Efficiency of the PCR reaction in each assay was assessed by a four point standard curve (125, 12.5, 1.25 and 0.125 ng) using DNA from pooled PBMCs. As relative amplification levels were considered the fold changes calculated using the equation 2-ΔΔCT, where $\Delta\Delta CT = (CT(MET)sample - CT(TOP3A)sample) - (CT(MET)reference\ DNA - CT(TOP3A)reference\ DNA)$; the reference sample consisted of DNA extracted from PBMCs of healthy volunteers. The cutoff values used to claim *MET* amplification were established from qPCR data of 14 normal lung formalin-fixed paraffin embedded samples. A tumor sample was considered as amplified when its fold change is over the mean fold change calculated from the 14 normal formalin-fixed paraffin embedded samples + two standard deviations. For all patients, triplicate cycle time (CT) values were averaged.

Immunohistochemistry

Immunostaining was performed on formalin-fixed paraffin wax embedded tissue sections, using UltraVision LP Large Volume Detection System AP Polymer (Thermo Fisher Scientific, Fremont, CA, USA). Primary antibodies for nuclear PTEN (Ms Mab, Clone 28H6 Catalog No. sc-56205; dilution 1:200, Santa Cruz, Biotechnology, USA), cytoplasmic PTEN (Ms Mab, Clone 17.A, Catalog No. MS1601; dilution 1:25, Neomarkers, Lab Vision, Fremont, CA, USA) and MET (Rb Pab, Clone C-28, Catalog No sc-161; dilution 1:100, Santa Cruz) were used. A step of microwave heating in a solution of sodium citrate was performed before

incubation with nuclear PTEN and MET primary antibodies; a step of EDTA antigen retrieval was used for cytoplasmic PTEN. Positive control slide was included for MET consisted of paraffin wax sections from a case of melanoma (membranous or cytoplasmic immunostaining was classified as positive). Paraffin wax sections of prostate cancer and endothelial cells were used as external and internal positive controls, respectively, for cytoplasmic PTEN. Nuclear immunostaining of lymphocytes and stroma cells served as internal positive control for nuclear PTEN. Negative control slides were prepared by omitting the primary antibody.

PTEN cytoplasmic and nuclear expression levels were scored semiquantitatively as previously described.[23,24] Intensity of immunoreactivity for both antibodies was determined as 0, negative; 1, weak; 2, moderate; and 3, strong. As regards the interpretation of PTEN cytoplasmic expression, one, two or three additional points were attributed if the percentage of positive cells was $<25\%$, $25-50\%$ or $>50\%$; tumors with a score <4 were considered to have loss of PTEN cytoplasmic expression. As regards the interpretation of PTEN nuclear expression, tumors with $<10\%$ of cancer cells stained with any intensity were considered to have loss of PTEN nuclear expression.

The MET immunoreactivity was predominantly localized in the cytoplasm and membrane, whereas in 5% of tumor specimens a nuclear immunoreactivity could also be detected in cancer cells. MET protein expression levels were arbitrarily scored dependent on the staining intensity as previously described; 0, negative; 1, weak; 2, moderate; and 3, strong.[25] Tumors with strong staining intensity were considered as MET overexpressed.

Statistical Analyses

Associations between *KRAS*, *BRAF*, *PIK3CA* mutations, *MET* gene status, PTEN and MET protein expression and clinicopathologic variables were assessed by χ^2-test or Fisher's exact test. Tested variables included factors related to the patient (age, sex, and performance status) and factors related to the primary tumor (tumor location, histologic type, invasion depth, lymph node status and histological grading). The association between primary tumors and related metastases for *KRAS*, *PIK3CA* and *BRAF* mutational status, *MET* gene status and PTEN and MET protein expression was evaluated by means of the Cohen's *k*-test, appropriate for the assessment of the concordance between two categorical measurements of the same individual. A moderate and good agreement was defined as the coefficient was $0.41 \le k \le 0.60$ and $0.61 \le k \le 0.80$, respectively.[26] The Spearman correlation coefficient testing was also used to determine the relationship of PTEN and MET expression levels between primary tumors and corresponding metastases. Cox proportional hazards models were used for univariate and multivariate analysis to estimate and test demographic characteristics, clinical features and biologic parameters for their associations with overall survival.

Variables associated with overall survival at univariate analysis were considered for multivariate analysis.

All statistical tests were two sided. Significance levels were set at $P \le 0.05$. All statistical analyses were carried out using the SPSS System V. 19 Software (SPSS Inc., Chicago, IL, USA)

Results

Patient's Clinicopathological Characteristics

Eighty-three patients with paired primary colorectal tumors and corresponding liver ($n = 71$; 86%), lung ($n = 6$; 7%) or other ($n = 6$; 7%) metastases were enrolled in the present study. Patient's demographics, clinical and pathological characteristics are listed in Supplementary Table S1. *KRAS* mutations were not associated with any of the pathological characteristics. On the contrary, a significant association between the *PIK3CA* mutations and mucinous adenocarcinoma ($P < 0.0001$) and the *BRAF* mutations and right-sided tumor localization ($P = 0.049$) was observed (Supplementary Table S1). In addition, loss of nuclear expression of PTEN was associated with right-sided tumors ($P = 0.011$) and high histological grade ($P = 0.024$), whereas MET overexpression was associated with mucinous adenocarcinoma ($P = 0.01$).

Survival and clinical data were available for 68 patients; none of the 68 patients received therapy before resection of the primary tumor. Fifty-one (75%) patients received all three active cytotoxic drugs (fluorouracil/leucovorin (5-FU/LV), irinotecan and oxaliplatin) and 17 (25%) received a regimen 5-FU/LV with either irinotecan or oxaliplatin. Thirty-four (50%) patients received combination chemotherapy with anti-EGFR monoclonal antibody (cetuximab or panitimumab) and 36 (53%) combination chemotherapy with anti-VEGF monoclonal antibody (bevacizumab). The median time interval between resection of the primary tumor and development of metachronous metastasis was 22 months. Fourteen (28%) patients with synchronous metastases and 14 (78%) with metachronous metastases received oxaliplatin or irinotecan based chemotherapy before biopsy or resection of metastases. At a median follow-up of 71.8 (4.63–136.7) months, 42(62%) patients had died. The median overall survival was 44.8 months (37.9 months for patients with synchronous ($n = 50$) and 79.6 for those with metachronous ($n = 18$) metastases ($P = 0.013$)).

Molecular Characteristics of Primary Colorectal Tumors

KRAS mutations were detected in 36 (43%), *BRAF* mutations in three (4%) and *PIK3CA* mutations in 11 (13%; seven in exon 9 and four in exon 20) out of 83 genotyped primary tumors, respectively (Figure 1). Among *KRAS* mutations identified,
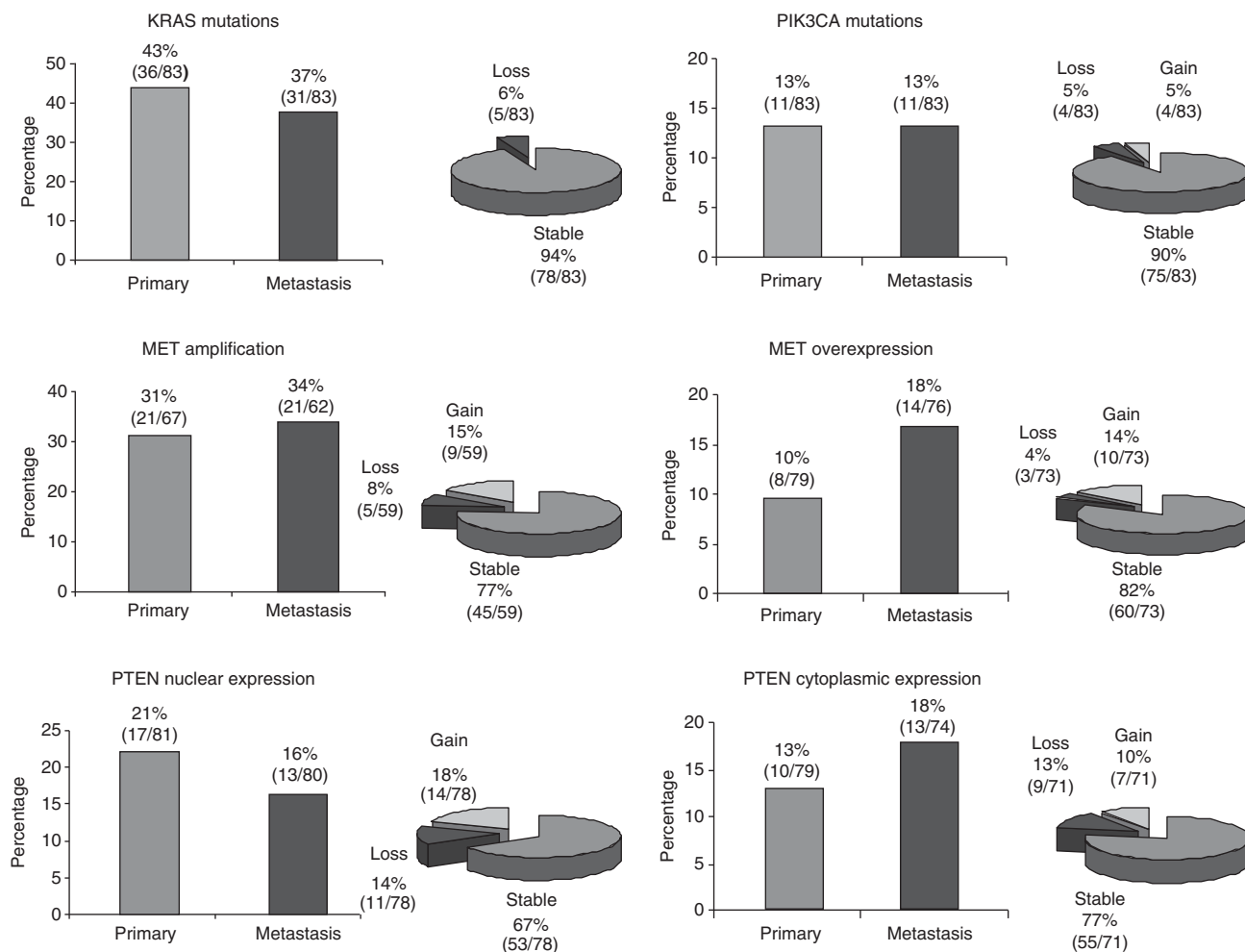
Figure 1 Proportion of the primary tumors and metastases with *KRAS* and *PIK3CA* mutations, high *MET* gene copy number, overexpression of MET and loss of nuclear and cytoplasmic expression of PTEN and distribution of these molecular alterations from primary tumor to metastases.

32 (89%) affected codon 12 and four (11%) codon 13. *KRAS* and *BRAF* mutations were mutually exclusive. One *BRAF* mutant primary tumor harbored the c.1633G>A (p.E545K) *PIK3CA* mutation. Nine (25%) of the 36 tumors with mutant *KRAS* and two (4%) of the 47 wild-type *KRAS* tumors carried *PIK3CA* mutations ($P = 0.008$, Figure 2e), indicating a significant association between the *PIK3CA* and *KRAS* mutations, which seems to be driven by the helical (exon 9) and not the kinase domain (exon 20) mutations ($P = 0.039$ *vs* $P = 0.312$); among the *PIK3CA* mutations co-occurring with *KRAS* mutations, three harbored the c.1624G>A (p.E542K), three the c.1633G>A (p.E545K) and three the c.3140A>G (p.H1047R). Overall, 40 (48%) patients harbored a mutation in any of the *KRAS, BRAF* and *PIK3CA* genes.
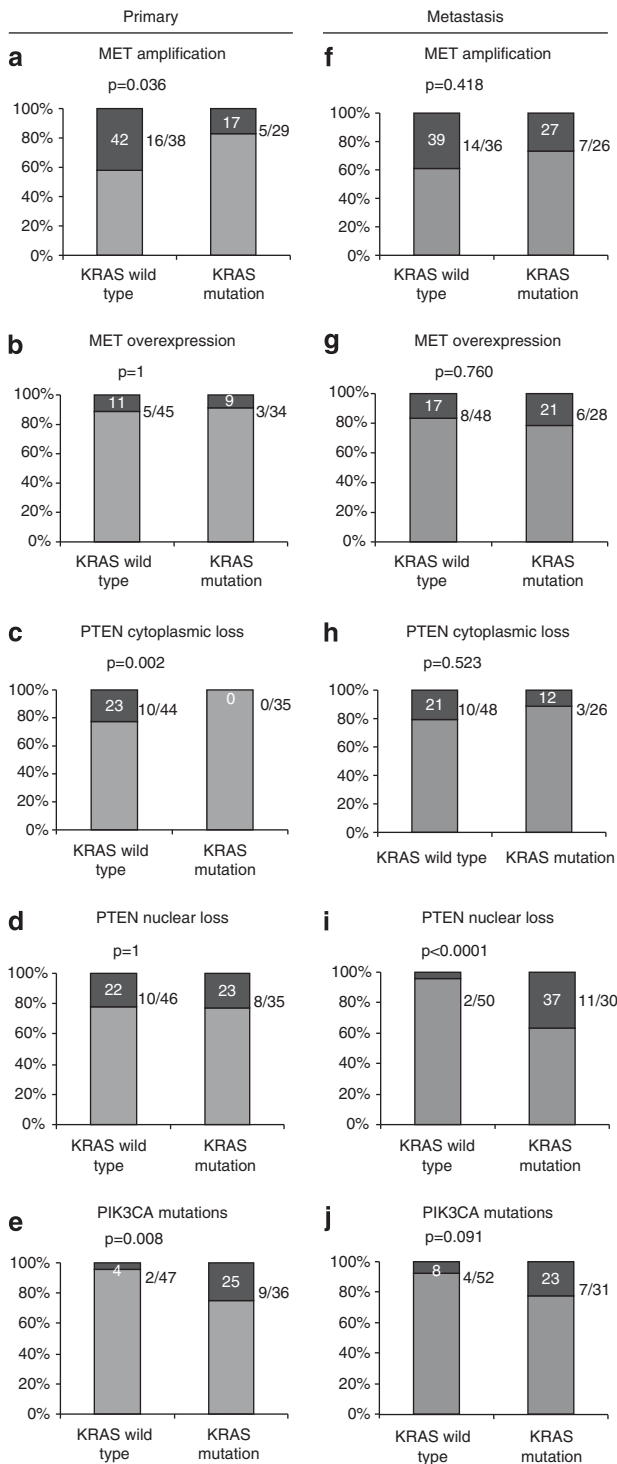
Loss of cytoplasmic and nuclear expression of PTEN was detected in 10 (13%) out of 79 and 17 (21%) out of 81 primary tumors, respectively; in addition, loss of both cytoplasmic and nuclear expression of PTEN was detected in only three

tumors ($P = 0.420$). All tumors with loss of cytoplasmic expression of PTEN were *KRAS* wild type ($P = 0.002$, Figure 2c).

MET overexpression and high gene copy number of *MET* were observed in eight (10%) of 79 and in 21 (31%) of 67 tumors, respectively (Figure 1). None of the eight MET-overexpressed primary tumors had high gene copy numbers of *MET* while 42% of *KRAS* wild-type primary tumors had high gene copy numbers of *MET* ($P = 0.036$; Figure 2a).

Molecular Characteristics of Metastases

KRAS mutations were detected in 31 (37%), *BRAF* mutations in three (3%) and *PIK3CA* mutations in 11 (13%; six in exon 9 and five in exon 20) out of 83 metastases, respectively (Figure 1). A trend for an increased incidence of *PIK3CA* mutations in *KRAS*-mutant metastases was observed ($P = 0.091$; Figure 2j). Overall, 38 (42%) patients harbored a

Figure 2 Percentage of high *MET* gene copy number (**a, f**), overexpression of MET (**b, g**), loss of cytoplasmic (**c, h**) and nuclear expression (**d, i**) of PTEN and *PIK3CA* mutations (**e, j**) in *KRAS* wild-type and *KRAS*-mutant primary tumors and metastases. *P*-values indicate the significance of the association between the above-mentioned molecular alterations and *KRAS* mutations.

mutation in any of the *KRAS, BRAF* and *PIK3CA* genes.

Loss of cytoplasmic and nuclear expression of PTEN was detected in 13 (18%) of 74 and 13 (16%)

of 80 metastases, respectively, while loss of both cytoplasmic and nuclear expression was detected in two metastases. Eleven (37%) *KRAS* mutant and two (4%) *KRAS* wild-type metastatic tumors were negative for nuclear expression of PTEN, indicating a significant positive association between the loss of nuclear expression of PTEN and *KRAS* mutations in metastases ($P < 0.0001$; Figure 2i). Among the eleven *KRAS*-mutant tumors with loss of nuclear expression of PTEN, five were metachronous and six synchronous metastases.

MET overexpression and high gene copy number of *MET* was observed in 14 (18%) of 76 and 21 (34%) of 62 metastases, respectively (Figure 1); four (7%) metastases had concurrent overexpression and high gene copy number of *MET*. None of the metastases with high gene copy number of *MET* had loss of nuclear expression of PTEN ($P = 0.012$). The incidence of either MET overexpression or high gene copy number of *MET* was not significantly different between synchronous (22%) and metachronous (6%) metastases ($P = 0.261$) although 10 out of 11 MET-overexpressed metastases were synchronous.

Heterogeneity between Primary Tumors and Corresponding Metastases

KRAS. *KRAS* mutations were detected by Sanger sequencing in 32 (39%) primary tumors and 22 (27%) related metastases; 10 (12%) patients had a *KRAS* mutation in their primary but not the metastatic tumor. The wild-type *KRAS* pairs (51 patients) and the 10 pairs with *KRAS* discordant results were re-evaluated by PNA Clamp allele-specific real-time PCR assay. Using this assay, 7 out of the 10 discordant paired specimens became concordant; moreover, *KRAS* mutations could be detected in four additional tumors, which had been characterized as *KRAS* wild type by Sanger sequencing (Table 1). Finally, five (6%) patients had *KRAS* mutations exclusively detected in their primary tumors but not in corresponding synchronous metastases (Cohen's $\kappa = 0.875$, $P < 0.001$; Figure 1).

BRAF. In all patients, the same classical *BRAF* c.1799T>A (p.V600E) point mutation was observed both in the primary tumor and the corresponding metastasis.

PIK3CA. *PIK3CA* mutation analysis by standard cycle sequencing revealed discrepancy between the primary tumors and the corresponding metastases in 12 (15%) patients. Following analysis of these 12 discordant paired specimens by ARMS allele-specific PCR combined with Scorpions probes (designed to detect c.1633G>A (p.E545K), c.1624G>A (p.E542K), c.3140A>G (p.H1047R), c.3140A>T (p.H1047L)), discrepancy was confirmed in six (7%) patients (Cohen's $\kappa = 0.581$, $P < 0.001$); in four of them the discrepancy

Table 1 *KRAS* mutation analysis by PNA clamp allele-specific PCR in specimens showing discordant results by sequencing between primary tumors and corresponding metastases

Case	KRAS mutations in primary tumors		KRAS mutations in metastases	
	Sequencing	PNA clamp PCR	Sequencing	PNA clamp PCR
3	c.35G>C (p.G12A)	c.35G>C (p.G12A)	wt	c.35G>C (p.G12A)
5	c.35G>A (p.G12D)	c.35G>A (p.G12D)	wt	c.35G>A (p.G12D)
11	c.34G>T (p.G12C)	c.34G>T (p.G12C)	wt	c.34G>T (p.G12C)
17	c.35G>T (p.G12V)	c.35G>T (p.G12V)	wt	c.35G>T (p.G12V)
31	c.35G>A (p.G12D)	c.35G>A (p.G12D)	wt	c.35G>A (p.G12D)
44	c.35G>A (p.G12D)	c.35G>A (p.G12D)	wt	c.35G>A (p.G12D)
65	c.38G>A (p.G13D)	c.38G>A (p.G13D)	wt	c.38G>A (p.G13D)
70	c.35G>T (p.G12V)	c.35G>T (p.G12V)	wt	wt
71	c.35G>A (p.G12D)	c.35G>A (p.G12D)	wt	wt
78	c.34G>T (p.G12C)	c.34G>T (p.G12C)	wt	wt
43	wt	c.35G>C (p.G12A)	wt	wt
67	wt	c.34G>T (p.G12C)	wt	wt
19	wt	c.35G>T (p.G12V)	wt	c.35G>T (p.G12V)
28	wt	c.35G>C (p.G12A)	wt	c.35 G>C (p.G12A)

Table 2 *PIK3CA* mutation analysis by the ARMS scorpion PCR in specimens showing discordant results by sequencing between primary tumors and corresponding metastases

Case	PIK3CA mutations in primary tumors		PIK3CA mutations in metastases	
	Sequencing	ARMS-scorpion assay	Sequencing	ARMS-scorpion assay
31	c.1624G>A (p.E542K)	c.1624G>A (p.E542K)	wt	c.1624G>A (p.E542K)
28	c.3140A>G (p.H1047R)	c.3140A>G (p.H1047R)	wt	c.3140A>G (p.H1047R)
65	c.3140A>G (p.H1047R)	c.3140A>G (p.H1047R)	wt	c.3140A>G (p.H1047R)
17	c.1633G>A (p.E545K)	c.1633G>A (p.E545K)	wt	c.1633G>A (p.E545K)
14	wt	wt	c.3140A>G (p.H1047R)	c.3140A>G (p.H1047R)
15	wt	wt	c.1633G>A (p.E545K)	c.1633G>A (p.E545K)
79	wt	wt	c.3139C>T (p.H1047Y)	ND
80	wt	wt	1634A>G (p.E545G)	ND
54	c.1633G>A (p.E545K)	c.1633G>A (p.E545K)	wt	wt
71	c.1633G>A (p.E545K)	c.1633G>A (p.E545K)	wt	wt
45	c.1624G>A (p.E542K)	c.1624G>A (p.E542K)	wt	wt
70	c.3140A>G (p.H1047R)	c.3140A>G (p.H1047R)	wt	wt

concerned the exon 9 and in two the exon 20 *PIK3CA* mutation status; however, the *PIK3CA* mutation status of the two primary tumors showing gain of the c.1634A>G (p.E545G) and c.3139C>T (p.H1047Y) in metastases could not be confirmed by the ARMS-Scorpion assay (Table 2). Among patients with *PIK3CA* discordant results, four (5%) changed genotype from wild type to mutant (two patients gained exon 9 and two exon 20 mutations) and four (5%) lost their mutations in metastases (Figure 1). Gain of *PIK3CA* mutations were identified only in synchronous metastases and loss in three synchronous and one metachronous metastases.

PTEN. PTEN cytoplasmic expression was successfully determined in 71 paired specimens. There was no correlation between PTEN cytoplasmic expression in the primary tumors and the corresponding metastases (Spearman's $\rho = 0.095$; $P = 0.431$). Nine (13%) tumors had lost cytoplasmic expression of PTEN and seven (10%) had gained expression in metastases (Cohen's $\kappa = 0.141$; $P = 0.233$).

PTEN nuclear expression was successfully determined in 78 paired specimens. There was no correlation of PTEN nuclear expression between primary tumors and corresponding metastases (Spearman's $\rho = 0.078$; $P = 0.495$). Eleven (14%) tumors had lost nuclear expression of PTEN and 14 (18%) had gained expression in metastases (Cohen's $\kappa = 0.056$; $P = 0.616$).

MET. The expression of MET was successfully determined in 73 paired specimens. There was a positive correlation between MET expression in primary tumors and corresponding metastases (Spearman's $\rho = 0.300$; $P = 0.01$) while the percentage of agreement was low (Cohen's $\kappa = 0.290$; $P = 0.007$). Three (4%) tumors had lost expression of MET and 10 (14%) had gained expression in metastases (Figure 1).

The level of gene copy number alterations of *MET* was assessed in 59 paired specimens; there was a significant positive correlation of gene copy number of *MET* between primary tumors and corresponding metastases (Spearman's $\rho = 0.516$; $P < 0.0001$), while the percentage of concordance was low (Cohen's $\kappa = 0.403$; $P = 0.002$). Five (9%) tumors lost the amplification of *MET* and nine (15%) changed genotype from non-amplified to amplified *MET* in metastases (Figure 1).

Intra-tumoral Heterogeneity

Using *KRAS* and *PIK3CA* mutation status, an exploratory analysis for intra-tumoral heterogeneity was performed in six paired specimens, classified as concordant by the mutant-enriched PCR assays; the mutation status of two different areas either of the primary tumors (three paired specimens) or metastases (three paired specimens) was examined both by Sanger sequencing and the mutant-enriched real-time PCR assay. Intra-tumoral heterogeneity was detected only in primary tumors; two *KRAS* c.35G>T (p.G12V) mutant tumors also comprised cancer cells harboring the *KRAS* c.37G>T (p.G13C) mutation or wild-type *KRAS,* respectively, and one *KRAS* and *PIK3CA* wild-type tumor also comprised cells harboring the c.38G>A (p.G13D) and c.3140A>G (p.H1047R) mutations (Table 3).

Association of Molecular and Pathological Tumor Characteristics with Survival

Univariate analysis revealed that the presence of synchronous metastases, the right-sided tumor location, administration of chemotherapy without biological compounds, loss of nuclear expression of PTEN in primary tumors, presence of *PIK3CA* mutations and overexpression of MET in metastases were significantly associated with shorter overall survival (Supplementary Table S2). The association of *MET* overexpression with overall survival remained significant even when univariate analysis was limited to only the subgroup of patients with synchronous metastases (24.1($n = 10$) *vs* 43.6($n = 36$) months; $P < 0.0001$). Notably, even when survival was measured from the date of biopsy/resection of metastases, MET overexpression was still associated with shorter survival (17($n = 11$) *vs* 37($n = 51$) months; $P < 0.0001$), whereas *PIK3CA* mutations (28($n = 10$) *vs* 34($n = 58$) months; $P = 0.101$) did not.

The multiple regression analysis revealed that the right-sided tumor localization and MET over-expression were independently and significantly associated with shorter overall survival (Table 4 and Figure 3).

Discussion

In the era of personalized cancer therapy, tailored treatment based on gene alterations and molecular
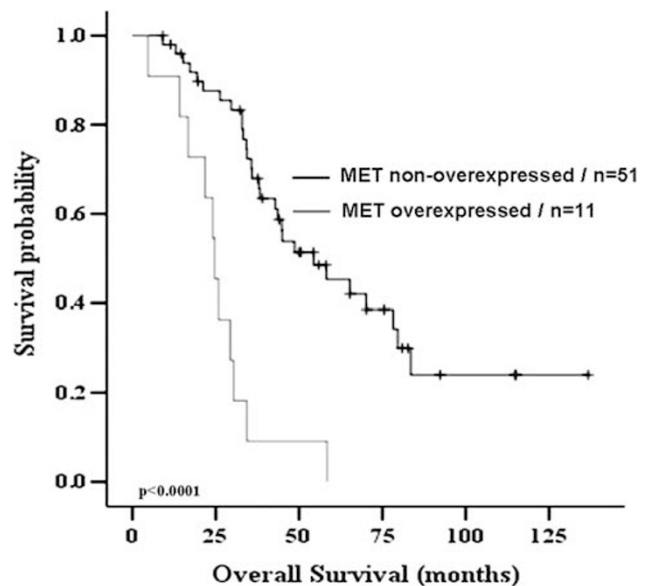
Figure 3 Kaplan–Meier plot to predict overall survival according to MET immunoexpression status in metastases.

Table 3 Mutation analysis of patients showing intra-tumoral heterogeneity

Case	Primary tumor area 1		Primary tumor area 2		Metastases	
	KRAS	*PIK3CA*	*KRAS*	*PIK3CA*	*KRAS*	*PIK3CA*
26	wt	wt	c.38G>A (p.G13D)	c.3140A>G (p.H1047R)	wt	wt
24	c.35G>T (p.G12V)	wt	c.37G>T (p.G13C)	wt	c.35G>T (p.G12V)	wt
21	c.35G>T (p.G12V)	c.1633G>A (p.E545K)	wt	wt	c.35G>T (p.G12V)	c.1633G>A (p.E545K)

Table 4 Multivariate Cox survival analyses

	HR (95% CI)	P (cox)
Site of primary tumor (proximal *vs* distal)	2.18 (1.05–4.49)	0.035
MET expression in metastases (overexpression *vs* non-overexpression)	4.59 (2.05–10.28)	<0.0001

marker expression has become a standard practice. In colorectal cancer, it is documented that *KRAS* mutations predict unresponsiveness to EGFR-targeted monoclonal antibody therapies; however, about 25% of patients not responding to EGFR-targeted therapy are a wild-type for *KRAS*, *BRAF*, *PIK3CA* and *PTEN* status. In these 'quadruple negative' patients, resistance to EGFR-targeted therapies may be mediated by the activation of parallel pathways such as the MET receptor tyrosine kinase signaling pathway. Moreover, the predictive *vs* the prognostic value of these molecular biomarkers is still a matter of controversy. Especially in metastatic colorectal cancer the degree of concordance between primary tumors and related metastases regarding the status of all these promising biomarkers may be of great importance for decision making and prediction of the efficacy of targeted therapies as metastasis mirrors the actual disease status.

To our knowledge, the current study is the first one reporting on the correlation of mutational status of *KRAS*, *BRAF* and *PIK3CA* with cytoplasmic and nuclear expression of PTEN, as well as the expression and the gene copy number of *MET* in primary colorectal tumors and corresponding metastases; moreover, this genetic characterization of primary and metastatic tumors was correlated with patients' clinical outcome.

Our findings demonstrate a high level of concordance between *KRAS* and *BRAF* mutation status in primary tumors and related metastases and point to a certain degree of intra-tumoral heterogeneity in primary tumors, which is in agreement with previous studies.[11,12] Furthermore, our data emphasize the importance of using sensitive molecular methods to ensure efficient mutation detection especially in metastases as the failure to detect a mutation using the Sanger sequencing may merely indicate that the mutation is present in <20% of the analyzed DNA template molecules. Nevertheless, different *KRAS* genotypes were observed in 6% of the paired specimens, indicating that in order to restrict EGFR-targeted therapy only to patients with wild-type *KRAS* tumors it could be more appropriate to perform *KRAS* genotyping in metastases rather than the primary tumors when biological material is available. It is obvious that the clinical utility of this remains to be proven in prospective well-designed clinical studies.

PIK3CA mutation status was found to be discordant in 10% of the analyzed paired specimens; a previous study in colorectal cancer reported a discordance rate of 5%, whereas in breast cancer the *PIK3CA* mutation status frequently changes during disease progression.[12,27,28] One important finding in the current study is the significant association of *KRAS* and *PIK3CA* mutations in primary tumors ($P = 0.008$), which seems to be driven by the helical (exon 9) mutations ($P = 0.039$) as kinase domain (exon 20) mutations alone do not

associate with *KRAS* mutations ($P = 0.312$). Conversely, a similar association could not be observed in metastases, despite the existence of a statistical trend ($P = 0.092$); this is in line with the observed discrepancy of *PIK3CA* mutation status in the paired specimens and could possibly have an impact in determining the optimal algorithm for mutation screening in metastatic colorectal cancer. However, the small number of patients harboring *PIK3CA* mutations in the present study precludes any definitive conclusion and these results remain hypothesis-generating. Recently, De Roock *et al*[29] studying a large cohort of patients with metastatic colorectal cancer arrived at the same conclusion concerning the association between *KRAS* and exon 9 *PIK3CA* mutations; in addition, the authors proposed that in *KRAS* wild-type tumors, only the *PIK3CA* exon 20 mutations have a negative effect on the efficacy of EGFR-targeted therapy.

PTEN expression and localization were heterogeneous between paired specimens. The detection of PTEN by immunohistochemistry instead of other molecular analyses was chosen because it covers both genetic and epigenetic mechanisms underlying loss of PTEN function.[30] Nuclear and cytoplasmic expression of PTEN were evaluated by using two different antibodies displaying predominantly nuclear and cytoplasmic staining, respectively.[31–34] Loss of PTEN cytoplasmic expression was observed only in primary tumors with *KRAS* wild-type status. Previous studies have shown that *RAS* mutations typically arise in PTEN wild-type genetic background, indicating that RAS activation and loss of PTEN probably serve the same function during tumorigenesis.[35–37] On the other hand, although *RAS* is a potent oncogene, its tumorigenicity depends on the cellular context and cooperative events and previous reports have shown that expression of PTEN is suppressed by the oncogenic RAS via the RAF/ERK/MEK signaling pathway giving rise to cells with greater malignant potential.[38–42] The localization of PTEN has not been assessed in these studies although accumulating genetic, pathologic and biochemical evidence suggests that the localization of PTEN either in the nucleus or cytoplasm affects the proliferation of tumor cells.[43–47] In the current study, it was found that loss of PTEN nuclear expression in metastases was significantly associated with *KRAS* mutations; this finding is in accordance with a mechanism of suppression of PTEN nuclear translocation through *KRAS* activation. Although the association of *KRAS* mutations with the loss of PTEN nuclear expression seems to be contradictory with the observed loss of PTEN cytoplasmic expression only in tumors with *KRAS* wild type, these findings could be explained if we hypothesize that the subcellular localization of PTEN is affected by different genetic or epigenetic alterations that, probably, may co-occur or not with *KRAS* mutations. However,

our results should be considered with caution as a common drawback of all studies evaluating expression of PTEN by immunohistochemistry is the lack of a robust universally-acceptable PTEN immunohistochemistry assay and because the differences observed between primary tumors and related metastases could be the result of previous medical treatments.[31,48]

Previous studies have shown that loss of PTEN nuclear expression and *BRAF* mutations were associated with poor clinical outcome while the prognostic significance of *PIK3CA* and *KRAS* mutations in colorectal cancer remains elusive.[24,49–58] In our series, loss of PTEN nuclear expression in primary tumors and the *PIK3CA* mutations in metastases were associated with shorter overall survival (Supplementary Table S2); however, neither PTEN expression nor *PIK3CA* mutations were emerged as independent prognostic factors in multivariate analysis (Table 4). Activation of RAS/PI3K signaling network by mutation of at least one of the *BRAF, KRAS* and *PI3KCA* was associated with shorter survival, as previously reported by Barault *et al* (data not shown).[54]

Aberrant activation of the MET signaling pathway has been implicated in the development and progression of colorectal cancer.[7,59–62] Our findings demonstrate that a high *MET* gene copy number in primary tumors was correlated with *KRAS* wild type while in metastases it was inversely correlated with the loss of nuclear PTEN expression. These data, combined with the positive association of *KRAS* mutations and loss of PTEN nuclear expression in metastases, seem to indicate a distinct role of MET signaling in *KRAS* wild-type metastases. Nevertheless, the most frequent cause of constitutive activation of MET in human tumors is increased protein expression in the absence of gene amplification;[63] accordingly, in the present study the expression of MET as measured by immunohistochemistry was not found to correlate with the amplification of *MET* as already has been described.[64]

However, the data reported on *MET* gene copy number are in disagreement with previous studies showing that *MET* amplification is a rare event in primary colorectal cancer tumors.[65,66] This discrepancy may be due to methodological differences as we used a quantitative RT-PCR assay instead of FISH and thus the reported increase in *MET* gene copy number could be due to a polysomy of chromosome 7 and not locus specific amplification of *MET*. Moreover, the choice of *TOP3A* as a reference gene in our experiments, which resides on chromosome 17 whose loss has been associated with transition to carcinoma, may be another reason for this discrepancy; however this possibility was investigated using *KRAS* as a reference gene and similar results were obtained (unpublished results).

In the current study, the *MET* gene copy number was not significantly associated with overall survival, whereas MET overexpression in metastases emerged as an independent factor associated with shorter overall survival. Several previous studies have shown that *MET* polysomy is correlated with high levels of *MET* mRNA and that high levels of *MET* mRNA were associated with tumor invasion; however, no prognostic significance has been reported for either *MET* polysomy or *MET* amplification.[25,67] Furthermore, previous studies have provided evidence for the prognostic value of MET protein expression in the early stages of carcinoma progression.[68,69] In this study the majority of patients had metastatic disease and they all received the appropriate chemotherapy although their access to EGFR- or VEGF-targeted therapy was not always based on molecular biomarker selection; therefore, the data reported herein highlight the value of MET expression in metastases as a biomarker that might be used to select advanced colorectal cancer patients who could benefit from targeted therapies.

In conclusion, the current study demonstrates (1) a certain degree of discordance between primary colorectal cancer tumors and related metastases regarding all examined biomarkers except *BRAF* mutations; (2) an association between *KRAS* and the helical *PIK3CA* mutations; (3) an association between *KRAS* mutations and loss of PTEN nuclear expression and (4) a possible prognostic value of MET overexpression in metastatic disease. Overall, it appears that metastatic lesions are the appropriate tissues to analyze in order to determine targeted therapies in metastatic colorectal cancer and the activation RAS/PI3K and MET signaling pathways defines distinct subsets of metastatic colorectal cancer patients.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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