Effect of KRAS and NRAS mutations on the prognosis of patients with synchronous metastatic colorectal cancer presenting with liver-only and lung-only metastases

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Abstract. It has been reported that 20-25% of patients with colorectal cancer (CRC) have metastases at the time of diagnosis. Liver and lung are the most common metastatic sites. The aim of the present study was to investigate the association of KRAS and NRAS mutations with clinicopathological features and prognosis of patients with initial liver-metastasis only (LiM-only) or lung-metastasis only (LuM-only) metastatic CRC (mCRC). Overall, 166 patients with CRC with initial LiM-only (n=124) and LuM-only (n=42) were retrospectively analyzed from January 2014 to December 2017. The median follow-up time was 19.2 months (1.0-57.1 months). Patient characteristics at diagnosis were collected. Genomic DNA was isolated from frozen primary CRC tissues for targeting KRAS and NRAS. Patients with LuM-only were significantly older compared with those with LiM-only (65.5 vs. 61.5 years; P=0.05). There was no significant differences between the LiM-only and LuM-only groups in terms of sex, location of the primary tumor, serum carcinoembryonic antigen level, histological grade and RAS mutation status. KRAS mutations were detected in 43 (41.0%) patients with LiM-only and 13 (35.1%) patients with LuM-only. The overall survival time (OS) of LuM-only was more favorable compared with that of patients with LiM-only (44.5 vs. 24.7 months); however, there was no significant difference (P=0.095). The progression-free survival (PFS) and OS in the RAS wild-type group were significantly improved compared with the RAS mutant cohorts (P=0.004 and P=0.031, respectively) in the LiM-only group. In patients with stage IV CRC, those with synchronous LiM-only mCRC had a higher incidence of metastasis but a less favorable PFS and OS compared with patients with LuM-only. RAS mutation status exhibited a significant association with the survival outcome in patients with LiM-only mCRC.

Introduction

According to GLOBOCAN 2018 data, colorectal cancer (CRC) is the fourth most common cancer and the third leading cause of cancer-associated death (1). It has been reported that 20-25% of patients have metastases at the time of diagnosis (2) and remains the primary reason of poor prognosis and cause of CRC-associated death (3). The most common sites of distant metastases from CRC are the liver and lung (4,5), which affects the prognosis and survival of patients with CRC (6). In recent years, it has been demonstrated that CRC treatment should be tailored to the individual patient due to the wide variety of risk factors, such as sex, age, tumor-node-metastases (TNM) stage and tumor location, genetic factors and surgical complexity (7). Therefore, it is important to identify clinicopathological features and genetic mutations in patients with CRC.

RAS gene mutations serve a role in the carcinogenesis of CRC (8). KRAS and NRAS are different mutant forms. KRAS mutations are observed in 43% of patients with metastatic CRC (mCRC) and have a less favorable prognosis in patients with mCRC. Amado et al (9) demonstrated the treatment effect of
anti-epidermal growth factor receptor (EGFR) monoclonal antibody (panitumumab) on progression-free survival (PFS) in the wild-type (WT) KRAS group was significantly greater compared with the mutant group. WT KRAS patients had longer overall survival (OS) (9). NRAS mutations affect patients with mCRC prognosis and predict lack of response to anti-epidermal growth factor receptors (10). The prognostic role of RAS mutations has been investigated previously and several studies have focused on stage II and III CRC (11-13); the effect of RAS mutations on the efficacy of mCRC treatment remains uncertain. Few studies have assessed the association between RAS gene mutation status, characteristics and survival outcomes of patients with the synchronous liver-metastasis only (LiM-only) and lung-metastasis only (LuM-only) mCRC.

EGFR is a transmembrane protein. Overexpression of EGFR has been described to have an association with disease progression, poor prognosis, metastatic spread and drug resistance in colorectal cancers (14-16). The efficacy of anti-EGFR monoclonal antibody (mAb) has been evaluated as monotherapy or combined with different types of chemotherapy in patients with mCRC (14). There were three methods to detect the EGFR status: Protein expression by immunohistochemistry (IHC), gene copy number by fluorescence in situ hybridization (FISH) and mutation analysis using the Scorpion amplification refractory mutation system (ARMS) (17). However, these 3 methods were closely related to each other (17). In the present study, the expression of EGFR was analyzed by immunohistochemical staining.

The aim of the present retrospective study was to investigate the clinicopathological and genetic characteristics and survival outcomes in patients with synchronous LiM- and LuM-only mCRC.

Materials and methods

Patient selection. According to the 7th edition of AJCC (American Joint Committee on Cancer staging system in 2010) (18), the retrospective cohort included 287 consecutive patients registered with a pathological proof stage IV mCRC at the Cancer Center of Kaohsiung Medical University Hospital (Kaohsiung, Taiwan) within a 4-year period (from January 2014 to December 2017). The inclusion criteria of this study were patients with mCRC with synchronous liver-only or lung-only metastasis and aimed to explore the effect of KRAS and NRAS mutations on the prognosis of patients with synchronous mCRC presenting with liver-only (LiM-only) and lung-only (LuM-only) metastases. Patients with >2 sites metastases (n=99), other sites of metastases other than the liver and lung, such as bone, spleen and brain (n=13), and peritoneal metastases only (n=9) were excluded. Ultimately, a total of 166 eligible patients were analyzed, including 124 synchronous LiM-only and 42 synchronous LuM-only patients with CRC (Fig. 1). There were 95 males and 71 females, with a median age of 63.3 years (range, 26-90 years). The clinical outcomes and survival status of the patients were regularly followed up every 3 months during a clinic visit either until February 2019 or until their death, with a median follow-up time of 19.2 months (1.0-57.1 months). The PFS and OS of the patients, according to RAS mutation status were investigated. For genetic analysis of KRAS and NRAS, all samples were collected immediately after surgical resection, frozen instantly in liquid nitrogen and then stored in -80˚C freezer until analyzed.

Clinicopathological features. The clinical and pathological records of each patient were obtained from medical records. The clinical information included demographic data (age and sex) and clinical parameters, such as the location of primary tumor, preoperative serum carcinoembryonic antigen (CEA) level, cancer cell differentiation according to pathological report (well differentiated, moderately differentiated and poorly differentiated), and duration of liver or lung metastases (Table I).

DNA extraction and KRAS/NRAS direct sequencing. Not all patients were routinely checked for their genetic profile, as some patients did not undergo surgery or biopsies. 105/124 (84.7%) LiM-only and 37/42 (88.1%) LuM-only patients received KRAS genetic testing, 101/124 (81.5%) LiM-only and 37/42 (88.1%) LuM-only patients received NRAS genetic testing. Genomic DNA was isolated from frozen primary CRC tissues using proteinase-K (Stratagene; Agilent Technologies, Inc.) digestion and phenol/chloroform extraction, as described previously (19). The designed sequences of the oligonucleotide primers for KRAS and NRAS exons 2-4 and the operational procedure for direct sequencing were based on those reported in our previous studies (20,21). For KRAS and NRAS genotyping, the frozen primary CRC tissues were fixed with 10% formalin for 24-48 h at room temperature, then deparaffinized (the procedure included 3 washes in xylene for 3 min followed by 3 washes in 99.8% ethanol for 3 min) and sliced into 5-µm tissue sections. After deparaffinization and rehydration, the DNA was isolated using a Gentra Puregene Tissue kit (Qiagen NV, Thermo Fisher Scientific Inc.) according to the manufacturer’s instructions. The primers used were designed using the Primer 3 v.0.4.0 free software program (http://primer3.ut.ee) and the sequences are as follows: KRAS forward, 5’-TCATATTATTGTATATAAGGCCTGCTGAA-3’ and reverse, 5’-CAAGACTGTGCTCGCCAGTGATAC-3’; NRAS forward, 5’-GATGTGGCTCGCAATTAACCA-3’ and reverse, 5’-GATATGGGTAAGATGATCAGGA-3’. Polymerase chain reaction (PCR) amplification of 0.5 µg DNA with 2.5µU of Pro Taq Plus DNA Polymerase (Protech Technology Enterprise Co., Ltd) in the presence of 200 µM dNTPs, 0.2 µM primers, and 1X reaction buffer was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems; Thermo Fisher Scientific Inc.). The PCR reaction volume was 40 µl and the PCR conditions for KRAS and NRAS were as follows: 94.0˚C for 10 min, 35 cycles of denaturation for 30 sec at 94.0˚C, annealing for 60 sec at 56.0˚C, primer extension for 30 sec at 72.0˚C and a final extension for 7 min at 72.0˚C. A fragment analysis of the PCR products was conducted to verify the KRAS and NRAS genotypes using automated capillary electrophoresis using an ABI PRISM 310 Genetic Analyzer system and Taqman Genotyper v.1.6 software (Applied Biosystems; Thermo Fisher Scientific, Inc.). According to RAS status, patients were categorized into the WT or mutant RAS group. There were 59 WT and 42 mutant RAS in the LiM-only group, and 22 WT and 15 mutant RAS in the LuM-only group.
Treatment protocol. For the WT RAS group, the majority of patients received biweekly cetuximab, at a dose of 500 mg/m² in a 2-h infusion, followed by folinic acid (leucovorin), fluorouracil (5-FU) and irinotecan (FOLFIRI) on day 1 of a 14-day cycle as the first-line treatment. The FOLFIRI regimen consisted of 180 mg/m² irinotecan as a 90-minute infusion, and 200 mg/m² leucovorin (LV), and 2,800 mg/m² 5-FU as a 46-h infusion injection on days 1 and 2. In contrast, for the mutant RAS group, the treatment regimen comprised of bevacizumab [5 mg/kg as a 120-min intravenous (IV) infusion] on day 1, followed by irinotecan (180 mg/m² as a 90-min IV infusion), LV (200 mg/m² as an IV infusion over 2 h) and 5-FU (2,800 mg/m² as an IV infusion over a 46-h period), which was repeated biweekly.

Immunohistochemical (IHC) analysis of epidermal growth factor receptor (EGFR) expression. The IHC analysis of EGFR expression was based on that from our previous studies (22,23). The tissues were fixed with 10% formalin for 24 h at room temperature. Following fixation, the tissues were paraffin embedded as follows (total duration, 16 h): 70% ethanol, two changes for 1 h each; 80% ethanol, one change for 1 h; 95% ethanol, one change for 1 h; 100% ethanol, three changes for 1.5 h each; paraffin wax, two changes for 2 h each at 60°C. Formalin-fixed and paraffin-embedded tissue blocks were cut into 3-µm sections to retrieve antigens. The sections were deparaffinized by performing 2-3 changes of xylene, 10 min each at room temperature. The tissues were rehydrated in a descending alcohol series which comprised of 2 changes of 100% ethanol for 3 min each followed by 95 and 80% ethanol for 1 min each. The sections were then rinsed with distilled water. Endogenous peroxidase activity was blocked with methanol containing 0.1% H₂O₂ for 30 min. After washing with a Tris-buffer solution, the sections were incubated for 30 min at room temperature with EGFR primary antibody (1:200; cat. no. NCL-L-EGFR-384; Leica Microsystems Trading Co., Ltd.) and then DAKO REAL EnVision Detection System-HRP (1:100 dilution; Agilent Technologies Inc.) was added for 30 min at room temperature according to the manufacturer's instructions. The slides were washed with Tris-buffered saline between incubations. Finally, the sections were incubated in 0.5% 3,3'-diaminobenzidine for 15-20 min at room temperature before being counterstained with Mayer's hematoxylin. Negative controls consisted of incubating the slides with negative control rabbit immunoglobulin (Agilent Technologies Inc.) in the absence of the primary antibody. The immunoreactivity of EGFR was evaluated by

Figure 1. CONSORT (Consolidated Standards of Reporting Trials) flow chart of the 293 patients initially diagnosed with stage IV metastatic colorectal cancer. Meta, metastasis.
two independent researchers blinded to the outcomes of the patients. The expression patterns of EGFR were determined in a semiquantitative manner using light microscopy (magnification, x100). Immunoreactivity for EGFR (membrane staining) was categorized according to the presence of tumor cell staining and staining intensity. The intensity of EGFR immunoreactivity was scored using a three-tier system as follows: 1+ (weak intensity, faint brown membranous staining); 2+ (moderate intensity, brown membranous staining of intermediate darkness producing a complete or incomplete circular outline of the neoplastic cell) and 3+ (strong intensity, dark brown or black membranous staining producing a thick outline, complete or incomplete of the neoplastic cell) (24). Negative EGFR expression was defined as the absence of membrane staining above the background in all tumor cells, whereas positive EGFR expression was defined the complete or incomplete IHC membrane staining of tumor cells, including intensities of 1+, 2+, or 3+ (Fig. 2).

Statistical analysis. All data were statistically analyzed using SPSS version 22.0 (IBM Corp.). Values are presented as the mean ± standard deviation for continuous variables and were compared using an unpaired Student’s t-test. The χ² test was used to determine the difference of clinicopathological characteristics, KRAS/NRAS mutation and EGFR overexpression status between the LiM-only and LuM-only groups. All probability values were two-tailed. The clinicopathological characteristics of the WT and mutant RAS/KRAS/NRAS groups were compared using the Pearson’s χ² test and the survival rates were calculated using the Kaplan-Meier method and log-rank tests were used to analyze survival distribution. P<0.05 was considered to indicate a statistically significant difference. The mutations identified in the present study were compared with that in the TCGA dataset (https://cancer.gov/tcga; accessed on 2019/12/31) (25,26). The selection criteria used for this dataset include colon cancer, colorectal cancer, KRAS, NRAS and BRAF.

Results

Clinical characteristics. The clinicopathological characteristics of the 166 patients, including 124 LiM-only and 42 LuM-only, are summarized in Table I. The incidence of LiM-only was nearly 3x that of LuM-only in stage IV CRC. Patients with LuM-only were significantly older compared with that in patients with LiM-only (P=0.050). The status of LiM- or LuM-only was not significantly associated with the location of the primary tumor, serum CEA level and differentiation of the primary tumor. Moreover, in 94 (75.8%) patients with LiM-only and 29 (69.0%) patients with LuM-only, the primary tumor originated from the left colon. A total of 84.7, 81.5 and 54.0% of patients with LiM-only and 88.1, 88.1 and 35.7% of patients with LuM-only were analyzed for KRAS, NRAS mutations and EGFR overexpression status, respectively. KRAS and NRAS mutations and EGFR overexpression rates were 41.0, 5.0 and 86.6% in patients with LiM-only, respectively, and 35.1, 38.1 and 80.0% in patients with LuM-only, respectively.

In the present study, the mutation spectra of RAS isoforms: KRAS and NRAS that mutationally activated at codons 12, 13 or 61 were studied. 69.6% (39/56) of KRAS mutations occur at codon 12 whereas 5.4% (3/56) mutations are observed at codon 61. In contrast, 57.1% (4/7) of NRAS tumors harbor mutations at codon 12 vs. 28.6% (2/7) at codon 12 (Table II). KRAS exon 2 mutations were detected in 39.4% (56/142) of patients, with both LiM and LuM-only CRC, which included 24 (42.9%) patients with G12D, 8 (14.3%) patients with G12V, 7 (12.5%) patients with A146T, 6 (10.7%) with G13D,
3 (5.4%) patients with G12C, 3 (5.4%) patients with codon 61, 2 (3.6%) patients with G12A, 1 (1.8%) patients with G13R, 1 (1.8%) patient with G12S and 1 (1.8%) patient with G12D, G13D mutations. NRAS mutation status was analyzed in 83.1% (138/166) of patients and only 5.8% (8/138) of patients harbored a mutation, including 3 (37.5%) patients with codon 61, 2 (25.0%) with codon 12, 1 (12.5%) patient with codon 146, 1 (12.5%) patient with Q61K and 1 (12.5%) patient with T58I and Q61L mutations (Table II). The TCGA data set showed that there were 176 KRAS mutations and 41 NRAS mutations among 10,202 papules. KRAS mutations included 49 (27.8%) with G12D, 33 (18.8%) with G12V, 31 (17.6%) with G13D, 13 (7.4%) with A146T, 10 (5.7%) with G12C, 8 (4.6%) with G12A, 7 (4.0%) with G12S and 3 (1.7%) with Q61K mutations. NRAS mutation included 4 (9.8%) with Q61K, 4 (9.8%) with G12D, 2 (4.9%) with G13R, and 1 (2.4%) with R164C, G12A, G12C, Q61R, G12V, NRAS L6L, E132K, E76K and Q61L mutations (Table II). Notably, the distributions of the different mutations were similar between the TCGA dataset and our study. 67/124 (54.0%) of LiM-only and 15/42 (35.7%) LuM-only patients underwent EGFR expression tests. The results demonstrated that 86.6% of LiM-only and 80.0% of LuM-only patients had EGFR overexpression. There was no significant difference between the LiM-only group and LuM-only group (P=0.515) (Table I).

Survival analysis. Survival data were collected for all patients. The median follow-up time was 19.2±5.3 months (1.0-57.1 months). The median PFS and OS times for LiM-only vs. LuM-only were 18.4±2.1 vs. 26.6±3.7 months (P=0.266) and 24.7±1.1 vs. 44.5±10.0 months (P=0.095), respectively (Fig. 3). Patients with LuM-only had a favorable PFS and OS compared with that in patients with LiM-only, however this difference was not significant (P=0.266 and P=0.095).

The results of the subgroup analysis of PFS and OS times conducted according to RAS, KRAS and NRAS mutation status in the LiM-only and LuM-only groups are presented in Tables III and IV. In the LiM-only group, the median PFS of the RAS WT group was significantly more favorable compared with that of the mutant group (27.1 vs. 12.9 months; P=0.004; Table III). The OS was also more favorable in the RAS WT group compared with the mutant group (36.8 vs. 20.7 months, P=0.031; Table IV). In the LuM-only group, the median PFS of the RAS WT group was not significantly more favorable compared with that of the mutant group (36.0 vs. 25.6 months, P=0.056; Table III) but was significantly more favorable in the KRAS WT group compared with patients with KRAS mutation (36.0 vs. 21.0 months, P=0.017; Table III).

The PFS and OS times in the RAS WT group were significantly improved compared with the RAS mutant cohorts (27.1 vs. 12.9 months, P=0.004 and 36.8 vs. 20.7 months, P=0.031, respectively) in the LiM-only group (Fig. 4A and B). Conversely, the RAS WT and mutant groups exhibited no significant PFS and OS difference in the LuM-only group (36.0 vs. 25.6 months, P=0.056 and 33.5 vs. 31.0 months, P=0.581, respectively; Fig. 5A and B). The PFS and OS times between the WT and mutant status for KRAS were 26.8 vs. 14.2 months (P=0.012) and 36.8 vs. 22.0 months (P=0.038), respectively in the LiM-only group (Tables III and IV; Fig. 6A and B). The KRAS WT and mutant groups showed significant PFS times (36.0 vs. 21.0 months, P=0.017) but not OS times (46.4 vs. 28.2 months, P=0.418) in the LuM-only group (Tables III and IV; Fig. 6C and D). Similarly, the PFS and OS times between the WT and mutant status for NRAS

Figure 2. Immunohistochemical staining of epidermal growth factor receptor in colorectal cancer (membrane staining). (A) Negative expression. (B) 1+ expression (weak intensity). (C) 2+ expression (moderate intensity). (D) 3+ expression (strong intensity). Magnification, x200.
were 21.4 vs. 6.6 months (P=0.032) and 30.1 vs. 7.8 months (P=0.002), respectively in the LiM-only group (Tables III, IV, Fig. 6E and F). The NRAS WT and mutant groups showed no significant PFS times (26.4 vs. 36.4 months, P=0.719) and OS times (33.5 vs. 46.4 months, P=0.719) in the LuM-only group (Tables III and IV; Fig. 6G and H).
KRAS mutations has been previously reported to have greater proportion of liver metastases (78.6 vs. 53.5%; P<0.001) when compared with mutant KRAS in the patients with lung metastases compared with those with liver and ovari or bladder metastases (P=0.039). Prasanna et al (4) also revealed that the incidence of LuM-only was higher with KRAS/RAS mutations (relative risk, 1.4: P=0.007) when compared with other site metastasis (including liver, lymph node, brain, and peritoneum). Recently, a retrospective analysis of 899 patients with mCRC demonstrated that WT KRAS had greater proportion of liver metastases (78.6 vs. 53.5%; P<0.001) when compared with mutant KRAS, whereas patients with mutant KRAS had greater proportion of lung metastases (23.3 vs. 8.7%; P=0.02) when compared with WT KRAS in the patients with left-sided tumors (34). In the present study, 75.8% (94/124) of patients with LiM-only and 69.0% (34/49) of those with LuM only mCRC metastasized from left-sided colon cancer, which was not significantly associated. The identification of KRAS mutational status as a predictive marker of response to anti-EGFR mAb has been one of the most significant and practice-changing recent advances in colorectal cancer (35). However, in a clinical setting, treatment with anti-EGFR mAb is not recommended for patients with mCRC and KRAS or NRAS mutant forms of RAS, in which case only anti-VEGF mAb therapy should be used (36). In addition, EGFR is a direct downstream target of RAS signaling; however, EGFR amplification (>2 copies) and protein overexpression in colon cancer tissues have not been established as reliable biomarkers for anti-EGFR agents (37,38). Several studies have demonstrated that KRAS mutations are associated with a higher incidence of CRC recurrence, metastatic spread and shorter OS time (39-41). Moreover, various metastatic sites and RAS mutation status exhibit distinct outcomes. For example, Prasanna et al (4) indicated that survival of patients with mCRC was associated with the site of metastases, with lung-only metastasis displaying a more favorable survival outcome compared with other single metastatic site diseases. The results from univariate analysis revealed that the median OS time was longer when metastases

Discussion

In the present study, data were collected over a 4-year period with a median follow-up period of 19.2 months (1.0-57.1 months) from 166 patients initially diagnosed with mCRC with LiM- or LuM-only. The results revealed that the incidence of LiM-only was ~3x that of LuM-only. Overall, the median OS was 19.2±13.7 months (1.0-57.1 months) in patients with LiM- and LuM-only mCRC. The association between RAS mutation status and clinicopathological features was also evaluated between the LiM- and LuM-only groups. The results demonstrated that RAS, KRAS and NRAS mutations were associated with both the PFS and OS time of patients with LiM-only. In contrast, RAS mutation did not affect the PFS and OS time of patients with LuM-only except for mutant KRAS in PFS. In addition, the association between EGFR overexpression and survival was examined; however, there was no significant difference between LiM- and LuM-only mCRC groups. To the best of our knowledge, the present study was the first to analyze RAS mutation status and survival in synchronous LiM-only and LuM-only patients with CRC.

RAS mutations lead to the constitutive activation of EGFR signaling through the oncogenic Ras/Raf/Mek/Erk pathway (27). All types of mutant KRAS are present in 35-45% of patients with CRC, and codons 12 and 13 are the two most common hotspots, accounting for ~95% of all mutation types (~80% occurring in codon 12 and 15% in codon 13) (28). The present study population was relatively homogenous, all of Han ethnicity and from the same geographic location.

Several clinical trials have demonstrated that active KRAS mutations are negative predictors of the clinical benefit of anti-EGFR therapies in patients with mCRC (21,29,30). The prognostic role of KRAS mutations has been previously investigated; however the prognostic value of RAS mutations remains uncertain with respect to the treatment of mCRC. Roth et al (11) prospectively collected 1,404 samples from patients with stage II and III CRC and demonstrated that KRAS mutations did not have a major prognostic value regarding the relapse-free survival or OS. There are several possible explanations for the differences in the results of these clinical trials, such as the study size and design, patient population and staging, tumor sampling (primary or metastatic site), use of archival vs. fresh or frozen material, laboratory methods, data analyses and distinct chemotherapy protocol and regimen. In terms of the metastatic sites in mCRC, numerous studies have reported an association with primary tumor location and RAS mutation status. For example, left-sided colon cancer is more likely to metastasize to the liver and lung (31,32). Kim et al (33) reported that RAS mutation rate was higher in patients with lung metastases compared with those with liver and ovari or bladder metastases (P=0.039). Prasanna et al (4) also revealed that the incidence of LuM-only was higher with KRAS/RAS mutations (relative risk, 1.4: P=0.007) when compared with other site metastasis (including liver, lymph node, brain, and peritoneum). Recently, a retrospective analysis of 899 patients with mCRC demonstrated that WT KRAS had greater proportion of liver metastases (78.6 vs. 53.5%; P<0.001) when compared with mutant KRAS, whereas patients with mutant KRAS had greater proportion of lung metastases (23.3 vs. 8.7%; P=0.02) when compared with WT KRAS in the patients with left-sided tumors (34). In the present study, 75.8% (94/124) of patients with LiM-only and 69.0% (34/49) of those with LuM only mCRC metastasized from left-sided colon cancer, which was not significantly associated.

The identification of KRAS mutational status as a predictive marker of response to anti-EGFR mAb has been one of the most significant and practice-changing recent advances in colorectal cancer (35). However, in a clinical setting, treatment with anti-EGFR mAb is not recommended for patients with mCRC and KRAS or NRAS mutant forms of RAS, in which case only anti-VEGF mAb therapy should be used (36). In addition, EGFR is a direct downstream target of RAS signaling; however, EGFR amplification (>2 copies) and protein overexpression in colon cancer tissues have not been established as reliable biomarkers for anti-EGFR agents (37,38). Several studies have demonstrated that KRAS mutations are associated with a higher incidence of CRC recurrence, metastatic spread and shorter OS time (39-41). Moreover, various metastatic sites and RAS mutation status exhibit distinct outcomes. For example, Prasanna et al (4) indicated that survival of patients with mCRC was associated with the site of metastases, with lung-only metastasis displaying a more favorable survival outcome compared with other single metastatic site diseases. The results from univariate analysis revealed that the median OS time was longer when metastases

Figure 3. Comparison of liver meta- and lung meta-only for (A) progression-free survival and (B) overall survival. Meta, metastasis.
were limited to the lung or liver and shorter in cases of brain, bone or peritoneal metastases (4).

The genetic analysis of somatic mutation hotspots in KRAS, NRAS and BRAF is now standard practice for selecting patients with mCRC eligible for anti-EGFR therapy (42). Mutations can be assessed using next-generation sequencing (NGS) or PCR-based assays. The number of analyzed targets, the speed of the assays and accuracy of the results are crucial. Several factors may affect the data produced, such as the quality of the DNA extracted from paraffin-embedded tissue, tumor heterogeneity, quality control of laboratories and different DNA polymerase enzymes. Nagakubo et al (42) compared mutation detection in KRAS and NRAS genes between the PCR-reverse sequence-specific oligonucleotide probe method and bridged nucleic acid-clamp PCR using Sanger sequencing. A total of three discordant results were obtained and the concordance rate was 94% between the two methods. All mutations identified using BNA-clamp PCR and Sanger sequencing were also identified using NGS. This suggested that BNA-clamp PCR using Sanger sequencing detects somatic mutations in KRAS, NRAS and BRAF with a high accuracy (42). Gilson et al (43) used DNA pipetted directly in the cartridge of the Idylla system, exhibiting a good sensitivity, specificity, reproducibility and limit of detection, and can be integrated in a laboratory workflow for samples with little tissue without compromising the further complete

### Table III. Gene mutation status and progression-free survival analysis of patients with metastatic colorectal cancer.

| Gene  | n  | Median PFS (months) | P-value | n  | Median PFS (months) | P-value |
|-------|----|---------------------|---------|----|---------------------|---------|
| RAS   |    |                     | 0.004   |    |                     | 0.056   |
|       | Wild-type | 59 | 27.1 | | 22 | 36.0 | |
|       | Mutant    | 42 | 12.9 | | 15 | 25.6 | |
| KRAS  |    |                     | 0.012   |    |                     | 0.017   |
|       | Wild-type | 62 | 26.8 | | 24 | 36.0 | |
|       | Mutant    | 43 | 14.2 | | 13 | 21.0 | |
| NRAS  |    |                     | 0.032   |    |                     | 0.719   |
|       | Wild-type | 96 | 21.4 | | 34 | 26.4 | |
|       | Mutant    | 5  | 6.6  | | 3  | 36.4 | |
| EGFR overexpression | | | 0.628 | | | 0.942 |
| Negative | 9 | 32.1 | 3 | 26.6 |
| Positive  | 58 | 23.4 | 12 | 36.0 |

Meta, metastasis; PFS, progression-free survival.

### Table IV. Gene mutation status and overall survival analysis of patients with metastatic colorectal cancer.

| Gene   | n  | Median OS (mon) | P-value | n  | Median OS (mon) | P-value |
|--------|----|-----------------|---------|----|-----------------|---------|
| RAS    |    |                 | 0.031   |    |                 | 0.581   |
| Wild type | 59 | 36.8 | 22 | 33.5 |
| Mutant | 42 | 20.7 | 15 | 31.0 |
| KRAS   |    |                 | 0.038   |    |                 | 0.418   |
| Wild type | 62 | 36.8 | 24 | 46.4 |
| Mutant | 43 | 22.0 | 13 | 28.2 |
| NRAS   |    |                 | 0.002   |    |                 | 0.719   |
| Wild type | 96 | 30.1 | 34 | 33.5 |
| Mutant | 5  | 7.8  | 3  | 46.4 |
| EGFR overexpression | | | 0.876 | | | 0.449 |
| Negative | 9 | 36.8 | 3 | 43.1 |
| Positive  | 58 | 31.1 | 12 | 36.6 |

Meta, metastasis; OS, overall survival.
tumor characterization using NGS. In the present study, the ABI PRISM 310 genetic analyzer system and Taqman genotyper v.1.6 software (Applied Biosystems; Thermo Fisher Scientific, Inc.) were used to perform accurate analysis using automated capillary electrophoresis for genotyping.

The present study was limited by its relatively small sample size and retrospective nature. Not all patients were routinely checked for their genetic profile. Furthermore, this study did not consider the severity of the patient's comorbidities and the performance status that may affect survival. Four patients hesitated to receive treatment, which affects the results. In addition, the small sample size did not permit evaluation of the effect of various KRAS mutation subtypes, such as G12D, G12V, G13D, and A146T.

In the present study, a population of patients with synchronous LiM- and LuM-only mCRC were evaluated. The clinicopathological characteristics and RAS mutation status between these two groups were compared. The results revealed a difference in the PFS and OS between patients with WT and mutant forms of KRAS in the LiM-only group but not in the LuM-only group. RAS mutation is a poor prognostic predictor of less favorable PFS and OS in synchronous patients with LiM- and LuM-only mCRC.

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Figure 6. Subgroup analysis of progression-free survival and overall survival conducted according to KRAS and NRAS mutation status in the LiM-only and LuM-only groups. (A and B) KRAS wild-type vs. mutant in LiM-only group. (C and D) KRAS wild-type vs. mutant in LuM-only group. (E and F) NRAS wild-type vs. mutant in LiM-only group. (G and H) NRAS wild-type vs. mutant in LuM-only group. LiM, liver metastasis; LuM, lung metastases.
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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions
SC conceived the study and wrote the manuscript. CH and CM performed the statistical analysis. YC performed the histological examinations. HT, TC, WS, WH and CK analyzed and interpreted the clinical data. JW conceived the study and revised the manuscript for important intellectual content. All authors read and approved the final manuscript.

Ethics approval and consent to participate
In compliance with the Helsinki Declaration, the present study was approved by The Institutional Review Board of the Kaohsiung Medical University Hospital (approval no. KMUHIRB-20130022). Written informed consent was provided by all patients prior to enrollment in the study.

Patient consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

References
1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA and Jemal A: Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA Cancer J Clin 68: 394-424, 2018.
2. Millikan KW, Staren ED and Doolas A: Invasive therapy of metastatic colorectal cancer to the liver. Surg Clin North Am 77: 27-48, 1997.
3. Nitzkorski JR, Farma JM, Watson JC, Siripurapu V, Zhu F, Matteotti RS and Sigurdson ER: Outcome and natural history of patients with stage IV colorectal cancer receiving chemotherapy without primary tumor resection. Ann Surg Oncol 19: 379-385, 2012.
4. Prasanna T, Karapetis CS, Roder D, Tie J, Padbury R, Price T, Wong R, Shapiro J, Nott L, Lee M, et al: The survival outcome of patients with metastatic colorectal cancer based on the site of metastases and the impact of molecular markers and site of primary cancer on metastatic pattern. Acta Oncol 57: 1438-1444, 2018.
5. Qiu M, Hu J, Yang D, Cosgrove DP and Xu R: Pattern of distant metastases in colorectal cancer: A SEER based study. Oncotarget 6: 38658-38666, 2015.
6. Zizzo M, Galeone C, Braglia L, Ugoletti L, Siciliani A, Nachira D, Margaritora S, Pedrazzoli C, Paci M, Lococo F, et al: Long-term outcomes after surgical resection for synchronous or metachronous hepatic and pulmonary colorectal cancer metastases. Digestion 101: 144-155, 2020.
7. Augestad KM, Merok MA and Ignatovic D: Tailored treatment of colorectal cancer: Surgical, molecular, and genetic considerations. Clin Med Insights Oncol 11: 1179554917690766, 2017.
8. Raskov H, Pommergaard HC, Burchart J and Rosenberg J: Colorectal carcinogenesis-update and perspectives. World J Gastroenterol 20: 18151-18164, 2014.
9. Amado RG, Wolf M, Peeters M, Van Cutsem E, Siena S, Freeman DJ, Juan T, Sikorski R, Suggs S, Radinsky R, et al: Wild-type KRAS is required for panitumumab efficacy in patients with metastatic colorectal cancer. J Clin Oncol 26: 1626-1634, 2008.
10. Schirripa M, Cremonlini C, Loupakis F, Morvillo M, Bergamo F, Zoratto F, Salvatore L, Antoniotti C, Marmorino F, Sensi E, et al: Role of NRAS mutations as prognostic and predictive markers in metastatic colorectal cancer. Int J Cancer 136: 83-90, 2015.
11. Roth AD, Teipar S, Delorenzi M, Yan P, Fiocca R, Klingbiel D, Dietrich D, Biesmans B, Bodoky G, Barone C, et al: Prognostic role of selected KRAS and BRAF in stage II and III colorectal cancer. Results of the translational study on the PetaCC-3, EORTC 40993, SAKK 60-00 trial. J Clin Oncol 28: 466-474, 2010.
12. Hutchins G, Southward K, HANDLEY K, Magill L, Beaumont C, Stalschmidt J, Richman S, Chambers P, Seymour M, Kerr R, et al: Value of mismatch repair deficiencies in predicting recurrence and benefits from chemotherapy in colorectal cancer. J Clin Oncol 29: 1261-1270, 2011.
13. Zhang Y, Ma J, Zhang S, Deng G, Wu X, He J, Pei H, Shen H and Zeng S: A prognostic analysis of 895 cases of stage III colon cancer in different colon subsites. Int J Colorectal Dis 30: 1173-1183, 2015.
14. Fakih M and Wong R: Efficacy of the monoclonal antibody EGFR inhibitors for the treatment of metastatic colorectal cancer. Curr Oncol 17 (Suppl 1): S3-S17, 2010.
15. Kenoo AP: Epidermal growth factor receptor-targeted treatment for advanced colorectal carcinoma. Cancer 103: 2435-2446, 2005.
16. Bibeau F, Boissiere-Michot F, Sabourin JC, Gourgou-Bourgade S, Radal M, Penault-Llorca F, Rochaix P, Arnould L, Bralet MP, Azria D and Ychou M: Assessment of epidermal growth factor receptor (EGFR) expression in primary colorectal carcinomas and their related metastases on tissue sections and tissue microarray. Virochim Arch 449: 281-287, 2006.
17. Liang Z, Zhang J, Zeng X, Gao J, Wu S and Liu T: Relationship between EGFR expression, copy number and mutation in lung adenocarcinomas. BMC Cancer 10: 376, 2010.
18. Edge SB and Compton CC: The American Joint Committee on Cancer: The 7th edition of the AJCC cancer staging manual and its future of TNM. Ann Surg Oncol 17: 1471-1474, 2010.
19. Sambrook J and Russell DW: The condensed protocols from molecular cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, p. p800, 2006.
20. Yen LC, Uen YH, Wu DC, Lu CY, Yu FJ, Wu IC, Lin SR and Wang JY: Activating KRAS mutations and overexpression of epidermal growth factor receptor as independent predictors in metastatic colorectal cancer patients treated with cetuximab. Ann Surg 251: 254-260, 2010.
21. Wang JY, Hsieh JS, Chen FM, Yeh CS, Alexanderks K, Huang TJ, Chen DC and Lin SR: High frequency of activated K-ras codon 15 mutant in colorectal carcinomas from Taiwanese patients. Int J Cancer 107: 387-393, 2003.
22. Huang CW, Chen YT, Tsai HL, Yeh YS, Su WC, Ma CJ, Tsai TN and Wang JY: EGFR expression in patients with stage III colorectal cancer after adjuvant chemotherapy and on cancer cell function. Oncotarget 8: 117463-117476, 2017.
23. Huang CW, Tsai HL, Chen YT, Huang CM, Ma CJ, Lu CY, Kuo CH, Wu DC, Chai CY, Chen YT, Su WC, Wu IC and Lin SR: Prognostic values of EGFR expression and KRAS mutation in patients with synchronous or metachronous metastatic colorectal cancer. BMC Cancer 13: 599, 2013.
24. Scartozzi M, Bearzi I, Berardi R, Mandolesi A, Fabris G and Cascini S: Epidermal growth factor receptor (EGFR) status in primary colorectal tumors does not correlate with EGFR expression in related metastatic sites: Implications for treatment with EGFR-targeted monoclonal antibodies. J Clin Oncol 1: 4772-4778, 2004.
25. Cancer Genome Atlas Network: Comprehensive molecular characterization of human colon and rectal cancer. Nature 487: 330-337, 2012.
26. Liu Y, Sethi NS, Hinoue T, Schneider BG, Cherniack AD, Sanchez-Vega F, Seoane JA, Farshidfar F, Bowby R, Islam M, et al: Comparative molecular analysis of gastrointestinal adenocarcinomas. Cancer Cell 33: 721-735.e8, 2018.
27. Wee P and Wang Z: Epidermal growth factor receptor cell proliferation signaling pathways. Cancer (Basel) 9: 52, 2017.
28. Tan C and Du X: KRAS mutation testing in metastatic colorectal cancer. World J Gastroenterol 18: 5171-5180, 2012.
29. Lo Nigro C, Ricci V, Vivenza D, Granetto C, Fabozzi T, Miraglio E and Merlano MC: Prognostic and predictive biomarkers in metastatic colorectal cancer anti-EGFR therapy. World J Gastroenterol 22: 6944-6954, 2016.
30. De Stefano A and Carlonmagno C: Beyond KRAS: Predictive factors of the efficacy of anti-EGFR monoclonal antibodies in the treatment of metastatic colorectal cancer. World J Gastroenterol 20: 9732-9743, 2014.
31. He XK, Wu W, Ding YE, Li Y, Sun LM and Si J: Different anatomical subsites of colon cancer and mortality: A population-based study. Gastroenterol Res Pract 2018: 7153685, 2018.
32. Engstrand J, Nilsson H, Stromberg C, Jonas E and Freedman J: Colorectal cancer liver metastases—a population-based study on incidence, management and survival. BMC Cancer 18: 78, 2018.
33. Kam SH, Heo JS, Lee J, Lee JY, Lee MY, Lim SH, Lee WY, Kim SH, Park YA, Cho YB, et al: The impact of KRAS mutations on prognosis in surgically resected colorectal cancer patients with liver and lung metastases: A retrospective analysis. BMC Cancer 16: 120, 2016.
34. Yong ZZ, Ching GT and Ching MTC: Metastatic profile of colorectal cancer: Interplay between primary tumor location and KRAS Status. J Surg Res 246: 325-334, 2020.
35. Shankaran V, Obel J and Benson AB III: Predicting response to EGFR inhibitors in metastatic colorectal cancer: Current practice and future directions. Oncologist 15: 157-167, 2010.
36. van Helden EJ, Menke-van der Houven van Oordt CW, Heymans MW, Ket JCF, van den Oord R and Verheul HMW: Optimal use of anti-EGFR monoclonal antibodies for patients with advanced colorectal cancer: A meta-analysis. Cancer Metastasis Rev 36: 395-406, 2017.