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

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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 outcome 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. Following 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'-TCATTATTTTTATTATAAGGCCTGCTG AA-3' and reverse, 5'-CAAAGACTGGTCCTGCACCAG TA-3'; *NRAS* forward, 5'-GATGTGGCTCGCCAATTAAC-3' and reverse, 5'-GAATATGGGTAAAGATGATCCGA-3'. Polymerase chain reaction (PCR) amplification of 0.5 μg DNA with 2.5U 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.

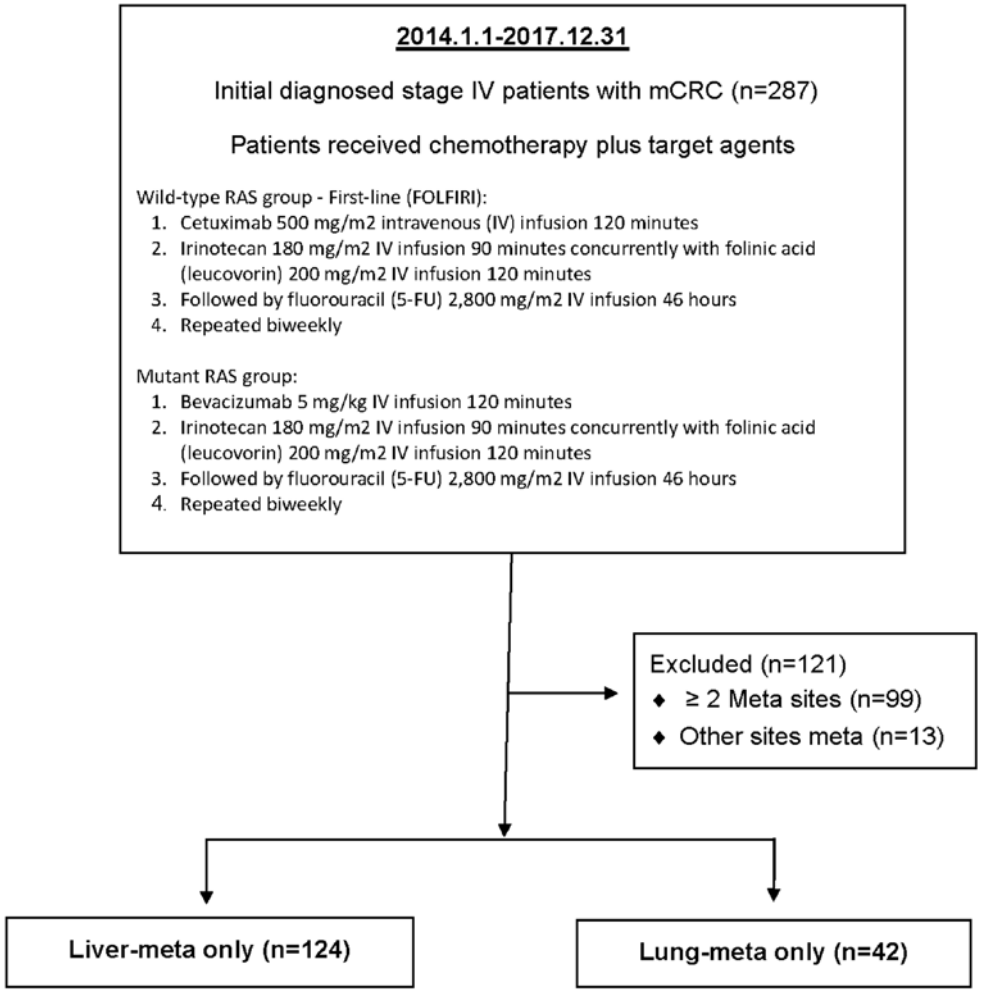

Figure 1. CONSORT (Consolidated Standards of Reporting Trials) flow chart of the 293 patients initially diagnosed with stage IV metastatic colorectal cancer. Meta, metastasis.

Treatment protocol. For the WT *RAS* group, the majority of patients received biweekly cetuximab, at a dose of 500 mg/m^2 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/m^2 irinotecan as a 90-minute infusion, and 200 mg/m^2 leucovorin (LV), and 2,800 mg/m^2 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/m^2 as a 90-min IV infusion), LV (200 mg/m^2 as an IV infusion over 2 h) and 5-FU (2,800 mg/m^2 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; xylene or xylene substitute, 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% H_2O_2 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

Table I. Characteristics of 166 patients with metastatic colorectal cancer at diagnosis and gene mutation profiles.

Characteristic	Liver-meta only (n=124)	Lung-meta only (n=42)	P-value
Median age, years (range)	62.2 (26-90)	66.6 (41-86)	0.050
Sex, male:female	76:48	19:23	0.069
Location of primary tumor, n			0.388
Right colon	30	13	
Left colon	94	29	
CEA level, ng/ml, n			0.589
<5	41	12	
≥5	83	30	
Differentiated, n			0.713
WD	9	1	
MD	89	34	
PD	9	3	
Not Determined	17	4	
KRAS mutation, % (n/total)	41.0 (43/105)	35.1 (13/37)	0.534
NRAS mutation, % (n/total)	5.0 (5/101)	8.1 (3/37)	0.482
EGFR overexpression, % (n/total)	86.6 (58/67)	80.0 (12/15)	0.515

WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

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 χ^2 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 χ^2 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 criterias 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 61 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,

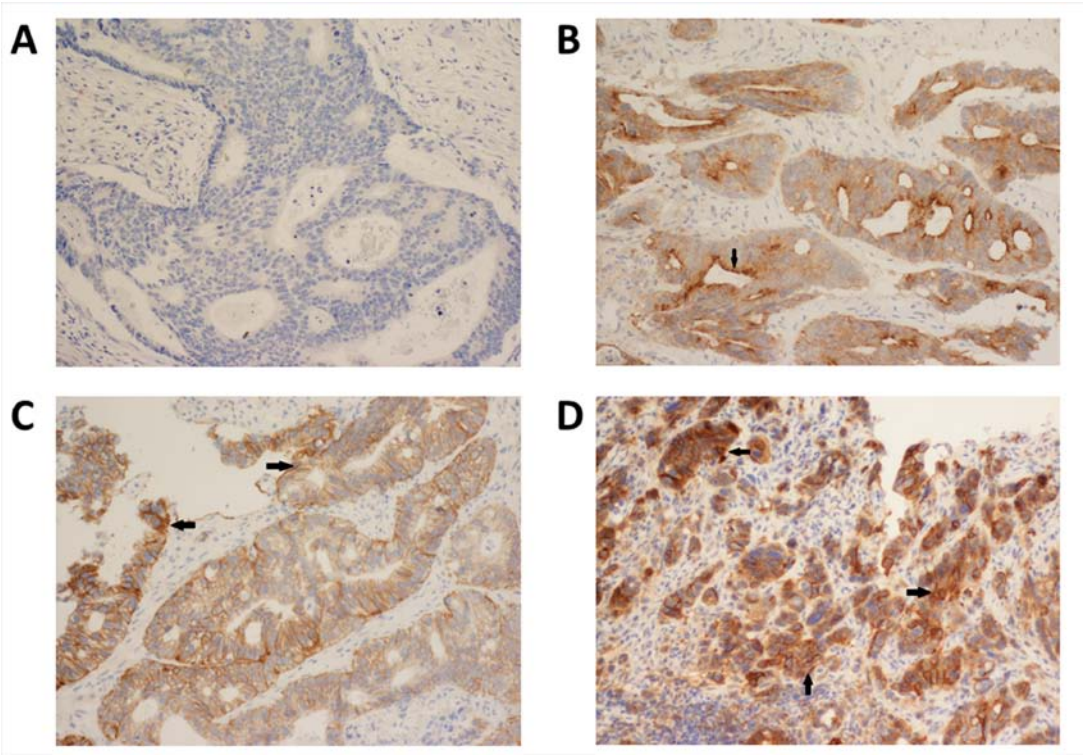

Figure 2. Immunohistochemical staining of epidermal growth factor receptor in colorectal cancer (membrane staining). (A) Negative expression. (B) 1+ expression (weak intensity). (C) 2+ expression (moderate intensity). (D) 3+ expression (strong intensity). Magnification, x200.

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*

Table II. Sites and occurrence of mutations in cases in the present study compared with The Cancer Genome Atlas dataset.

A, LiM-only, n=124		
KRAS mut 41.0% (43/105)	*NRAS* mut 5.0% (5/101)	*BRAF* mut 7.1% (6/85)
Mut site (n, %)	Mut site (n, %)	Mut site (n, %)
G12D (20, 46.5)	Codon 61 (1, 1)	Codon V600E (5, 5.9)
G12V (7, 16.3)	Q61K (1, 1)	Codon V601E (1, 1.2)
G13D (5, 11.6)	Codon 12 (1, 1)	-
A146T (4, 9.3)	Codon 146 (1,1)	-
G12C (2, 4.7)	T58I and Q61L (1, 1)	-
G12A (2, 4.7)	-	-
G12D. G13D (1, 2.3)	-	-
G12S (1, 2.3)	-	-
G13R (1, 2.3)	-	-
B, LuM-only (n=42)		
KRAS mut 35.1% (13/37)	*NRAS* mut 8.1% (3/37)	*BRAF* mut 3.2% (1/31)
Mut site (n, %)	Mut site (n, %)	Mut site (n, %)
G12D (4, 10.8)	Codon 61 (2, 5.4)	Codon V600E (1, 3.2)
A146T (3, 8.1)	Codon 12 (1, 2.7)	-
Codon 61 (3, 8.1)	-	-
G12C (1, 2.7)	-	-
G12V (1, 2.7)	-	-
G13D (1, 2.7)	-	-
C, The Cancer Genome Atlas dataset (N=10,202)		
KRAS mut (n=176)	*NRAS* mut (n=41)	*BRAF* mut
Mut site (n, %)	Mut site (n, %)	
G12D (49, 27.8)	Q61K (4, 9.8)	-
G12V (33, 18.8)	G12D (4, 9.8)	-
G13D (31, 17.6)	G13R (2, 4.9)	-
A146T (13, 7.4)	R164C (1, 2.4)	-
G12C (10, 5.7)	G12A (1, 2.4)	-
G12A (8, 4.6)	G12C (1, 2.4)	-
G12S (7, 4.0)	Q61R (1, 2.4)	-
Q61K (3, 1.7)	G12V (1, 2.4)	-
-	NRAS L6L (1, 2.4)	-
-	E132K (1, 2.4)	-
-	E76K (1, 2.4)	-
-	Q61L (1, 2.4)	-

Mut, mutation.

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).

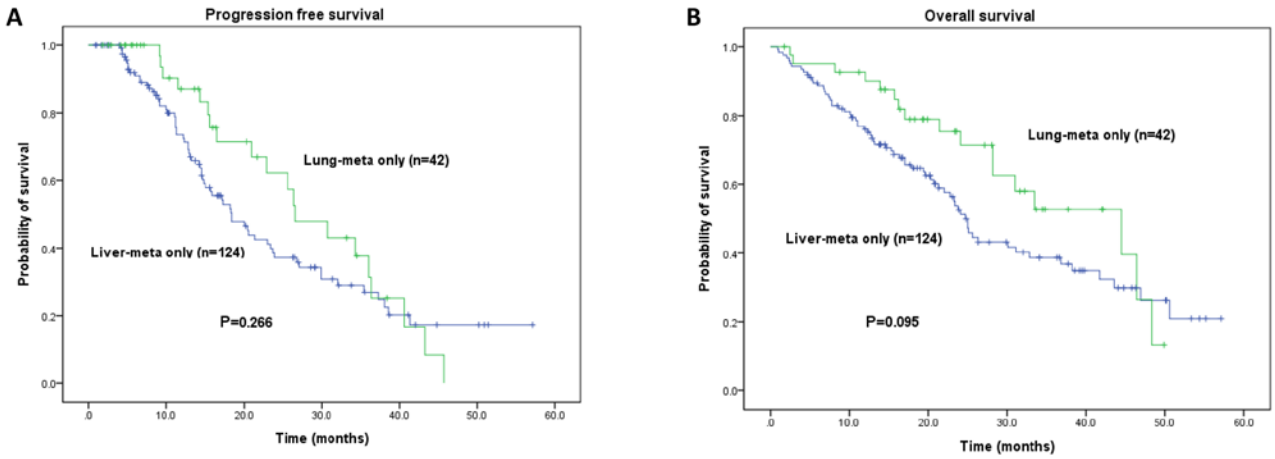

Figure 3. Comparison of liver meta- and lung meta-only for (A) progression-free survival and (B) overall survival. Meta, metastasis.

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 ovary 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, bone 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% (29/42) 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

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

	Liver-meta only			Lung-meta only		
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.

	Liver-meta only			Lung-meta only		
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.

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

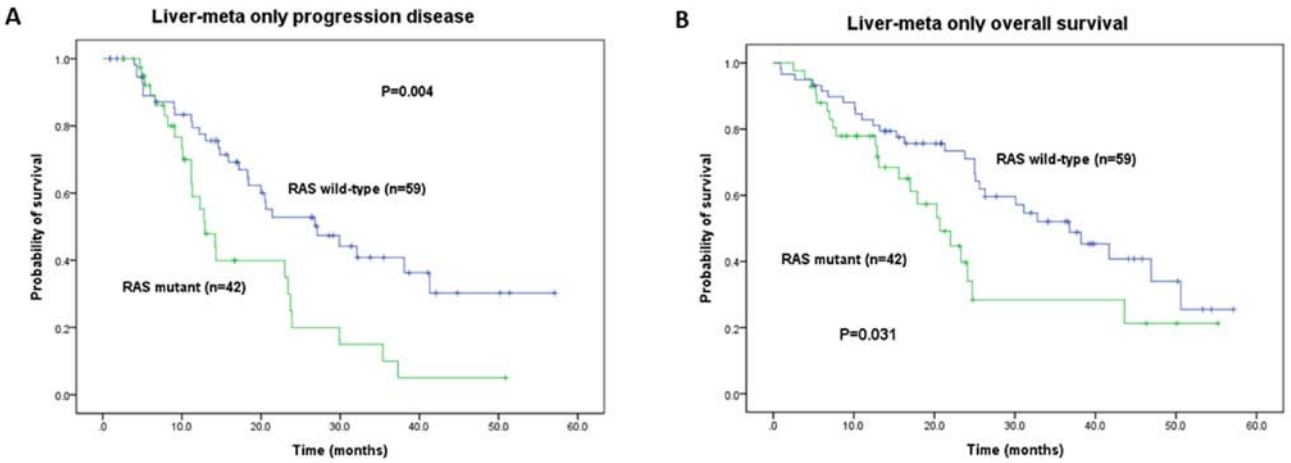

Figure 4. The Kaplan-Meier survival curves for patients with colorectal cancer with liver meta-only divided according to *RAS* mutation status. Patients in the wild-type group had significantly longer (A) progression-free survival and (B) overall survival compared with that in the mutant group. Meta, metastasis.

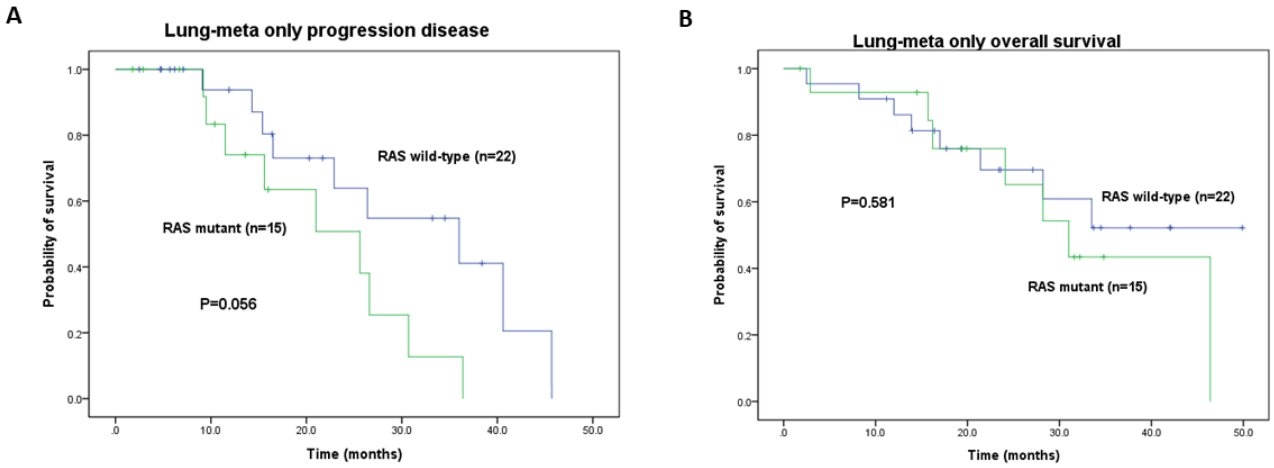

Figure 5. Comparison of patients with colorectal cancer with lung meta-only classified according to *RAS* mutation status (wild-type vs. mutant). (A) Progression-free survival. (B) Overall survival. Meta, metastasis.

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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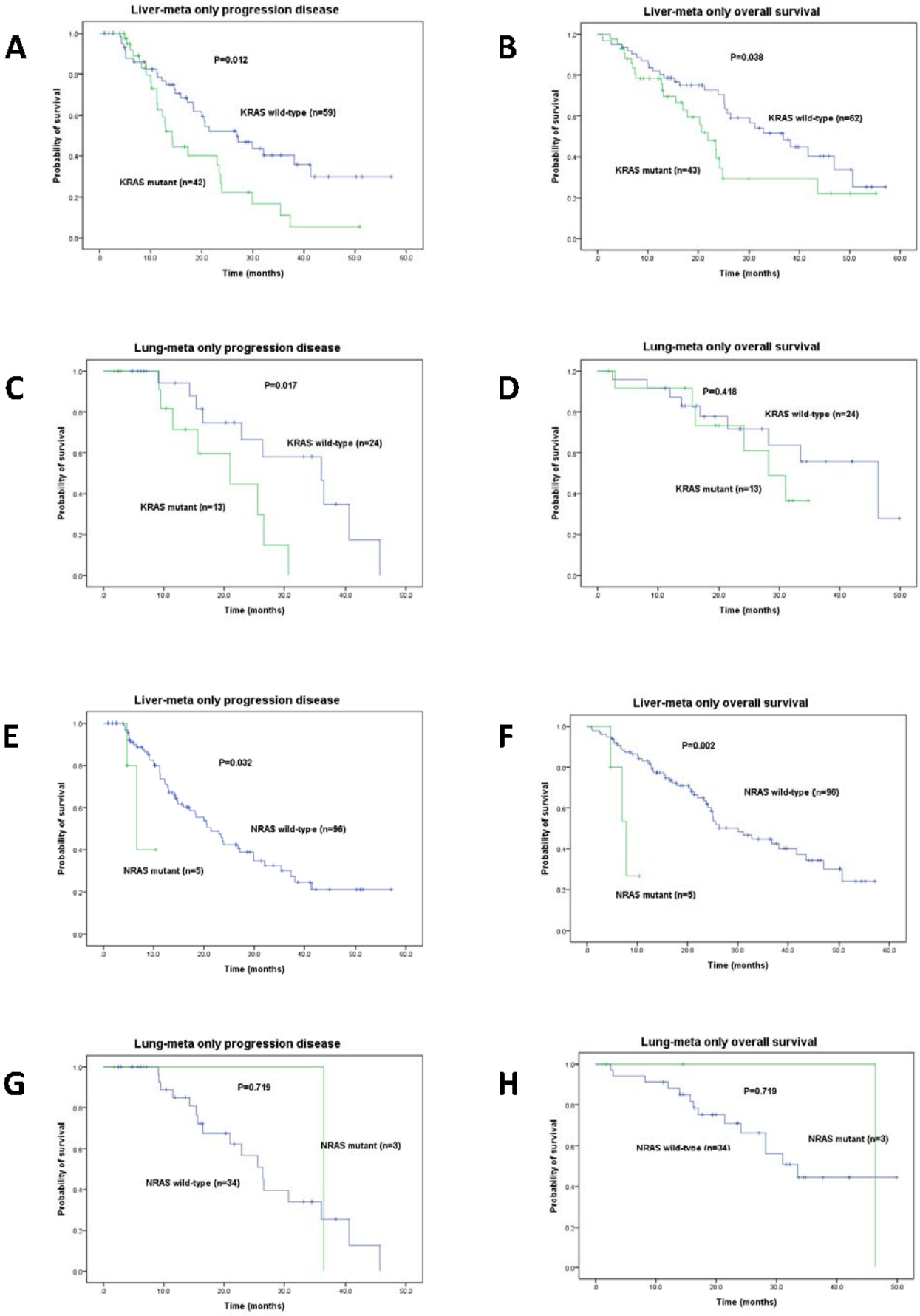

Figure 6. Subgroup analysis of progression-free survival and overall survival conducted according to *KRAS* and *NRAS* mutation status in the LiM-only and LuM-only groups. (A and B) *KRAS* wild-type vs. mutant in LiM-only group. (C and D) *KRAS* wild-type vs. mutant in LuM-only group. (E and F) *NRAS* 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.

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