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KRAS mutant allele-specific imbalance in lung adenocarcinoma

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The significance of *KRAS* mutant allele-specific imbalance (MASI) in lung adenocarcinomas is unknown. *KRAS* MASI was defined as predominance of the mutant allele over the wild-type allele. We assessed the frequency of *KRAS* MASI by comparing peak heights of mutant and wild-type alleles on sequencing electropherograms and by *KRAS* fluorescence *in situ* hybridization (FISH). A review of sequencing electropherograms of 207 *KRAS*-mutated lung adenocarcinomas demonstrated 23 (11%) cases with the mutant allele peak higher than the wild-type allele peak and 15 (7%) cases with the mutant allele peak equal to the wild-type allele peak. Of 17 cases with the mutant allele peak higher or equal to the wild-type allele peak, 8 (47%) showed *KRAS* amplification by FISH. *KRAS* FISH analysis of 36 *KRAS*-mutated lung adenocarcinomas with the mutant allele peak lower than the wild-type allele peak, 21 *KRAS* and *EGFR* wild-type and 16 *EGFR*-mutated adenocarcinomas showed no *KRAS* amplification. *KRAS* MASI was associated with selective amplification of the *KRAS* mutant allele ($P<0.001$). Patients with *KRAS* MASI showed worse overall survival. The cumulative proportion surviving at 17 months for *KRAS* MASI group was 35% compared with 84.1% for patients with *KRAS* mutant allele peak lower than wild-type allele peak ($P=0.012$). The adverse prognostic significance of *KRAS* MASI was independent of clinical stage and was maintained among stage I patients. The detection of *KRAS* MASI in lung adenocarcinomas by sequencing electropherograms may identify patients with more aggressive disease.
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About 30% of lung adenocarcinomas in western population harbor *KRAS* mutations.[1,2] In addition to activating point mutations, the oncogenic potential of *KRAS* can be achieved by increase in copy number of the *KRAS* gene.[3,4] Recent studies have shown that in a subset of cases, *KRAS* mutations were strongly associated with higher *KRAS* gene copy number.[5–7] The combination of these genetic events may result in an imbalance between the wild-type allele and the mutant allele.

The scenario with mutant allele being predominant over the wild-type allele was defined as mutant allele-specific imbalance (MASI).[8–10] The incomplete dominance of the mutant allele over the wild-type allele may result from selective amplification of the mutant allele (partial MASI) and is likely to be identified by fluorescence *in situ* hybridization (FISH). In some cases, the mutant allele may be predominant in the absence of the wild-type allele (complete MASI), a phenomenon most likely to arise through acquired uniparental disomy.[11]

Recently, the combination of *KRAS* mutation and copy number gain was correlated with worse clinical outcome in lung adenocarcinoma patients.[10,12]

KRAS mutation analysis is usually achieved by the automated chain termination method of direct DNA sequencing. When *KRAS* mutations are present, review of the sequencing electropherogram allows visualization of both the mutant allele and the wild-type allele. We hypothesized that mutant allele/wild-type allele peak height ratio on sequencing electropherogram is representative of the actual *KRAS* mutant allele/wild-type allele ratio in the tumor and may offer additional clinical prognostic information in an otherwise relatively homogenous population of *KRAS*-mutated lung adenocarcinomas. This hypothesis was tested by characterizing *KRAS*

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MASI in 207 prospectively accrued lung adenocarcinomas that harbored *KRAS* mutations. Furthermore, we correlated *KRAS* MASI with clinicopathological parameters such as *KRAS* amplification determined by FISH, clinical stage and overall survival.

Materials and methods

Clinicopathological Characteristics of Studied Patients

A total of 244 lung adenocarcinomas prospectively tested for *EGFR* and *KRAS* mutations at the University of Pittsburgh Medical Center were included in this study (207 *KRAS* mutated, 16 *EGFR* mutated and 21 *EGFR/KRAS* wild types). The clinicopathological features of the patients with *KRAS*-mutated adenocarcinomas are summarized in Table 1. There were 124 women and 83 men. All cases were staged according to the seventh edition of the American Joint Committee on Cancer manual.[13] Patients with *KRAS*-positive adenocarcinomas included 201 Caucasians and six African Americans. Only four patients received neoadjuvant chemotherapy before *KRAS* testing. Information on previous treatment with tyrosine kinase inhibitors was not available. The median follow-up was 17 months. Adequate smoking history was available for 172 patient (65—current smokers, 95—former smokers and 12—never smokers). The high prevalence of smokers in this cohort is consistent with the previously reported high smoking rate among patients treated at the University of Pittsburgh Medical Center (41 never smokers and 296 smokers).[14] This study was approved by the University of Pittsburgh Medical Center Institutional Review Board (IRB no. PRO08040162).

Table 1 Clinical and laboratory characteristics of patients with lung adenocarcinoma positive for *KRAS* mutation, overall and specific for *KRAS* mutant allele-specific imbalance

Feature	*Overall* (n = *207*)	KRAS *MASI* (n = *38*)[a]	KRAS *non-MASI* (n = *169*)
F/M	1.5	1.1	1.6
Age, mean, years	66.3	66	66.3
Clinical stage[b]			
I	94	12	82
II	33	9	24
III	46	8	38
IV	32	8	24
Procedure[c]			
Lobectomy	101	15	86
Biopsy	48	16	32
Wedge resection	30	3	27
Segmental	23	4	19
KRAS/CEP12 FISH[d]			
>2	8	8	0
<2	45	9	36
Cumulative proportion surviving at 18 months	NA	72.4%[e]	84.1%

CEP, chromosome enumeration probe; F, female; FISH, fluorescence *in situ* hybridization; M, male; MASI, mutant allele-specific imbalance.

[a]Mutant allele peak height was equal to wild-type allele peak height in 15 cases.

[b]Clinical stage was unknown for two patients. *KRAS* MASI was not associated with clinical stage.

[c]Pneumonectomy, $n = 5$.

[d]*KRAS* FISH was performed on 53 cases of lung adenocarcinomas positive for *KRAS* mutation. Of 38 *KRAS* MASI cases, 17 cases had sufficient material for FISH. One *KRAS* MASI case without amplification by FISH showed chromosome 12 hyperploidy. Chromosome 12 hyperploidy is not associated with *KRAS* MASI.

[e]Cumulative proportion of patients surviving at 18 months was 35% for patients with *KRAS* mutant allele peak higher than wild-type allele peak.

KRAS and *EGFR* Mutation Analysis

KRAS and *EGFR* mutation analysis was performed as previously described.[15] Briefly, guided by hematoxylin and eosin (H&E)-stained slides, tumor targets containing more than 70% tumor cells were manually microdissected from the 4-μm unstained histological sections. DNA was isolated from each target using the DNeasy tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions.

For the detection of mutations, DNA was amplified (40 cycles) with primers flanking exon 2 of the *KRAS* gene (forward primer 5′-GGTGAGTTTGT ATTAAAAGGTACTGG-3′ and reverse primer 5′-TCC TGCACCAGTAATATGCA-3′), exon 19 of the *EGFR* gene (forward primer 5′-CCCAGCAATATCAGCCTT AGGTG-3′ and reverse primer 5′-CCACTAGAGCTA GAAAGGGAAAGAC-3′) and exon 21 of the *EGFR* gene (forward primer 5′-CCTCACAGCAGGGTCTT CTC-3′ and reverse primer 5′-CCTGGTGTCAGGAA AATGCT-3′). PCR products were sequenced in both the sense and antisense directions using the BigDye Terminator v3.1 cycle sequencing kit on an ABI 3130 automated sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The sequences were analyzed using Mutation Surveyor software (SoftGenetics, LLC, State College, PA, USA). Cases were classified as mutated or wild type for the *KRAS* and *EGFR* mutation based on the sequencing results.

KRAS Fluorescence *In Situ* Hybridization

Dual-color *KRAS* FISH analysis was performed using a Spectrum Green-labeled chromosome enumeration probe 12 (CEP12; Abbott Molecular, Des Plaines, IL) and a Spectrum Orange-labeled, locus-specific *KRAS* (RP11-295I5, CHORI, Oakland, CA) probe (Figure 1).[16] In brief, paraffin sections were deparaffinized, dehydrated in ethanol and air dried. Sections were digested with protease (0.5 mg/ml) at 37°C for 28 min. The slides were denatured at 75°C for 5 min in 70% formamide

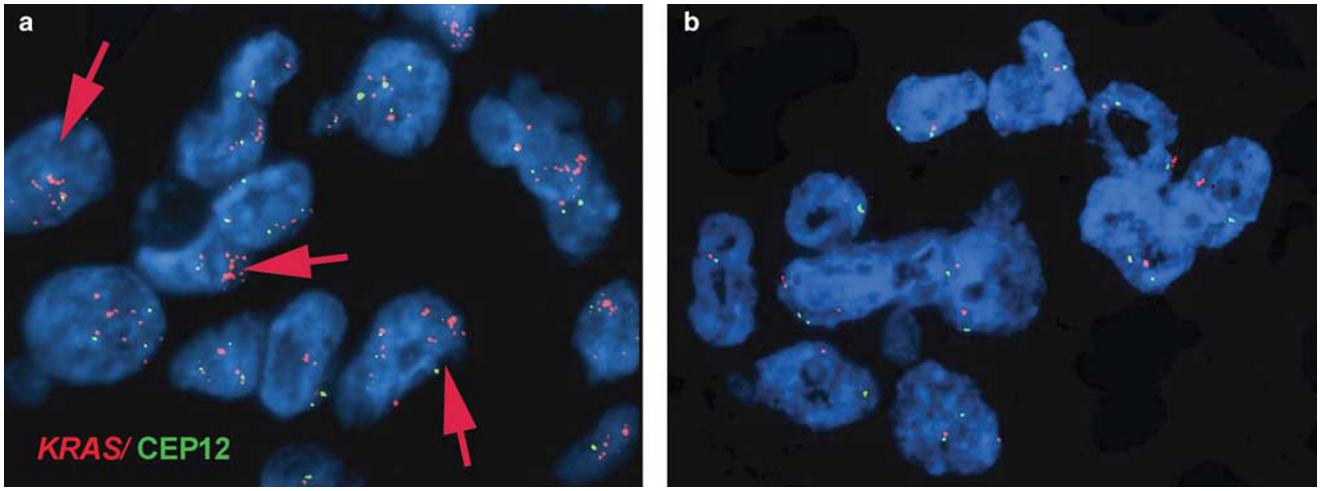

Figure 1 Representative case of lung adenocarcinoma characterized by fluorescence *in situ* hybridization (FISH). (**a**) *KRAS*/chromosome enumeration probe 12 (CEP12) ratio of >2 indicating *KRAS* amplification. (**b**) *KRAS* FISH-negative control—normal lung tissue showing two CEP12 and two *KRAS* signals per nucleus. Arrows indicate clusters of amplified *KRAS*.

(Chemicon, Billerica, MA) and dehydrated in ethanol. The probes were denatured for 5 min at 75°C before hybridization. Slides were hybridized overnight at 37°C and washed in 2XSSC/0.3% Igepal (Sigma, St Louis, MO) at 72°C for 2 min. Nuclei were counterstained with DAPI/antifade (Abbott Molecular). Analyses were performed using a fluorescence microscope (Nikon Eclipse E600) and Cytovysion Workstation (Applied Imaging, Santa Clara, CA) equipped with filter sets with single- and dual-band excitors for Spectrum Green, Spectrum Orange and DAPI (UV 360 nm). The histological areas previously selected on the H&E-stained sections were identified on the FISH-treated slides. Only individual and well-delineated cells were scored. Overlapping cells were excluded from the analysis. Amplification was defined as a *KRAS*/CEP12 ratio of >2.

Semiquantitative Assessment of *KRAS* MASI by Comparing Mutant Allele and Wild-Type Allele Peak Heights on Sequencing Electropherograms

Peak heights of mutant and wild-type alleles were compared and grouped into three categories: mutant allele higher than wild-type allele, mutant allele equal to wild-type allele and mutant allele lower than wild-type allele (Figure 2). Any increase in the mutant allele over wild-type allele and cases with equal peaks were defined as MASI.

Statistical Analysis

Overall survival was measured from the date of surgery to the date of death. Only patients who survived for at least 3 months after surgery were included in survival analysis. Living patients were censored at the date of the last available clinical information. Survival probabilities were calculated using the Kaplan–Meier method and compared among different groups and subgroups (log-rank test and Mantel–Cox). Statistical analysis was performed using SPSS 19 (Somers, NY, USA).

Results

Identifying *KRAS* MASI: Comparing Sequencing Electropherograms and FISH

A review of sequencing electropherograms of 207 *KRAS*-mutated lung adenocarcinomas identified 15 cases with the mutant allele peak equal to the wild-type allele peak and 23 cases with the mutant allele peak higher than the wild-type allele peak (overall *KRAS* MASI incidence—38/207, 18%). Sufficient material for *KRAS* FISH was available in 17 of 38 cases with *KRAS* MASI. In all, 2 of 7 (29%) *KRAS* cases with equal peaks and 6 of 10 (60%) *KRAS* cases with the mutant allele peak higher than the wild-type allele peak showed *KRAS* amplification. Overall, 8 of 17 (47%) cases with *KRAS* MASI by sequencing electropherogram showed a *KRAS*/CEP12 FISH ratio of >2, indicating that amplification of the *KRAS* mutant allele is a common mechanism of *KRAS* MASI. None of 36 *KRAS* cases with the mutant allele peak lower than the wild-type allele peak showed amplification. Therefore, *KRAS* MASI on sequencing electropherogram is associated with higher incidence of *KRAS* amplification (0 *vs* 47%; $P<0.001$, Fisher exact probability test) (Table 1).

KRAS FISH was also performed on a randomly selected group of *KRAS* wild-type adenocarcinomas, including 21 *KRAS* and *EGFR* wild-type cases and 16 cases harboring *EGFR* mutations. None of the

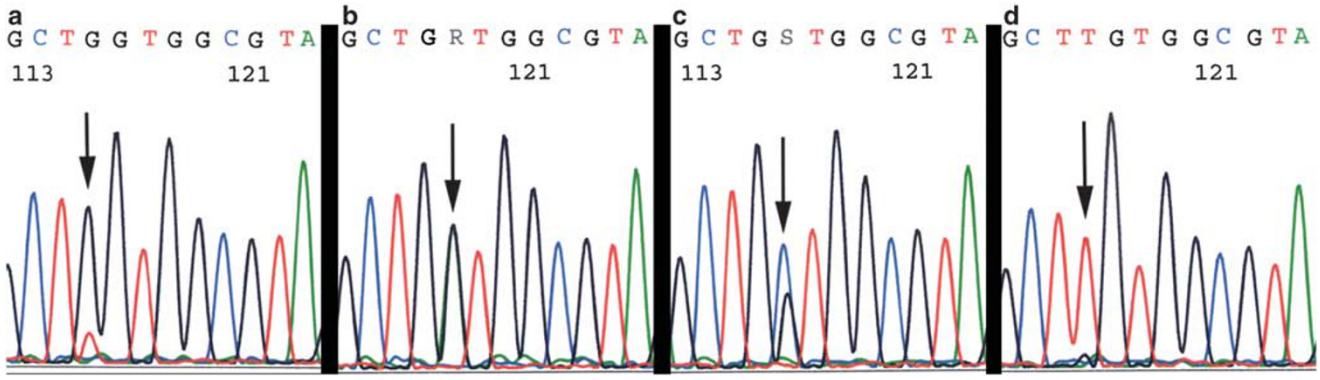

Figure 2 Scanned segments of sequencing electropherograms of representative cases of lung adenocarcinoma harboring *KRAS* mutations. The variation in peak height of wild-type allele and mutant allele is illustrated. The top part of each panel shows nucleotide sequence of the dominant PCR product. Point mutation is indicated by an arrow. (**a**) G to T nucleotide substitution; height of the mutant allele peak (T) is lower than that of the wild-type allele (G). (**b**) G to A substitution; heights of mutant and wild-type alleles are equal. (**c**) G to C substitution; mutant allele peak (C) is higher than the wild-type allele peak (G), consistent with partial mutant allele-specific imbalance. (**d**) G to T substitution; wild-type allele peak is virtually absent, consistent with complete MASI. Of note, this case showed no *KRAS* amplification or chromosome 12 hyperploidy by FISH, raising the possibility of an acquired uniparental disomy or homozygous mutation.

KRAS and *EGFR* wild-type or *EGFR*-mutated cases showed *KRAS* amplification.

Clinicopathological Correlates of *KRAS* MASI

The presence of *KRAS* MASI was not associated with patients' gender, age, smoking history, type of *KRAS* mutation, tumor morphology or clinical stage (Table 1). Consistent with the previous report, patients with *KRAS* amplification by FISH were older than patients without *KRAS* amplification (69 *vs* 66 years of age);[12] however, this difference did not reach statistical significance.

Male patients had worse overall survival; however, this trend did not reach statistical significance ($P=0.063$). Patients' age, smoking history and type of *KRAS* mutation did not correlate with overall survival. Patients with *KRAS* amplification by FISH showed trend to worse overall survival ($P=0.182$).

The prognostic value of clinical stage is shown in Figure 3a.

Overall, this cohort of patients with *KRAS*-mutated lung adenocarcinoma was quite homogenous, as most of the well-recognized prognostic factors (ie, age and gender) did not correlate with overall survival.

The difference in overall survival was most striking when *KRAS*-mutated cases with the mutant allele peak higher than the wild-type allele peak were analyzed separately from *KRAS*-mutated cases with equal peaks (Figure 3b). Among patients with the *KRAS* mutant allele peak higher than the wild-type allele peak, the cumulative proportion surviving at 17 months was 35%, and for patients with the *KRAS* mutant allele peak lower than the wild-type allele peak, it was 84.1% ($P=0.012$).

The cases with equal height of mutant and wild-type allele peaks were grouped together with cases showing mutant allele peak higher than wild-type allele peak for the following reasons: (1) Two of seven cases with equal mutant and wild-type allele peaks, but none of the cases with the *KRAS* mutant allele peak lower than the wild-type allele peak showed *KRAS* amplification; (2) clinical outcome of patients with equal mutant and wild-type allele peaks resembled that of patients with *KRAS* mutant allele higher than wild-type allele peak (Figure 3b); and (3) combining 'mutant allele higher than wild-type allele peak' cases with 'mutant allele equal to wild-type allele peak' group allowed statistical subgroup analysis of the possible relationship between clinical stage and *KRAS* MASI.

Patients with adenocarcinomas characterized by *KRAS* MASI showed worse overall survival. The mortality among *KRAS* MASI patients was 12/34 (35.3%) *vs* 29/158 (18.4%) among patients with *KRAS* mutant allele peak lower than wild-type allele peak ($P=0.07$). The cumulative proportion surviving at 18 months was 84.1% in *KRAS* non-MASI cases and 72.4% in *KRAS* MASI cases ($P=0.003$) (Figure 3c).

To determine whether adverse prognostic significance of *KRAS* MASI is independent of clinical stage, a subgroup analysis was performed. The adverse prognostic effect of *KRAS* MASI was maintained among patients with stage I disease (Figure 3d). On the other hand, the prognostic value of clinical stage was maintained only among patients with *KRAS* mutant allele peak lower than wild-type allele peak.

Discussion

The significance of *KRAS* MASI stems from the fact that the allelic imbalance is maintained after

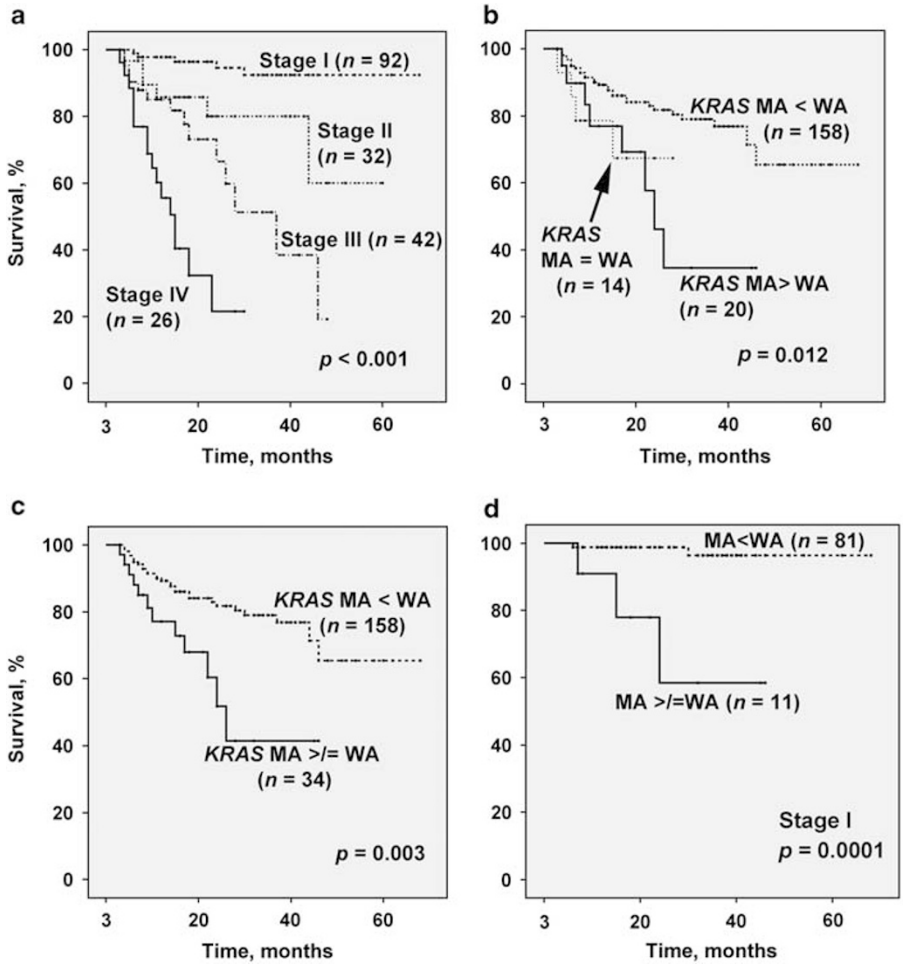

Figure 3 Overall survival analysis, Kaplan–Meier method. (**a**) Overall survival by clinical stage. (**b**) Overall survival and *KRAS* mutant allele (MA) to wild-type allele (WA) peak height comparison as seen on sequencing electropherogram. The survival curve for patients with equal mutant and wild-type allele peak heights (MA = WA) resemble that of patients with mutant allele higher than wild-type allele (MA > WA). (**c**) Overall survival and *KRAS* mutant allele-specific imbalance (MA ⩾ WA). (**d**) To control for clinical stage, the overall survival analysis was performed among patients grouped by clinical stage, stage I through IV. Stage I *KRAS* MA > WA patients showed worse overall survival. No such relationship was identified for patients with stages II, III or IV.

transcription, affects the dosage of *KRAS* mutant allele and the level of its kinase activity.[7,10] Recently, it was shown that reactivation of p53 in a $KRAS^{G12D}$ mouse model of non-small-cell lung carcinoma lead to tumor regression only in tumors with *KRAS* signal elevated through the amplification of the mutant allele and loss of wild-type allele.[17,18]

In this larger series of *KRAS*-mutated lung adenocarcinomas, we confirm and expand the finding that *KRAS* MASI correlates with worse clinical outcome. In this clinically homogenous cohort of *KRAS*-mutated lung adenocarcinomas, clinical stage was the only other prognostic factor that maintained its significance. We believe that the adverse prognostic significance of *KRAS* MASI is independent of the clinical stage for several reasons. First, cases characterized by *KRAS* MASI were not associated with more advanced clinical stage. Second, in a subgroup analysis, the presence of *KRAS* MASI maintained its adverse prog-

Table 2 Summary of reported incidence of *KRAS* mutation, amplification and mutant allele-specific imbalance in lung adenocarcinomas

KRAS-*mutated adeno-carcinoma,* n	*Analysis of* KRAS *copy number, method*	*Mutated and amplified adeno-carcinoma,* n	*Clinical correlates of KRAS mutation and amplification*	*Ref.*
34[a]	qPCR, FISH	8 (qPCR), 6 (FISH)	Worse overall survival	12
3[a]	FISH	3/71 (4%)	None	16
18	qPCR, FISH, SE	7	None	7
46	qPCR, SNP array	6/46 (13%)	Worse clinical outcome in stage I–III patients	10

Ref., reference; FISH, fluorescence *in situ* hydridization; qPCR, quantitative polymerase chain reaction; SE, sequencing electropherogram; SNP, single nucleotide polymorphism.
[a]In this study, cases with non-adenocarcinomatous histology and wild-type *KRAS* were included as well.

nostic value even within stage I patients. Most interestingly, clinical stage predicted outcome only among patients with the *KRAS* mutant allele peak lower than the wild-type allele peak.

The quantitative nature of the direct sequencing and its reliability in assessing allelic imbalance was previously shown both on cell lines and in clinical tumor samples.[7,10,19] In addition to sequencing electropherogram and FISH, previous studies of *KRAS* allelic imbalance in lung adenocarcinomas used such methods as qPCR and SNP (Table 2). The number of clinical samples was lower, and the ethnicity of studied patients was distinct from the present study. Nevertheless, our results are in agreement with the previously reported adverse prognostic impact of the combined *KRAS* mutation and increased *KRAS* gene copy number.[10,12]

In this study, we employed *KRAS* FISH to analyze two known mechanisms of *KRAS* MASI—amplification and chromosome 12 hyperploidy. It appears that these two events are rare and insufficient to explain the adverse prognostic impact of *KRAS* MASI. The role of two additional MASI mechanisms, chromosome 12 uniparental disomy and *KRAS* homozygous mutation, should be addressed in future studies.

The reported incidence of *KRAS* amplification in non-small-cell lung carcinomas ranges from 2.5%[12] to 7%[16] and its variation among different histological subtypes of non-small-cell lung carcinoma is unclear. The current study was not designed to establish the incidence of *KRAS* amplification in a general cohort of non-small-cell lung carcinomas—our study was histologically limited to lung adenocarcinomas only. Of the *KRAS*-mutated adenocarcinomas, *KRAS* FISH was performed on all cases with *KRAS* MASI with available material and on additional 36 randomly selected *KRAS*-mutated adenocarcinomas with the mutant allele peak lower than the wild-type allele peak. This *KRAS* MASI-centric approach resulted in a 15% (8/53) incidence of *KRAS* amplification in *KRAS*-mutated adenocarcinomas and in an 8.8% (8/90) incidence of *KRAS* amplification in combined *KRAS/EGFR* wild-type, *EGFR*- or *KRAS*-mutated adenocarcinomas.

In summary, *KRAS* MASI as identified on sequencing electropherograms was seen in 11% (mutant allele peak higher than wild-type allele peak) to 18% (equal mutant and wild-type allele peaks) of 207 patients with *KRAS*-mutated lung adenocarcinoma. Forty-seven percent of lung adenocarcinomas with *KRAS* MASI by sequencing electropherogram showed *KRAS* amplification by FISH. *KRAS* MASI appears to identify a subset of patients with worse overall survival. On the basis of a subgroup analysis, the adverse prognostic significance of *KRAS* MASI is independent of clinical stage. The results presented in this study require further validation in larger cohorts including patients from different ethnic groups and with more extensive treatment history.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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