

Coexistence of two missense mutations in the KRAS gene in adenocarcinoma of the lung: a possible indicator of poor prognosis

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Summary

Background. KRAS mutations are present in up to 30% of patients with lung adenocarcinoma. The two most common KRAS mutations in non-small cell lung cancer (NSCLC) are G12C (~40%) and G12V (~22%). We describe the case of a 63-year-old Asian male patient with a very aggressive lung adenocarcinoma harbouring two coexisting missense mutations in the same exon.

Methods. The patient presented with a 6 cm spiculated lung mass and bilateral mediastinal lymphadenopathy on imaging. A cytology sample was obtained from EBUS-TBNA of mediastinal lymph nodes, and mutation screening was performed by next-generation sequencing using the Ion Torrent Cancer Hotspot panel.

Results. Cytological examination and immunocytochemistry confirmed the presence of metastatic lung adenocarcinoma. The molecular analysis revealed the coexistence of two missense mutations: c.34G > T; p.(Gly12Cys) and c.38G > T; A; p.(Gly13Asp) in exon 2 of the KRAS gene. The two independent variants were confirmed on Integrative Genomic Viewer (IGV), suggesting molecularly independent clones. The patient was treated with palliative care and died within two months of the diagnosis.

Conclusions. The present case showed aggressive clinical behaviour. It is questionable whether this aggressive course was due to the coexistence of multiple mutations or to a specific single mutation. Data in the literature regarding the outcome of polyclonal KRAS polyclonal lung adenocarcinomas are scarce, but some evidence seems to indicate that specific mutations may have prognostic value, possibly depending on the disease setting.

Key words: NSCLC, KRAS, molecular pathology

Introduction

Lung cancer represents the leading cause of cancer-related deaths worldwide, with an overall 5-years survival rate of less than 20% ¹.

Every year the UK accounts for more than 39,000 new lung cancer cases, and every year more than 35,000 patients die of this condition ². About 90% of lung cancers are caused by smoking, and since smoking habit has increased in females and decreased in males in the last two decades, lung cancer is now one of the leading causes of cancer deaths in both sexes ³. Unfortunately, only a minority of cases are currently treated with success, and the cure rate is modest compared with other malignancies such as *colorectal cancer* (CRC) and breast cancer ²⁻⁴. Amongst lung cancers, *non-small cell carcinomas* (NSCLC) account for more than 85% of all lung cancers, the most frequent histotypes being *adenocarcinoma* (ADC) and *squamous cell carcinoma* (SCC) ³. The poor overall survival (OS) of

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patients with NSCLC lead to several efforts to improve early diagnosis, identify fundamental cellular and molecular pathways involved in tumourigenesis and develop target therapies³⁻⁵. Molecular profiling is becoming increasingly important in the management and therapy of patients with ADC, with several oncogenes emerging as tools for prognostic and predictive decisions¹. Mutations in epidermal growth factor receptor (EGFR) and rearrangements in anaplastic lymphoma receptor tyrosine kinase (ALK), mutations in proto-oncogene tyrosine-protein kinase ROS (ROS) and in rearranged during transfection (RET) oncogenes are currently considered as essential predictive factors and therapy targets in patients with NSCLC^{1,6}. Overall, oncogenic driver mutations are identified in up to 60% of lung ADCs, and some have shown to have both prognostic and predictive significance^{3,7,8}. Kirsten rat sarcoma viral oncogene homolog (KRAS) is a hydrolase enzyme that binds to the nucleotide guanosine triphosphate (GTP) and hydrolyze it to guanosine diphosphate (GDP). This GTP-ase belongs to the wider rat sarcoma (RAS) family which also includes, for humans, Neuroblastoma rat sarcoma (NRAS) and Harvey rat sarcoma (HRAS). KRAS is the most commonly altered isoform in human cancers, with a general incidence of mutations over 80%¹. The RAS family genes are involved in different processes such as cellular proliferation, differentiation, migration and survival⁶. Deregulation of KRAS plays therefore a crucial role in cancer progression. The available data in literature show that KRAS mutant cancers have a worse outcome compared with correspondent non-mutated tumours^{1,9}. KRAS also represents a predictive factor for the use of EGFR-targeted therapies in lung and colorectal carcinomas^{6,9}.

KRAS alterations appear to be influenced by ethnicity (more common in the European population than Asians: 30% vs 10-15%) and smoking habit^{3,4}. Most of the mutations involve codons 12 and 13^{4,10} and usually consist of single amino acid substitutions in codon 12, followed by codons 13 and 61^{3,4}. The RAS family genes encode four related proteins (HRAS, NRAS and two isoforms of KRAS) having GTP-ase function. Extracellular stimuli can promote the binding of specific ligands such as epidermal growth factor (EGF) to their tyrosine kinase receptors (for example, EGF-receptor) and work as triggers promoting the receptor's dimerization and phosphorylation. This causes the activation of the GTP-ase from RAS/GDP to RAS/GTP^{11,12}. The activated complex conveys EGFR signal activation to multiple downstream intracellular pathways that regulate cellular functions, including cellular proliferation, mobility and survival^{3,11}, see Figure 1¹.

KRAS is now considered one of the most important cancer-related molecules and plays a crucial role in

aggressive tumours, including NSCLC and CRC. Mutation of KRAS are frequently encountered in NSCLC, and although the last decade has seen some therapeutic improvements, the median survival rate for lung cancers is still low (5-year survival rate < 5%)⁸.

The molecular alterations found in KRAS are also gaining increasing prognostic and predictive value^{5,6}. Patients with CRC with wild type KRAS benefit from anti-EGFR therapies, whereas this has not been seen in NSCLC. The reason could be the unique role that smoking has in lung tumourigenesis resulting in a specific type of KRAS mutation, thus contributing to differences in the therapeutic response and outcome^{5,6,8}. We report here a rare and unusual combination of two KRAS mutations associated with a particularly aggressive tumour behaviour.

Materials and methods

A 63-year-old Asian male patient with history of end-stage renal failure treated with renal transplant, presented with respiratory symptoms and weight loss during his follow-up visit. Patient was an ex-smoker (20 cigarettes/day) but quit in 2003 and a minimal alcohol consumer.

Prior to the transplant he was known to have bilateral pulmonary nodules which were dismissed as likely infective in origin.

A CT chest was performed and revealed a 6 cm spiculated lesion in left lower lobe of the lung, highly suspicious for cancer, associated with bilateral necrotic-looking lymph nodes in the mediastinum and multiple lytic bone lesions. The subsequent PET provisionally staged the disease as T3N3M1b. Diagnostic endoscopic ultrasound-guided aspiration (EBUS-FNA) of the lymph nodes was performed and the material was submitted to the cellular pathology laboratory.

Three air-dried MGG stained slides and a PAP stained cytospin from 16 ml of turbid red fluid were prepared. A cell block was also obtained from the fluid and used to perform a panel of immunocytochemistry (TTF-1 (Leica PA0364), CK7 (Leica PA0942), CK20 (Leica PA0022), CDX2 (Leica PA0375), and p63 (Leica PA0103) and ancillary studies including cytogenetic analysis of ALK and Next-generation sequencing. ALK translocation was analysed through fluorescence in situ hybridisation (FISH) with probe ALK (2p23) break (Kreatech Diagnostics). The analysis required a tumour content of 30% in the cell block. The interphase FISH analysis was undertaken on the specimen incorporating at least 100 cells from three separate regions of the sample. DNA was extracted from formalin fixed paraffin embedded tissue using the Qiagen QI Symphony DSP DNA Minikit.

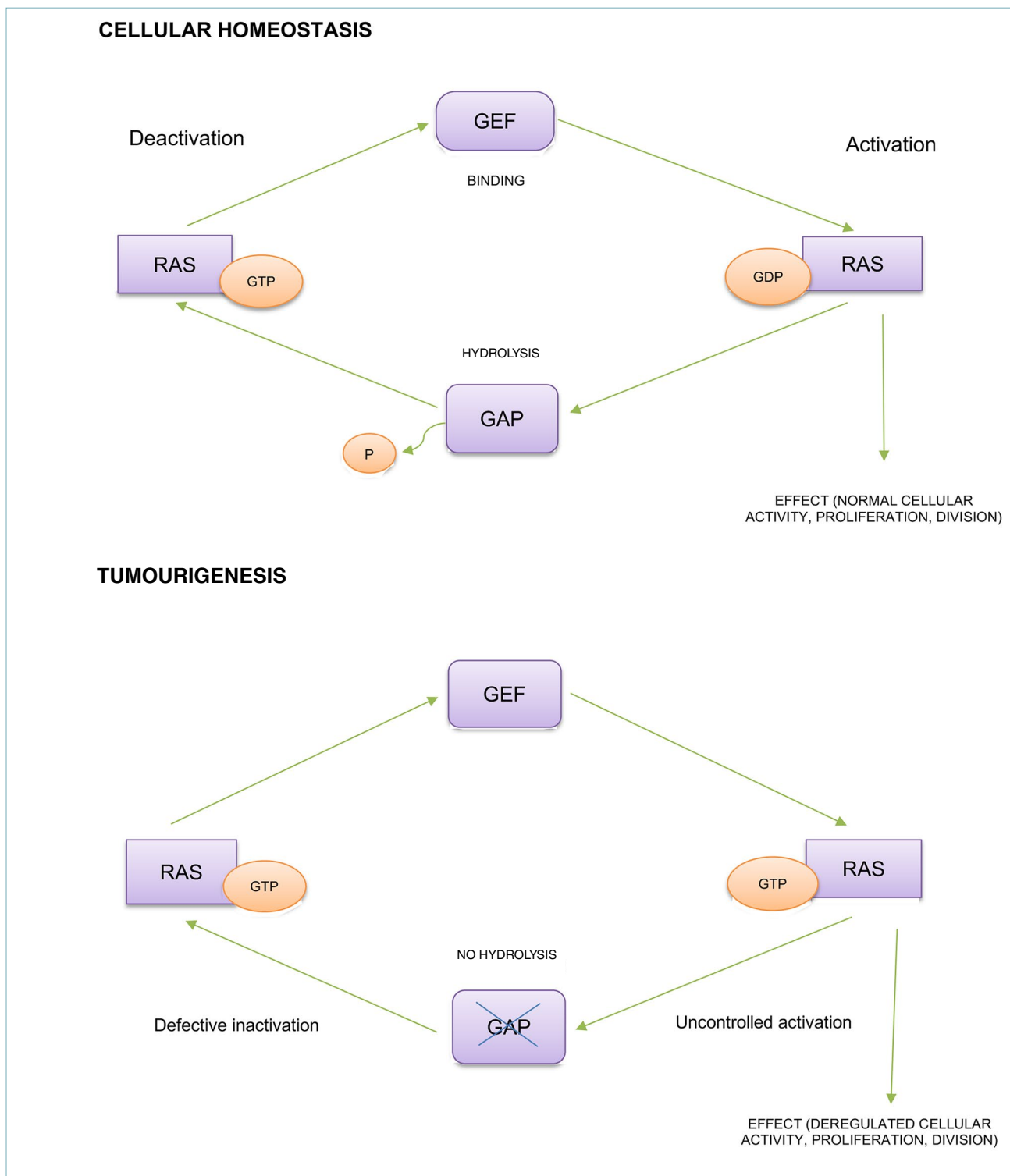


Figure 1. Schematic representation of normal and abnormal functional states of KRAS gene. GEFs and GAPs control the switch from GTP- and GDP-bound states of KRAS. GEF promotes the GDP to GTP passage resulting in activation of KRAS. GAP has hydrolytic activity and causes the return of KRAS to inactive status. Mutations in KRAS modify its binding with GAP and cause the blockage of hydrolysis, which ultimately results in deregulated KRAS activation effects. RAS: Rat sarcoma proto-oncogene; GTP: Guanosine-triphosphate; GDP: Guanosine-diphosphate; GEF: Guanine-nucleotide exchange factor; GAP: GTP-ase activation protein.

The mutational screening was performed by next-generation sequencing using the ION Torrent Cancer Hotspot panel (ThermoFisherScientific, USA). Reference Sequences NM_002524.4, NM_004985.3, NM_004333.4, NM_005228.3 and NM_006218.2 were used to screen the NRAS, KRAS, BRAF, EGFR, PIK3CA genes, respectively. For the molecular analysis, the assay comprised 207 amplicons in 50 oncogenes frequently mutated in solid tumours.

Results

The smears showed clusters of pleomorphic epithelial cells with abundant pale/vacuolated cytoplasm, enlarged nuclei and coarse chromatin. The background featured neutrophils and normal looking lymphocytes compatible with nodal sampling. Immunocytochemistry revealed that tumour cells were positive for TTF-1 and CK7 and negative for p63, CK20 and CDX2, thus confirming a metastatic adenocarcinoma. The morphological features and immunoprofile were in line with a metastatic adenocarcinoma favouring a lung primary (Fig. 2).

ALK FISH revealed intact fusion signals only, with no evidence of ALK rearrangement in any of the analysed cells. The patient was, therefore, unlikely to benefit

from ALK inhibiting targeted therapies (Crizotinib). Next generation sequencing (NGS) revealed wild type EGFR, with no evidence of mutation in exons 18-21; therefore, the patient was not eligible for tyrosine kinase inhibitor (TKI) therapies. There was no evidence of mutations in BRAF exons 11 and 15, NRAS exons 2-4 and PIK3CA exons 10, 14 and 2. Two missense mutations, c.34G > T; p.(Gly12Cys) and c.38G > A; p.(Gly13Asp), were detected in exon 2 of the KRAS gene. The two independent variants were confirmed on IGV, suggesting molecularly independent clones (Tab. I).

PDL1 was not performed as the patient was clinically

Table I. Molecular profiling of the present case.

Oncogene	Mutated	Non mutated	Mutation
ALK		*	
EGFR		*	
BRAF		*	
PIK3CA		*	
NRAS		*	
KRAS	*		C.34G>t;p(Gly12Cys) C.38G>A;p(Gly13Asp)

ALK: Anaplastic lymphoma receptor tyrosine kinase. EGFR: Epidermal growth factor receptor. BRAF: v-raf murine sarcoma viral oncogene homolog B1. NRAS: Neuroblastoma rat sarcoma. PIK3CA: phosphatidylinositol-3 kinase.. KRAS: Kirsten rat sarcoma

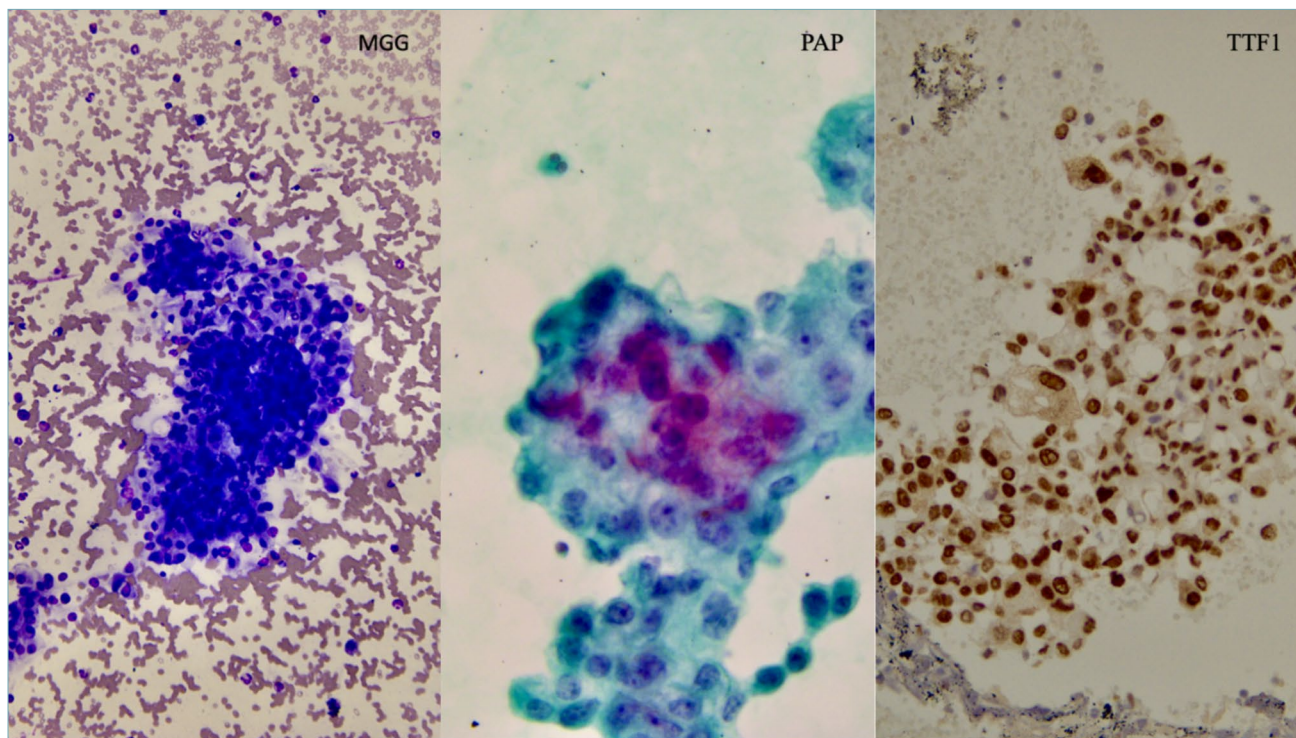


Figure 2. Left and centre: Cytomorphological details of malignant cells [May-Grunwald-Giemsa (MGG), 10X and Papanicolaou (PAP) stain, 20X]. Right: Immunohistochemical stain for TTF-1 confirming the pulmonary origin [10X].

not eligible for immunotherapy and given his immunosuppression status chemotherapy was also not considered.

The patient was then referred to palliative radiotherapy and then discharged to palliative care. Sadly, he deteriorated very rapidly and died within four months from presentation.

Discussion

We report a rare case of a very aggressive lung ADC featuring two co-existent mutations in KRAS oncogene. RAS proteins control different pathways that regulate several cellular functions, such as cellular differentiation and proliferation^{1,6,11}. The constitutively activated RAS oncoproteins promote cellular cascades, which result in uncontrolled cellular proliferation and abnormal cell survival^{4,6}. KRAS is one of the most investigated oncogenes because it is one of the first discovered and is frequently mutated in many tumours⁶. The most frequent mutation of KRAS encountered in NSCLC involves codons 12 and 13; Glycine 12 to Cysteine (G12C) and Glycine 12 to Valine (G12V) are the most common alterations found in patient with smoking history, whereas Glycine 12 to Aspartic acid (G12D) is more common in non smokers^{1,4,10}. Since early 2000s several studies have shown that there is a strong coincidence of G to T transversion hotspots in lung cancers and sites of preferential formation of polycyclic aromatic hydrocarbons (PAH) adducts, even in non-smoker patients who had some sort of exposure.

PAH molecules are present in tobacco smoke, diesel exhausts, and smoked foods, as well as particulate matter; PAH-derived reactive metabolites are significant contributors to lung cancer development¹².

These reactive metabolites interact with DNA to form DNA adducts, which are linked to pulmonary carcinogenesis. Also, it has been shown in studies on Asian, non-smokers, lung cancer patients that KRAS mutation G > T are consistent with exposure to smoky coal¹³.

KRAS mutant lung cancers often present other co-occurring genetic alterations involving tumour protein p53 (p53) or thyroid transcription factor-1 (TTF-1) and Serine/Threonine kinase 11 (STK-11)^{1,3,4,6}.

Interestingly, De Marini et al. reported G > T transversions at either KRAS (86%) or TP53 (76%) in their cohort of non-smoker patients consistent with an exposure to PAH, and postulated that mutations in the TP53 and KRAS genes can reflect a specific environmental exposure and a peculiar subset of lung cancer¹⁴.

Our patient presented two mutations at codon 12 and 13, which are G to T transversion, and we wonder if one of the mutations was previously acquired with his smoking history while the second acquired later.

Different amino acid substitutions may have distinct binding affinities and may influence in other ways the effects of mutated KRAS in terms of biological behaviour, overall survival (OS) and disease-free survival (DFS)^{15,16}.

Mutations in the KRAS oncogene have been extensively investigated in the past few decades for (OS) and (DFS) in patients affected by lung cancer with controversial results. Mascaux et al. carried out a large meta-analysis of 28 studies to assess the prognostic value of KRAS alterations on the survival in lung ADCs¹⁶ confirming an adverse prognostic role for OS in ADCs but not in SCCs. At present, the primary utility of KRAS testing in NSCLC is to exclude the presence of mutations in other oncogenic drivers as these alterations are mutually exclusive^{15,16,17}.

Currently few inhibitors are being investigated in clinical trials for lung cancer to target specific mutations such as G12C⁶, unlike colorectal cancer where EGFR monoclonal antibodies are available and have shown significant efficacy in patients with KRAS-wild type tumours^{18,19}.

PFS is reported to be higher in patients with KRAS mutation of the type non-G12C than G12C. G12C is the most common mutation of KRAS and represents a negative prognostic factor for EGFR-TKIs treatment, whereas other KRAS mutations have not predicted treatment failure compared with wild-type KRAS status.

Regarding the presence and significance of multiple mutations in KRAS, there are only few reports in the literature of cancers with double KRAS mutations and the significance is unclear^{6,9,21}. We checked our archive and did not find these mutations in any other lung cancers, although a few gastrointestinal and haematological malignancies presented this phenotypes. Charkiewicz et al. in 2013²² with two mutations in codons 12 and 13. The alterations were mono-allelic mutations not associated with EGFR alterations. This case, like ours, represents a rare example of KRAS gene molecular mosaicism and heterogeneity in lung primary ADC. Some authors have postulated that double mutations represent the expression of a specific subset of genetic alterations which may potentially be used as screening tools for the identification of tumours with particularly aggressive behaviour and resistance to EGFR monoclonal antibodies (predictive role)^{6,21,23} but to date, there is no evidence of a prognostic nor predictive significance of double mutations compared to ADCs harbouring single mutations.

Investigations of prognostic and predictive roles of mutant KRAS in lung ADCs should be extended to analyse the potential role of specific allelic variants and/or the significance of multiple mutations. Our patient was severely immunosuppressed and thus did not qualify for immunotherapy even though he would be eligible based on immunocytochemistry results. Once again this emphasises the need for further studies and ad hoc drugs to target KRAS mutated lung cancers

Conclusions

We present a case of lung cancer harbouring a double KRAS mutation characterised by an aggressive clinical course. It is questionable whether this aggressive course was due to the coexistence of multiple mutations or to a specific single mutation. Data in the literature regarding the role of KRAS-mutations in lung tumours are conflicting, but they are increasingly indicating that specific mutations may have crucial prognostic and predictive role.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

FUNDING

The authors hereby declare that no funding was provided for the present manuscript.

ETHICAL CONSIDERATION

No ethical considerations were necessary for the manuscript as there was no confidentiality breach or patient's involvement.

AUTHOR CONTRIBUTIONS

All authors contributed to ideation, preparation of manuscript, method, result and discussion and review of literature.

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