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New monoallelic combination of *KRAS* gene mutations in codons 12 and 13 in the lung adenocarcinoma

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ABSTRACT

Purpose: In a retrospective analysis of the prevalence of *KRAS* mutations in patients with advanced non-small cell lung cancer (NSCLC), we detected a unique and not earlier described case of a double combination of mutations at codons 12 and 13 of the *KRAS* gene in a patient with lung adenocarcinoma.
Material/Methods: To determine the molecular characteristics of the infrequent mutation and the mutational status of the *KRAS* gene in metastatic brain tumors in the same patient, we performed morphological and molecular tests.
Results: Molecular analysis of the nature of the double mutation showed that the unique combination of variants is a monoallelic mutation. This type of changes has not yet been registered in the Catalogue of Somatic Mutations in Cancer database. Molecular assessment of the *KRAS* mutation status in the brain metastatic site in the same patient, showed no mutations in codons 12 and 13. Moreover, we did not find mutation at exon 19 and 21 of *EGFR* gene, both in primary tumor as well as in secondary metastatic foci in the brain.
Conclusions: The presented case shows an example of *KRAS* gene molecular mosaicism and heterogeneity of lung adenocarcinoma primary and metastatic tumors. Molecular heterogeneity of lung adenocarcinoma tumors can significantly affect eligibility of patients for targeted therapies.

Key words: adenocarcinoma of lung, *KRAS*, mutation, heterozygosity, molecular heterogeneity.

INTRODUCTION

Lung cancer is one of the most common cancers in the world and its mortality rate is still the highest among all cancers [1-3]. At diagnosis, over 70% of patients with non-small cell lung cancer (NSCLC) present with a locally advanced or metastatic disease [4,5]. NSCLC is characterized by formation and accumulation of many different genetic and molecular changes with the progression of the disease [6,7]. Mutations of the RAS family genes (*HRAS*, *NRAS* and *KRAS*) are among the most common oncogenic mutations detected in many cancers in human [8]. Point mutations in the v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*KRAS*) gene critical coding sequences ('hotspots') detected in 10-30% of NSCLC cases mainly relate to codon 12 and less often to codons 13 and 61 [9]. Mutational changes at codon 12 are predominant molecular abnormalities, which constitute about 95% of *KRAS* mutations in patients with adenocarcinoma of the lung [10]. Unlike, mutational variants of codon 13 are observed very rarely and represent less than

2% of all mutations in the *KRAS* gene [10]. For comparison, mutations at codon 61 of exon 2 are fewer than 1% of the molecular changes in the *KRAS* gene [9]. The coexistence of two or more mutations located in the 'hotspot' codons of the *KRAS* gene is observed extremely rarely in tumors. Until now, only a few cases the simultaneous presence of several types of *KRAS* mutations have been described in gastric and colon tumors [11,12]. Coexisting mutations were monoallelic [12], either originated from one or more clonal populations of tumor cells. Study of *KRAS* mutational status in NSCLC has never revealed the simultaneous presence of two types of mutations within a single tumor. According to our knowledge, this report as the first one describes simultaneous existence of two types of activating mutations within the same allele of *KRAS* gene in lung adenocarcinoma. Moreover, the presented case is an example of molecular diversity of *KRAS* gene primary lung adenocarcinoma and metastatic tumors.

MATERIALS AND METHODS

Clinical history

The study was performed in an NSCLC patient with brain metastases. A head CT scan performed in a 46-year-old men, heavy smoker (50 pack years – 2 packs a day for 25 years) revealed a brain tumor in the right parieto-occipital region. A chest x-ray showed a tumor in the sixth segment of the left lung. A right side craniotomy was performed with resection of the brain tumor. Histopathological examination revealed a metastatic adenocarcinoma probably originating from the lung. Left pneumonectomy with lymphadenectomy was also accomplished. No postoperative complications were observed. Histopathology revealed adenocarcinoma G3 with focal features of mucus production and secretion, infiltrating a pulmonary vein with tumor cells present in the intrapulmonary blood vessels. In clinico-histopathological staging the tumor was classified as pT4N0M1.

Clinical materials

Specimens for morphological and molecular examination were selected from surgically resected tissue of the metastatic brain tumor and lung tumor. The resected tissue specimens were fixed for 24h in 10% buffered formalin. Resected tissue samples were embedded into paraffin blocks following routine procedures. Tissue sections 5-µm-thick were used for haematoxylin and eosin (H&E) stain and immunohistochemical stains.

Immunohistochemistry

Immunohistochemical (IHC) staining for TTF-1, SpA, SpB was performed using the following antibodies: FLEX Monoclonal Mouse Anti-Thyroid Transcription Factor (TTF-1) Clone 8G7G3/1 (1:200 dilution), Ready-to-Use, (Dako Corporation, Carpinteria, CA, USA), Novocastra™ Lyophilized Mouse Monoclonal Antibodies Surfactant Protein A Clone 32E12 (1:200 dilution), Novocastra™ Lyophilized Mouse Monoclonal Antibodies Surfactant Precursor Protein B Clone 19H7 (1:50 dilution) (Leica Biosystems Newcastle Ltd, UK), respectively. We used NovoLink™ Polymer Detection System (Novocastra Laboratories Ltd., UK) for SpA and SpB staining and EnVision™ FLEX+, Mouse, High pH (Dako Corporation, Carpinteria, CA, USA) for TTF-1. IHC staining was accomplished according to the manufacturer's instructions. For a positive control of TTF-1, SpA and SpB we used samples from sites of bronchiolization with proliferation of type II respiratory epithelial cells. For negative control, the primary antibody was omitted.

DNA isolation

DNA isolation from paraffin-embedded lung adenocarcinoma tumor specimens (primary tumor and brain metastatic site) was prepared using 10-30-µm-thick tissue sections after macrodissection, resulting in selection of at least 80% of tumor cells. DNA was extracted using The NucliSens easyMAG platform (bioMérieux) for automated nucleic acid extraction.

KRAS and *EGFR* mutation analysis

Mutation analysis at codons 12 and 13 of the *KRAS* gene was carried out by direct sequencing of amplified PCR products using specific primers: forward FS 5'-TCATTATTTTTATTATAAGGCCTGCTG-3', reverse RS 5'-CAAGATTTACCTCTATTGTTGGATCA-3'. The method was described elsewhere [13]. Molecular assessment of the *EGFR* gene status was performed using direct sequencing technique described previously by Sun et al. [14]. The primer sets, used in order to amplification of segments of exons 19 and 21, had the following sequences: *EGFR* exon 19: 5'-AGCATGTGGCACCATCTCAC-3', 5'-GCAGGGTCTAGAGCAGAGCAG-3'; *EGFR* exon 21: 5'-CTGAATTCGGATGCAGAGCTT-3', 5'-CTAGTGGGAAGGCAGCCTGGT-3'. Sequencing reactions were analysed on an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, USA).

Enriched PCR-RFLP analysis for *KRAS* codon 12 mutations detection

Detection of *KRAS* mutations at codon 12 was executed by enriched PCR-RFLP method as described by Banerjee et al., though with some modification [15]. The conditions of PCR and the restriction digestion were described elsewhere [16]. This method combined with direct sequencing of appropriate PCR products, obtained after restriction digestion, enabled detection of heterozygosity of the *KRAS* codons 12 and 13 mutations. It is based on two-step DNA amplification and restriction digestion *(Fig. 1)*. The first-round PCR mismatched upstream primer K1 (5'-ACTGAATATAAACTTGTGGTAGTTGGACCT-3')

introduced G to C substitution at the codon 11 of *KRAS* in order to create a *BstOI* restriction site (5'-CCTGG-3'). The restriction site covers the first two nucleotides of codon 12. The sequence of the downstream PCR primer DD5P is as follows: 5'-TCATGAAAATGGTCAGAGAA-3' *(Fig. 1a).* The first step PCR products were digested by a restriction enzyme. The restriction endonuclease *BstOI* recognizes the sequence 5'-CCTGG-3' which is present in *KRAS* codon 12 wild type PCR products, but is absent from the mutant ones. As a result, only the wild type molecules are digested (*Fig. 1b*). The second step PCR was performed using the downstream K2 primer (5'-TCAAAGAATGGTCCTGGACC-3'), which is modified at base 16 (C to G substitution) to create another *BstOI* restriction site in all second PCR products which serves as internal control for completion of the digestion (*Fig. 1c*). Products of second amplification were also digested with *BstOI* (*Fig. 1d*). The first and second digestion products were electrophoresed on a 3% agarose gel. The DNA products after the first and second digestion were subjected to direct sequencing with appropriate primers (*Fig. 1e-g*). The analysis enabled determining whether codon 13 mutation was on the same allele as codon 12 mutation.

RESULTS

Histopathology and immunohistochemistry (IHC)

Primary tumor was composed of variably sized nests of frequently vacuolized pleomorphic cells arranged in cribriform pattern. At the periphery of the infiltrate tubular structures infiltrating desmoplastic stroma were observed. Significant proportion (30%) of the neoplastic nests showed marked discohesion with cells acquiring signet-ring appearance (*Fig. 2a*). Mucocellular component smoothly merged with areas with organoid architecture. The tumor was diagnosed as acinar-predominat type of adenocarcinoma with mucocellular component. Metastatic tumor was better differentiated with predominance of tubular structures and focal papillary pattern (*Fig. 2b*). Better differentiation manifested in lower grade of cellular and nuclear atypia and lack of discohesive mucocellular component. Both tumors stained positive with TTF-1, SP-A and SP-B (*Fig. 3*).

Molecular genetic analysis

Molecular analysis of the DNA from the tissue of lung adenocarcinoma primary tumor revealed the presence of two mutations in the *KRAS* codons 12 and 13–G12V (35 G→T)

Figure 1. **Scheme of two-step enriched PCR-RFLP analysis for *KRAS* codon 12 mutations detection in combination with direct sequencing of appropriate PCR products, obtained after restriction digestion. The white square shows the recognition site of the restriction enzyme *BstOI*, the gray square indicates the codon 13 sequences and the black square shows the codon 12 mutation in *KRAS* exon 1. A) First-step PCR. B) First round digestion for codon 12. C) Second-step PCR. D) RFLP analysis for codon 12. E-G) Direct sequencing of corresponding DNA fragments. bp, Base pairs.**

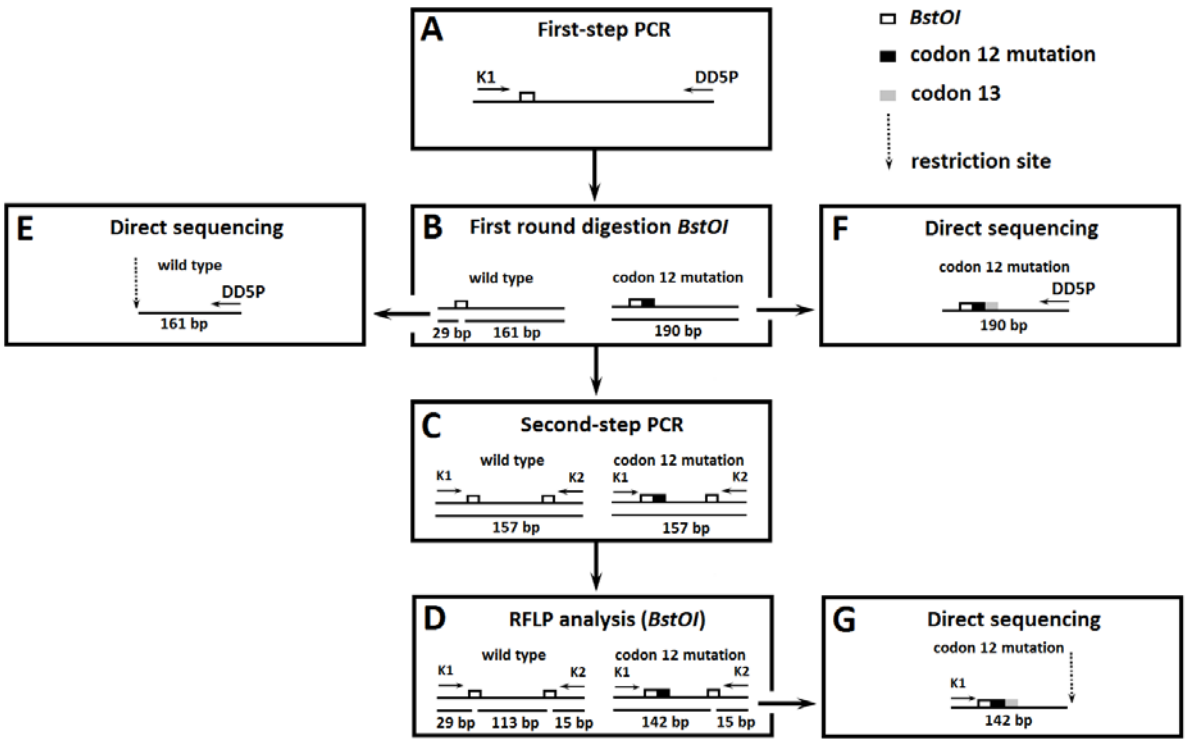


Figure 2. **Histomorphologic appearance of lung adenocarcinoma primary tumor (A) and metastatic tumor in the brain (B). H&E, magnification×200.**

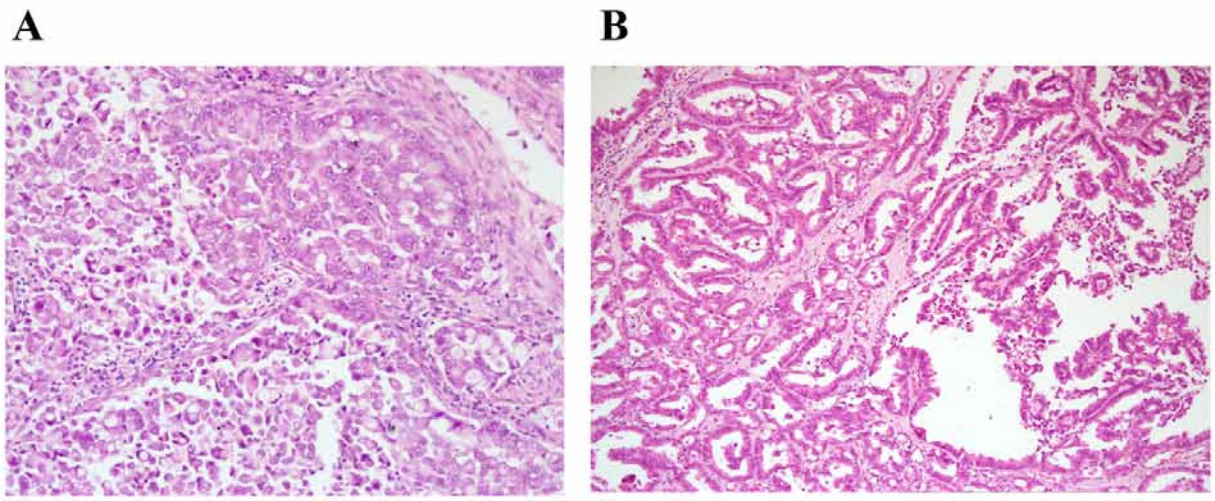
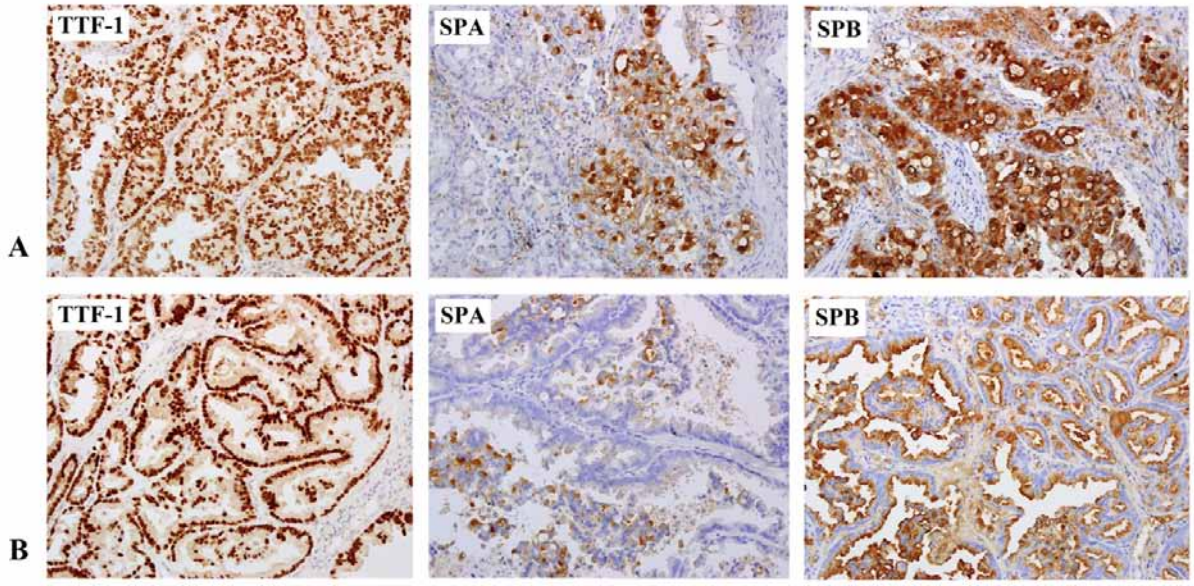


Figure 3. **Immunohistochemical staining for thyroid transcription factor-1 (TTF-1), surfactant apoprotein A (SPA), surfactant apoprotein B (SPB) in primary tumor of the lung (A) and metastatic tumor in brain (B). Magnification×200.**



and G13C (37 G→T) (*Fig. 4a*). Direct sequencing did not detect the heterozygosity of the two mutations. In order to detect it, we used the enriched PCR-RFLP method for *KRAS* codon 12, and combined it with the direct sequencing of PCR products that were digested with the restriction enzymes *BstOI*. The restriction digestion allowed the separation of DNA fragments with the mutation at codon 12 from DNA amplicons without mutation. The separated fragments were then subjected to direct sequencing in order to determine the location of mutations at codon 13. Using the two methods enabled identification of both substitutions on one allele (*Fig. 5a-b*). Monoallelic character of missense mutations caused a change in the sequence of two neighboring amino acids in the KRAS protein (G12V and G13C). Molecular assessment of *KRAS* mutation status in the brain metastatic site showed no mutations in codons 12 and 13 (*Fig. 4b*). Evaluation of the molecular status in exon 19 and 21 of the *EGFR* gene, did not show mutations in both the primary tumors as well as in metastatic foci in the brain.

DISCUSSION

The described case presents a unique combination of mutations at codons 12 and 13 of the *KRAS* gene in the lung adenocarcinoma primary tumor. Molecular assessment of

Figure 4. **Analysis of the *KRAS* gene mutation status. A) The sequence of reverse strand DNA shows two neighboring mutations of the *KRAS* codons 12 and 13 - G12V and G13C in the primary tumor of the lung. The arrows show substitution at positions 35 G→T and 37 G→T. B) DNA sequence electropherogram of the brain metastatic site – no *KRAS* mutations (WT-*KRAS* – wild type *KRAS*). "N" means the positions of point mutations.**

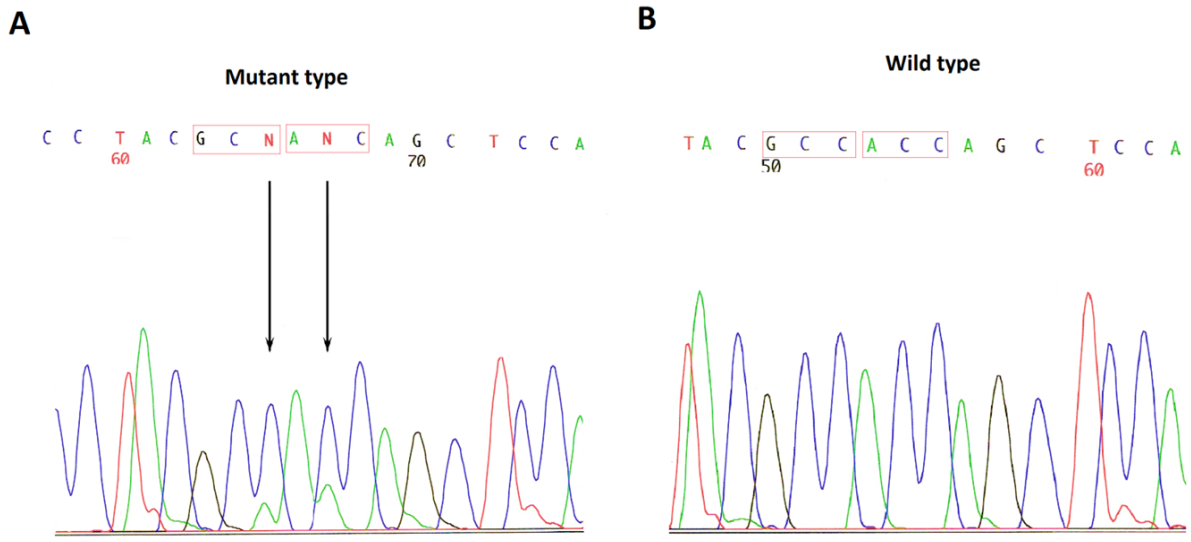
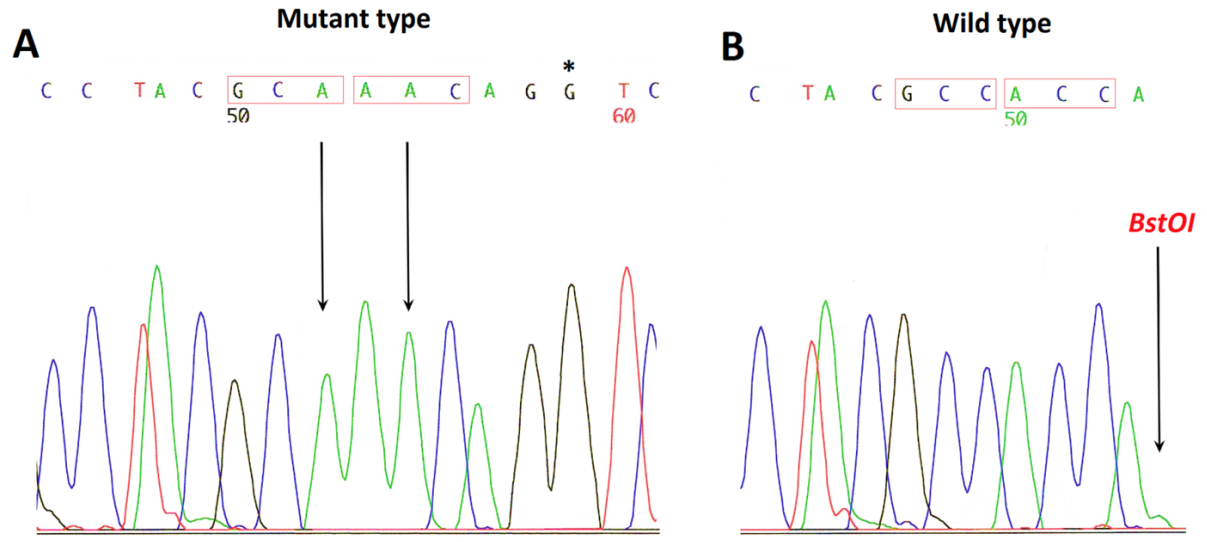


Figure 5. ***KRAS*** **mutation profile analysis using enriched PCR-RFLP and direct sequencing for identification of heterozygosity in the lung adenocarcinoma primary tumor. Sequences are shown in reverse direction. A) Direct sequencing was carried out on first-digested PCR product being fragments of DNA with mutation at codon 12 in *KRAS* gene. DNA sequence electropherogram shows the mutation at codon 12 - G12V (35 G→T) and the mutation at codon 13 - G13C (37 G→T) in *KRAS* exon 1. The arrow indicates a point mutation from C to A at nucleotide position 35 (codon 12) and C to A at nucleotide position 37 (codon 13). The asterisk indicates the mismatched nucleotide introduced with K1 primer in order to create the restriction site for *BstOI*. B) Direct sequencing of PCR product, obtained after first digestion, being fragments of DNA without mutation at codon 12 in *KRAS* exon 1, shows no mutation at codon 12 or 13 (wild-type *KRAS*) and the cutting site of enzyme *BstOI*.**



heterozygosity of mutations revealed a monoallelic character of the two changes. Interestingly, mutations of the *KRAS* gene codon 13 in the NSCLC tumors are extremely rare (<2%) [10]. Coexistence of mutations at codons 12 and 13 has been previously described in gastric and colorectal cancer, however, the monoallelic nature of changes has never been found [11,12]. The described case presents a new monoallelic combination of mutations of the *KRAS* gene codons 12 and 13 which has not yet been registered in the Catalogue of Somatic Mutations in Cancer database. The double nature of mutation caused a change in the sequence of two neighboring amino acids of the KRAS protein (G12V and G13C). The

changed protein has not been subjected to functional tests. The coexistence of two substitutions at adjacent codons, treated separately as activating *KRAS* mutations, can cause a loss of biological features of KRAS. It is possible, that this combination of *KRAS* mutations, reducing the oncogenic potential of tumor, lowered the mutation-bearing tumor cell clone's ability to metastasize. Further studies assessing functionality of KRAS protein are needed.

The described case also presents a discordance between *KRAS* mutation pattern in the primary tumor and in the brain metastatic site. Molecular analysis of *KRAS* in the metastatic site showed no mutations. Recently published studies revealed a high degree inconsistency of the *KRAS* mutation pattern in the primary tumors and in the recurrent sites of NSCLC – discordance ratio 24% [17]. Badalian et al., presented the results of the analysis of *KRAS* mutation status in a group of 11 patients with NSCLC, showing that the *KRAS* mutation status in the primary tumor does not always predict the status of bone metastasis – discordance reaching 64% [18]. Explanation of molecular heterogeneity in primary tumors and metastases of NSCLC is currently based on several models of metastatic progression. The first model presupposes that the tumor cells acquire the metastatic phenotype as a result of genetic drift or clonal selection during a multistage tumor progression [19]. It is possible that the NSCLC primary tumor can metastasize in its early stage [20], which leads to autonomous courses of oncogenesis at individual sites. The second model demonstrates the presence of diverse tumor cell clones with different invasive and metastatic potential, based on i.a. the presence of *EGFR* and/or *KRAS* mutations in the heterogeneous environment of the tumor tissue [21]. Discordance in the *KRAS* mutation pattern in the primary and secondary tumor presented in this study can be explained by means of the models described above.

Kalikaki et al. [17], states that chemotherapy may intensify the phenomenon of clonal selection. In the presented case, the *KRAS* genotype found in the metastasis site is different to the one in the primary tumor and it has no relationship with the administered chemotherapy. Molecular analysis of *KRAS* was conducted in tumor tissues collected before the decision about administering appropriate treatment. This case was diagnosed before the era of using EGFR-TKIs treatment.

Currently, there are no data on the correlation between the occurrence of *KRAS* mutations and subtype of adenocarcinoma of the lung. Moreover, there have not been clearly estimated the relationship between the type of mutation *KRAS* and morphological features of cells carrying these disorders. In our case, histopathological evaluation showed the presence of signet ring cell components in primary tumor and its absence in metastatic focus. Additionaly, molecular analysis demonstratred the unique combination of *KRAS* mutations in primary tumor and the lack of these variants of mutations in metastatic tumor. The above observations may suggest the occurrence of *KRAS* mutations in signet ring cells. Similary, Wistuba et al. found the existence of a distinct pattern of *KRAS* mutation (transversion A:T at codon 61) in Signet Ring Cell Colorectal Carcinoma (SRCCC) [22].

KRAS mutation status is a potential predictive factor of primary resistance to EGFR-TKI treatment [23]. A significant percentage of patients with NSCLC qualified for treatment (present EGFR-activating mutations and no *KRAS* mutations) (5%–25%), show a very low or no response to EGFR-TKI treatment [24]. The significance of *KRAS* mutation status in the prediction of TKI therapy and prognosis of patients with NSCLC remains controversial. Molecular heterogeneity of the *KRAS* gene in the NSCLC primary tumors and metastases needs further studies on the significance of mutation status discordance in the assessment of clinical response to EGFR-TKIs. Considering, that the targeted therapy with the EGFR-TKIs is used for the treatment of advanced NSCLC, specimens from distant metastases should be resected for a mutation status analysis of the main predictive markers. The discordance between *KRAS* and *EGFR* mutation status in the metastases could be an important element in the selection process of patients with NSCLC for EGFR-TKI therapy.

CONCLUSIONS

The presented case shows an example of *KRAS* gene molecular mosaicism and heterogeneity of lung adenocarcinoma primary and metastatic tumors. Molecular heterogeneity of lung adenocarcinoma tumors can significantly affect eligibility of patients for targeted therapies.

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The protocol of study and informed consent were in compliance with the Helsinki Convention and were approved by local Ethics Committee.

No Conflict of Interest has been declared by the authors.

REFERENCES

1. Molina JR, Yang P, Cassivi SD, Schild SE, Adjei AA. Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc. 2008 May;83(5):584-94.

2. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer statistics, 2009. CA Cancer J Clin. 2009 Jul-Aug;59(4):225-49.

3. Siegel R, Ward E, Brawley O, Jemal A. Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. CA Cancer J Clin. 2011 Jul-Aug;61(4):212-36.

4. Perry MC, Ihde DC, Herndon JE 2nd, Grossbard ML, Grethein SJ, Atkins JN, Vokes EE, Green MR. Paclitaxel/ifosfamide or navelbine/ifosfamide chemotherapy for advanced non-small cell lung cancer: CALGB 9532. Lung Cancer. 2000 Apr;28(1):63-8.

5. Yang P, Allen MS, Aubry MC, Wampfler JA, Marks RS, Edell ES, Thibodeau S, Adjei AA, Jett J, Deschamps C. Clinical features of 5,628 primary lung cancer patients: experience at Mayo Clinic from 1997 to 2003. Chest. 2005 Jul;128(1):452-62.

6. Petersen S, Aninat-Meyer M, Schlüns K, Gellert K, Dietel M, Petersen I. Chromosomal alterations in the clonal evolution to the metastatic stage of squamous cell carcinomas of the lung. Br J Cancer. 2000 Jan;82(1):65-73.

7. Li H, Schmid-Bindert G, Wang D, Zhao Y, Yang X, Su B, Zhou C. Blocking the PI3K/AKT and MEK/ERK signaling pathways can overcome gefitinib-resistance in non-small cell lung cancer cell lines. Adv Med Sci. 2011;56(2):275-84.

8. Bos JL. Ras oncogenes in human cancer: a review. Cancer Res. 1989 Sep 1;49(17):4682-9.

9. Forbes S, Clements J, Dawson E, Bamford S, Webb T, Dogan A, Flanagan A, Teague J, Wooster R, Futreal PA, Stratton MR. COSMIC 2005. Br J Cancer. 2006 Jan 30;94(2):318-22.

10. Huncharek M, Muscat J, Geschwind JF. K-ras oncogene mutation as a prognostic marker in non-small cell lung cancer: a combined analysis of 881 cases. Carcinogenesis. 1999 Aug;20(8):1507-10.

11. Kimura K, Nagasaka T, Hoshizima N, Sasamoto H, Notohara K, Takeda M, Kominami K, Iishii T, Tanaka N, Matsubara N. No duplicate KRAS mutation is identified on the same allele in gastric or colorectal cancer cells with multiple KRAS mutations. J Int Med Res. 2007 Jul-Aug;35(4):450-7.

12. Miyakura Y, Sugano K, Fukayama N, Konishi F, Nagai H. Concurrent mutations of K-ras oncogene at codons 12 and 22 in colon cancer. Jpn J Clin Oncol. 2002 Jun;32(6):219-21.

13. Kosakowska EA, Stec R, Charkiewicz R, Skoczek M, Chyczewski L. Molecular differences in the KRAS gene mutation between a primary tumor and related metastatic sites–case report and a literature review. Folia Histochem Cytobiol. 2010 Dec;48(4):597-602.

14. Sun L, Zhang Q, Luan H, Zhan Z, Wang C, Sun B. Comparison of KRAS and EGFR gene status between primary non-small cell lung cancer and local lymph node metastases: implications for clinical practice. J Exp Clin Cancer Res. 2011 Mar 17;30:30.

15. Banerjee SK, Makdisi WF, Weston AP, Campbell DR. A two-step enriched-nested PCR technique enhances sensitivity for detection of codon 12 K-ras mutations in pancreatic adenocarcinoma. Pancreas. 1997 Jul;15(1):16-24.

16. Dobrzycka B, Terlikowski SJ, Mazurek A, Kowalczuk O, Niklińska W, Chyczewski L, Kulikowski M. Mutations of the KRAS oncogene in endometrial hyperplasia and carcinoma. Folia Histochem Cytobiol. 2009;47(1):65-8.

17. Kalikaki A, Koutsopoulos A, Trypaki M, Souglakos J, Stathopoulos E, Georgoulias V, Mavroudis D, Voutsina A. Comparison of EGFR and K-RAS gene status between primary tumours and corresponding metastases in NSCLC. Br J Cancer. 2008 Sep 16;99(6):923-9.

18. Badalian G, Barbai T, Rásó E, Derecskei K, Szendrôi M, Tímár J. Phenotype of bone metastases of non-small cell lung cancer: epidermal growth factor receptor expression and K-RAS mutational status. Pathol Oncol Res. 2007;13(2):99-104.

19. Sasatomi E, Finkelstein SD, Woods JD, Bakker A, Swalsky PA, Luketich JD, Fernando HC, Yousem SA. Comparison of accumulated allele loss between primary tumor and lymph node metastasis in stage II non-small cell lung carcinoma: implications for the timing of lymph node metastasis and prognostic value. Cancer Res. 2002 May 1;62(9):2681-9.

20. Izbicki JR, Passlick B, Hosch SB, Kubuschock B, Schneider C, Busch C, Knoefel WT, Thetter O, Pantel K. Mode of spread in the early phase of lymphatic metastasis in non-small-cell lung cancer: significance of nodal micrometastasis. J Thorac Cardiovasc Surg. 1996 Sep;112(3):623-30.

21. Nakano H, Soda H, Takasu M, Tomonaga N, Yamaguchi H, Nakatomi K, Fujino S, Hayashi T, Nakamura Y, Tsukamoto K, Kohno S. Heterogeneity of epidermal growth factor receptor mutations within a mixed adenocarcinoma lung nodule. Lung Cancer. 2008 Apr;60(1):136-40.

22. Wistuba II, Behrens C, Albores-Saavedra J, Delgado R, Lopez F, Gazdar AF. Distinct K-ras mutation pattern characterizes signet ring cell colorectal carcinoma. Clin Cancer Res. 2003 Sep 1;9(10 Pt 1):3615-9.

23. Pesek M, Benesova L, Belsanova B, Mukensnabl P, Bruha F, Minarik M. Dominance of EGFR and insignificant KRAS mutations in prediction of tyrosine-kinase therapy for NSCLC patients stratified by tumor subtype and smoking status. Anticancer Res. 2009 Jul;29(7):2767-73.

24. Sharma SV, Bell DW, Settleman J, Haber DA. Epidermal growth factor receptor mutations in lung cancer. Nat Rev Cancer. 2007 Mar;7(3):169-81.