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.
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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), NovocastraTM Lyophilized Mouse Monoclonal Antibodies Surfactant Protein A Clone 32E12 (1:200 dilution), NovocastraTM 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'-TCATTATTTTTAATTAAAGGCCTGCTG-3', reverse RS 5'-CAAGATTTACCTCTATTTTGATCA-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'-AGCATGTGGGACCCACCTCAC-3', 5'-GCAGGGTCTAGAGCAGAGCAG-3'; EGFR exon 21: 5'-CTGAATTTCTCGAGTCAAGCTC-3', 5'-CTAGTGGGAAGGCAGCTTGTTG-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'-ACTGAATATAAACTTGTGGTAGTTGGA-CT-3')
introduced G to C substitution at the codon 11 of KRAS in order to create a BstO1 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 BstO1 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 BstO1 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 BstO1 (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-predominant 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 BstO1, 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.
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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 BstI. 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
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).
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. Additionally, molecular analysis demonstrated 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


