

Report on mutation in exon 15 of the APC gene in a case of brain metastasis

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Abstract The study analyzes exon 15 of the adenomatous polyposis coli gene (APC) in a 49-year-old male patient with brain metastasis. The primary site was lung carcinoma. PCR method and direct DNA sequencing of the metastasis and autologous lymphocyte samples identified the presence of a somatic mutation. The substitution was at position 5883 G–A in the metastasis tissue. The mutation was confirmed by RFLP analysis using Msp I endonuclease, since the mutation strikes the Msp I restriction site. Immunohistochemical analysis revealed the lack of protein expression of this tumor suppressor gene. The main molecular activator of the wnt pathway, beta-catenin, was expressed, and located in the nucleus. The mutation is a silent mutation that might have consequences in the creation of a new splice site. Different single-base substitutions in APC exons need not only be evaluated by the predicted change in amino acid sequence, but rather at the nucleotide level itself. In our opinion, such silent mutations should

also be incorporated in mutation detection rate and validation.

Keywords Adenomatous polyposis coli gene · Mutation · Brain metastasis · G–A transversion

Introduction

One of the most common metastatic sites for lung cancer is the brain. We investigated exon 15 of the APC gene in a patient with brain metastasis. The primary site was lung carcinoma. The APC gene (chromosome 5q21) is 8,535 bp long and is organized in 15 [1] translated exons. The gene encodes a large multidomain 2,843 amino acid protein that is expressed in a number of fetal and adult human tissues [2, 3]. The protein of this tumor suppressor gene has many cellular functions: as a component of the wnt signal transduction pathway [4, 5], as a component of adherens junctions, and as a component of the cytoskeleton stabilization. Mutation analysis of the APC gene revealed more than 400 different germline mutations [3, 6] responsible for familial adenomatous polyposis coli (FAP), but the overall number of detected mutations, germline and somatic, is more than 700 according to the Human Gene Mutation Database (HGMD, <http://www.hgmd.org>). The majority of detected mutations result in a truncated (shorter) protein product. Novel mutational reports reveal a great variety of private mutations [7], and also single-base substitutions that result in silent DNA variants having a role in RNA splicing regulation [1].

Exon 15 comprises more than 75% of the coding sequence of the APC gene and is the most common target for both germline and somatic mutation [3, 8]. Although extensive somatic mutations were observed throughout the

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APC gene, the majority of somatic mutations in colon would be clustered within a small region of exon 15, less than 10% of the coding region, called mutation cluster region or MCR (from codons 1,250–1,513) [9]. On the other hand, reports on somatic APC mutations in human tumors other than colon indicate that the concept of a mutation cluster region is not applicable to all cancers, for instance breast cancer [10].

It has been well documented that the APC gene is implicated in tumorigenesis [11–13]. The involvement of APC as a general tumor suppressor gene in a great variety of human tumors has been known for a long time. Moreover, its involvement in lung cancer is not an unknown event. Frequent loss of heterozygosity has been observed in 70–80% of small cell lung carcinomas (SCLCs) [14] and in 30–80% of non-small cell lung carcinomas (NSCLCs) [15]. Novel reports indicate that APC mutations although infrequent can still be identified in lung carcinogenesis [16, 17].

Materials and methods

DNA extraction

A case of brain metastasis, together with autologous blood tissue was collected from the Department of Neurosurgery and Department of Pathology, University Hospital “Sisters of Charity”, Zagreb, Croatia. The metastasis tissue was frozen in liquid nitrogen and transported to the laboratory, where it was immediately transferred at -75°C . The peripheral blood sample was collected in EDTA and processed immediately.

The local Ethical Committee approved our study and the patient gave his informed consent.

Approximately 0.5 g of tumor tissue was homogenized with 1 ml extraction buffer (10 mM Tris HCl, pH 8.0; 0.1 M EDTA, pH 8.0; 0.5% sodium dodecyl sulfate) and incubated with proteinase K (100 $\mu\text{g}/\text{ml}$; Sigma, USA) overnight at 37°C . Phenol chloroform extraction and ethanol precipitation followed.

Blood was used to extract leukocyte DNA. Five ml of blood was lysed with 7 ml distilled water and centrifuged (15 min/5,000g). The pellet was then processed as for DNA extraction from the tissue samples.

Polymerase chain reaction

To amplify fragment of the exon 15 of the APC gene we used in a volume of 50 μl : 5 pmol of each primer (5'-ATGATGTTGACCTTCCAGGG-3' and 5'-CTTTTTGGCA TTGCGGAGCT-3'), 200 ng template DNA, 5 μl $10\times$ buffer II, 1.5 mM MgCl_2 , 2.5 mM of each dNTP, 1 μl (5 U) of

Taq polymerase. PCR conditions were: initial denaturation, 5 min/ 95°C ; denaturation, 30 s/ 95°C ; annealing, 30 s/ 57°C ; extension, 45+1 s/ 72°C ; final extension, $72^{\circ}\text{C}/7$ min, 30 cycles. The PCR products were analyzed on 2% agarose gels.

Sequencing

PCR products were separated by agarose gel electrophoresis and visualized by ethidium bromide staining. DNA fragments were cut out and isolated from agarose gel (QIAquick gel extraction Kit; QIAGEN, Hilden, Germany). Sequencing reactions were performed with appropriate primers in a BigDye Terminator v1.1; Applied Biosystems, Foster City, USA, and analyzed on ABI PRISM 310. All sequences were confirmed by bidirectional sequencing of PCR products generated by at least two independent reactions. The sequencing of the observed sequence alteration in metastasis was repeated after Msp I restriction fragment length polymorphism (RFLP) allele separation. The allelic band suspected to be mutated was extracted from the gel and sequenced separately.

Restriction fragment length polymorphism (RFLP)

The exon 15 of APC gene was also investigated on the basis of RFLP of the PCR products, since the mutation was observed in an Msp I polymorphic site. The amplified fragment of exon 15 is 550 bp long and is cleaved with the Msp I endonuclease to two 250-bp fragments if the restriction site is present. Heterozygous patients demonstrated two bands (550 + 250 bp). PCR aliquots from metastasis and blood (10–15 μl) were digested with 6 U Msp I (Gibco, USA) overnight at 37°C and were electrophoresed on 2.5% agarose gels and/or Spreadex gels (Elchrom Scientific, Switzerland).

Immunohistochemistry

Immunohistochemistry was performed in order to determine the presence of APC protein in our sample. The sample was formalin-fixed, paraffin-embedded, and 4-mm-thick sections were placed on Capillary gap microscope slides (DakoCytomation, Denmark). The sections were immunostained using the biotin–avidin–horseradish peroxidase method as described previously [13]. The primary antibodies at optimized dilutions were applied for 30 min at room temperature. The antibodies used for APC protein detection were: APC-1 monoclonal mouse anti-human antibody that recognizes amino terminal epitope (diluted 1:50) (Calbiochem, USA) and APC-2 monoclonal rabbit anti-human antibody (ready-to-use) that recognizes carboxy terminal epitope (Spring Bioscience, USA). Monoclonals

used for beta-catenin detection were: monoclonal mouse anti-human antibody (diluted 1:200) from Dako Corporation, USA. All chemicals were from DakoCytomation. Negative controls were samples that underwent same staining procedure with the exclusion of the primary antibodies. Normal skin served as positive control. Two independent observers, i.e., blinded pathologists, experts in the field on an Olympus BH-2 microscope, performed the analysis of the labeling.

Case report

A 49-year-old male patient from Osijek in continental Croatia with brain metastasis (*carcinoma metastaticum cerebri regionis parietalis sinistrae*) was admitted to our hospital due to operative procedure of the brain tumor lesion. The metastasis infiltrated the dura. Using magnetic resonance imaging (MRI) a tumor lesion was found in the left parietal region. During the operative procedure, the tumor was maximally reduced using a microneurosurgical technique. Pathology provided the histopathological diagnosis of metastasis. The diagnosis of the primary tumor from which the metastasis originated was established during the patient's first visit to the hospital in January 2003. The chest radiograph and chest computed tomography (CT) scan revealed a large mass in the right upper lobe bronchus with bilateral mediastinal lymph node swelling which was confirmed by bronchoscopic examination. Biopsies were taken and histological evaluation revealed a small cell lung carcinoma (SCLC). At his first visit, the patient was already stage IV (T4N2M1), but was in good physical condition. Laboratory findings were normal. CT scan revealed metastases in the lymph nodes and skin of the neck, in the bone, in the mediastinal lymph nodes, and brain. The patient had no family history of tumors but was a habitual smoker. He was started on chemotherapy with adriamycine (100 mg i.v.), endoxane (cyclophosphamide, 1,000 mg i.v.), oncovin (1 mg i.v.), and zofran (ondansetron hydrochloride, 8 mg i.v.) followed by further radiotherapy. The first response to the therapy was evaluated as good partial response. Altogether, the patient was given three courses of the same combination of chemotherapy that was administered in his first visit. In September 2003, chemotherapy treatment was not successful. The patient was admitted to the hospital in May 2004 after which followed cerebral coma and respiratory arrest.

The results of DNA sequencing of the PCR product from the metastasis revealed a G to A change at position 5,883, codon 1,961 (Fig. 1a). At the first sequencing attempt, the single base substitution G/A was visible only when sequencing was performed in one direction, i.e., with one primer. The nucleotide sequence of the other allele was

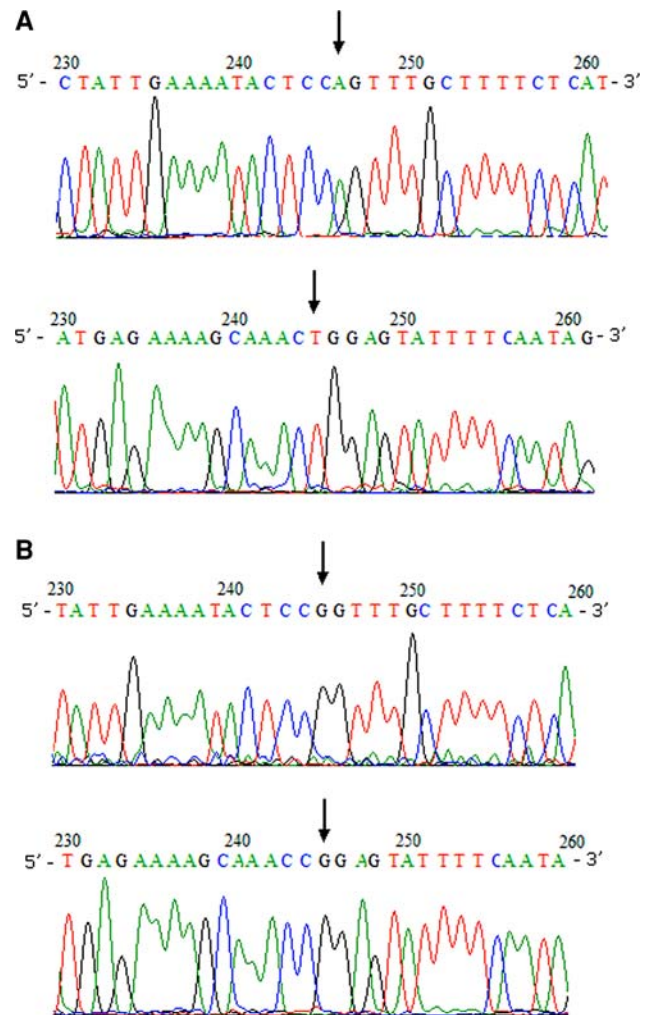


Fig. 1 Sequence for the exon 15 of the APC gene. 550 bp fragment was sequenced with appropriate primers, and analyzed on ABI PRISM 310. **a** Metastasis DNA, forward and reverse. **b** Autologous lymphocyte DNA, forward and reverse. The *arrow* points to the mutant nucleotide

impairing the analysis. Therefore, we suspected that only one allele was hit with the mutation. We decided to separate the alleles from the total PCR volume of 50 μ l and to sequence them separately. After this procedure, it was obvious that one allele had CCAG while the other retained CCGG sequence. Both alleles from the autologous blood sample had CCGG sequence as shown in the result of sequencing (Fig. 1b).

The mutation was confirmed by RFLP analysis using Msp I endonuclease, since the mutation strikes the Msp I restriction site. The analysis of Msp I polymorphic site in APC's exon 15 of our proband's blood sample showed that the patient was homozygous for this polymorphism, i.e., both alleles had CCGG Msp I restriction site and only one band appeared on the gel. In contrast to this, our metastasis sample showed an additional band (550 + 250 bp)

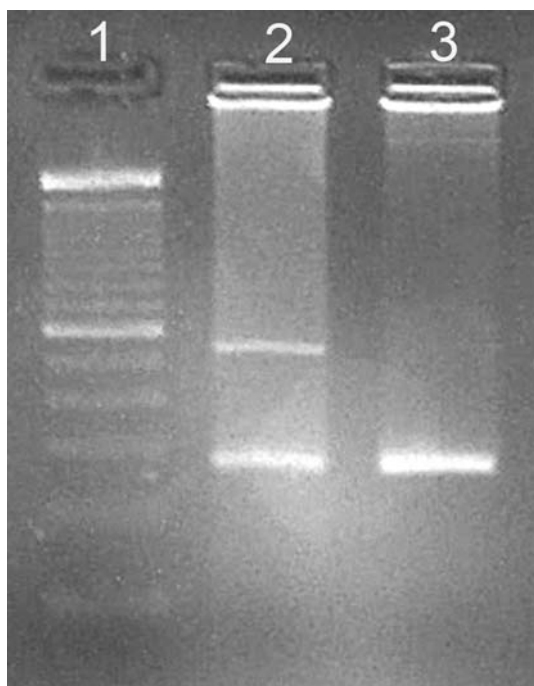


Fig. 2 RFLP/MspI of APC gene's exon 15. *Lane 1* Molecular marker (100 bp ladder), *lane 2* brain metastasis sample, *lane 3* corresponding blood sample

(Fig. 2). This finding led us to conclude that our sample comprised a mutation in the polymorphic site in exon 15 of the APC gene.

Discussion

Exon 15 starts at nucleotide 1,958, and is 6,577 bp long [18]. After direct sequencing the fragment of exon 15, we found that the sequence at codon 1,961 (position 5,883) changed in metastasis tissue in CCAG. Since the change affected only one allele, the digestion resulted in two bands. We therefore separately sequenced alleles from metastasis tissue, and confirmed that the uncut allele had the above-mentioned substitution.

Since CCG codes for proline, and CCA codes for the same amino acid, it is possible that our silent mutation has no functional consequences. On the other hand, the silent mutation present in the metastasis sample and not found in the constitutive DNA of the same patient may indicate an important regulatory element change at the DNA level. Different single-base substitutions in APC exons need to be evaluated not only by the predicted change in amino acid sequence, but rather at the nucleotide level itself. The introduction of a novel splice site is one explanation.

Heinimann et al. [19] reported on mutations within regulatory elements of the APC gene. These authors also found many silent mutations within the exon 15 of the APC

gene. Although they categorized the silent mutations as nonpathogenic polymorphisms they did not exclude their possible effects on transcription and splicing efficiency. Montera et al. [20] report on a silent mutation in exon 14 of the APC gene that causes exon skipping. They stress the importance of investigating the significance of silent mutations. According to those findings, new models of mutation consequences via changes in exonic regulatory elements are proposed. Recent studies indicate that efficient splicing requires sequence elements distinct from the splice sites. These elements may affect splice site recognition during constitutive and alternative splicing. They are found within coding exons and are called exonic splicing enhancers (ESE) [21]. A silent genomic DNA variation might modulate the splicing and affect RNA.

Silent mutations not leading to amino acid changes are generally considered to be normal variants, and are thought to have no role in disease. However, exonic silent mutations that are able to induce exon skipping have been described in association to the diseases in MLH1 [22], fibrilin-1 [23] and human phenylalanine hydroxylase genes [24].

Aretz and co-workers [1] revealed the importance of rare exonic single-base substitutions of APC gene that are predicted to result in silent (SNP) variants. The authors demonstrated that traditional functional consequence due exclusively to amino acid changes can no longer be regarded as the only responsible pathological event. Changes at the nucleotide DNA level are equally important. Silent mutations can affect classic consensus splice-site signals, can strike internal exonic sites such as ESE motifs, or can introduce a novel cryptic, less active, in-frame splice site.

In our case, this assumption is additionally strengthened by immunostaining that showed the lack of APC protein expression in our sample (Fig. 3). We can speculate that APC protein was absent by inactivation of both alleles of the APC gene. One allele could be deleted at another gene locus, and the remaining one might have suffered from point mutation or a small deletion. Another possible explanation of the APC protein absence is the instability of the corresponding mRNA or truncated protein. This finding is in accordance with Kartheuser et al. [25] who report on a rare 3' exon 15 APC mutation at nucleotide 5960delA that was predicted to lead to a truncated protein of about 220 kDa. However, the western blot analysis they performed failed to show a truncated protein. The same result was also obtained by van der Liugt et al. [26].

Contrary to APC protein status, beta-catenin was present in metastasis tissue and located in the nuclei of the majority of the cells. This finding is logical since APC acts as a negative regulator of Wnt signaling, and is a critical component of the beta-catenin destruction machinery leading to the proteasome. In response to Wnt signaling, or

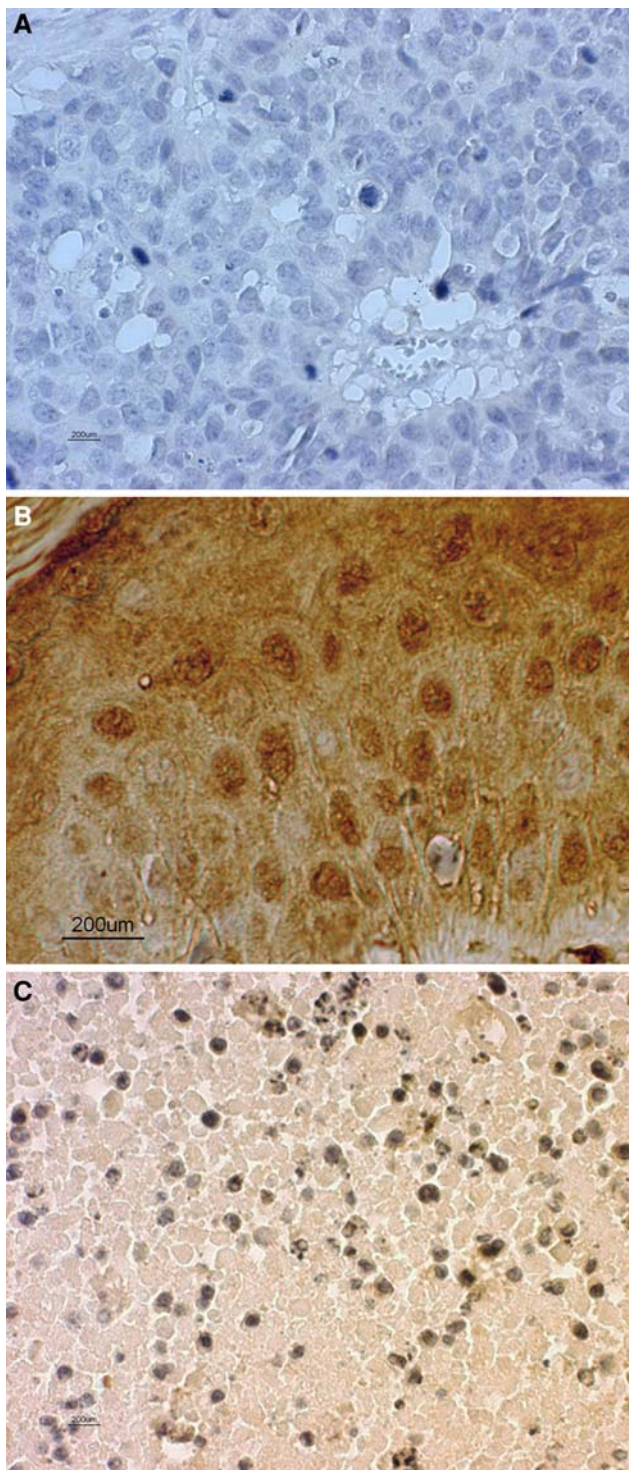


Fig. 3 Brain metastasis immunohistochemically stained for detection of **a** APC protein: patient was negative for the expression of APC protein when tested both with Ab-1 (monoclonal mouse anti-human antibody; Calbiochem, USA) that recognizes the amino terminus, and with Ab-2 (monoclonal rabbit anti-human antibody; Spring Bioscience, USA) that recognizes the carboxy terminus; **b** positive control for APC detection; **c** beta-catenin protein: patient with brain metastasis demonstrating nuclear localization of beta-catenin tested with monoclonal mouse anti-human antibody (Dako Corporation, USA)

under the circumstances of mutated or missing APC, beta-catenin is stabilized, accumulates in the cytoplasm, and enters the nucleus where it activates the expression of target genes, among others, c-myc and cyclin D1 [12]. Our finding on beta-catenin's nuclear staining in metastasis derived from lung cancer may be indicative of beta-catenin's oncogenic activity.

The nucleotide position of the detected mutation is located in the central region of the APC protein that contains a series of seven 20-amino acid repeat motifs that bind beta-catenin. The majority of truncated mutant proteins lack all or most of the 20-amino acid repeats, suggesting that this area is a target for elimination during tumorigenesis [3, 9].

Recent research found beta-catenin mutations in a small subset of human and rat lung carcinomas, suggesting the involvement of the Wnt pathway in lung tumorigenesis [17]. Although LOH of the APC gene is a common finding in lung cancer, point mutations of this tumor suppressor have not been frequent [14, 15]. Contrary to the previous reports, novel findings indicate the presence of point mutations of the APC gene in lung cancers. Oghaki et al. [16] found two somatic mutations in squamous cell carcinoma, a 2-bp deletion in codon 1,465, and a missense mutation in codon 1,317. The same authors also recorded one case of small-cell lung carcinoma with somatic missense mutation in codon 1,284. They also found nuclear beta-catenin staining in the mutated SCLC.

In our opinion, silent mutations should also be incorporated in mutation detection rate and validation. We wanted to inform you on this silent mutation which may contribute to a better understanding of the brain metastasis genetic profile.

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