Mutational analysis of driver genes defines the colorectal adenoma: in situ carcinoma transition

Jiri Jungwirth1,2,15, Marketa Urbanova1,3,15, Arnoud Boot4, Petr Hosek5, Petra Bendova3,5, Anna Siskova1,3, Jiri Svec6,7, Milan Kment8, Daniela Tumova9, Sandra Summerova10, Zdenek Benes10, Tomas Buchler11, Pavel Kohout10, Tomas Hucl12, Radoslav Matej13,14, Ludmila Vodickova1,3,5, Tom van Wezel4, Pavel Vodicka1,3,5 & Veronika Vymetalkova1,3,5

A large proportion of colorectal carcinomas (CRC) evolve from colorectal adenomas. However, not all individuals with colonic adenomas have a risk of CRC substantially higher than those of the general population. The aim of the study was to determine the differences or similarities of mutation profile among low- and high-grade adenomas and in situ carcinoma with detailed follow up. We have investigated the mutation spectrum of well-known genes involved in CRC (such as APC, BRAF, EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53) in a large, well-defined series of 96 adenomas and in situ carcinomas using a high-throughput genotyping technique. Besides, the microsatellite instability and APC and MLH1 promoter methylation were studied as well. We observed a high frequency of pathogenic variants in the studied genes. The APC, KRAS and TP53 mutation frequencies were slightly lower in adenoma samples than in in situ carcinoma samples. Further, when we stratified mutation frequency based on the grade, the frequency distribution was as follows: low-grade adenoma—high-grade adenomas—in situ carcinoma: APC gene 42.9–56.0–54.5%; KRAS gene 32.7–32.0–45.5%; TP53 gene 8.2–20.0–18.2%. The occurrence of KRAS mutation was associated with the presence of villous histology and methylation of the APC promoter was significantly associated with the presence of POLE genetic variations. However, no association was noticed with the presence of any singular mutation and occurrence of subsequent adenoma or CRC. Our data supports the multistep model of gradual accumulation of mutations, especially in the driver genes, such as APC, TP53 and KRAS.

Colorectal cancer (CRC), the third most common cancer and the fourth most frequent cause of cancer death worldwide1, represents an ideal model to investigate and dissect the genetic alterations involved in tumor initiation and progression. It has been known for some time that the majority of CRCs arises and progresses through
a series of well-defined molecular and histopathological changes, the so-called adenoma-carcinoma sequence\textsuperscript{2,3}, first described by Fearon and Vogelstein\textsuperscript{4}.

The adenoma-carcinoma sequence was described as a gradual transformation of colorectal epithelium to adenomatous lesions and ultimately to an adenocarcinoma and a metastatic tumor. Even though most neoplastic adenomas will not give rise to cancer, it is well accepted that most colorectal carcinomas evolve from adenomatous polyps\textsuperscript{5}. However, it cannot currently be predicted which of the early lesions will develop into cancer\textsuperscript{6}. Molecular alterations that play a role in the initiation and progression of CRC suggest a heterogeneous adenoma-carcinoma sequence that comprises several distinct molecular pathways. These include chromosomal instability, microsatellite instability (MSI), and CpG island methylator phenotype (CIMP) pathways that all of which are responsible for genetic and epigenetic instability in CRC\textsuperscript{2}. These genetic and epigenetic alterations affect different pathways that regulate multiple biological processes critical to cancer development\textsuperscript{7}.

Genetic mutations enriched in both adenomas and carcinomas are likely to represent early driver events. Mutations present predominantly in the carcinomas may constitute later driver mutations involved in tumor progression. Genes mutated in adenomas and not mutated in associated carcinoma tissue comprise either random mutation events not important for cancer initiation, or rare events that were not identified in the carcinomas\textsuperscript{8}.

The aim of the present study was to explore the genetic heterogeneity of adenomas and early carcinomas by analyzing the mutation spectrum of well-known genes involved in colorectal carcinogenesis (\textit{APC, BRAF, EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53}) in a large, well-defined series of adenomas and in situ carcinomas with follow up using a next generation sequencing approach.

In this context, we have analyzed each gene for the number and type of mutations present in the adenomas and in situ carcinomas and their specific relationship to MSI status. In addition, we have analyzed the levels of methylation of CRC-related genes by methylation-sensitive high-resolution melting (MS-HRM). We investigated the tumor suppressor adenomatous polyposis coli gene (\textit{APC}), which encodes a key protein in the WNT signaling pathway and is indicated as an early event in carcinogenesis\textsuperscript{9}, and \textit{MLH1} gene whose aberrant promoter methylation is responsible for the loss of mismatch repair activity.

Results
Patient’s characteristics. The studied set included 96 patients, out of which 74 were patients with adenomas and 22 with in situ carcinoma. The clinic-pathological characteristics are presented in Table 1.

|                | All n = 96 (%) | Adenomas n = 74 (%) | In situ carcinomas n = 22 (%) | p value (test) for difference between adenomas and in situ carcinomas |
|----------------|---------------|---------------------|-----------------------------|---------------------------------------------------------------|
| Age (mean ± SD) years | 65.6 ± 10.4   | 65.1 ± 10.6         | 67.0 ± 9.9                  | 0.48 (t test)                                               |
| Sex            |               |                     |                             |                                                              |
| Men            | 58 (60.4)     | 46 (62.2)           | 12 (54.5)                   | 0.62 (Fisher’s)                                             |
| Women          | 38 (39.6)     | 28 (37.8)           | 10 (45.5)                   |                                                              |
| Lesion site    |               |                     |                             |                                                              |
| Colon          | 54 (56.2)     | 46 (62.2)           | 8 (36.4)                    | 0.05 (Fisher’s)                                             |
| Rectum         | 42 (43.8)     | 28 (37.8)           | 14 (63.6)                   |                                                              |
| Polyp type     |               |                     |                             |                                                              |
| Tubular        | 46 (47.9)     | 37 (50)             | 9 (40.9)                    | 0.62 (Fisher’s)                                             |
| Tubulo-villous | 38 (39.6)     | 29 (39.2)           | 9 (40.9)                    |                                                              |
| Villous        | 12 (12.5)     | 8 (10.8)            | 4 (18.2)                    |                                                              |
| Grade*         |               |                     |                             |                                                              |
| Low            |               | 49 (66.2)           |                             |                                                              |
| High           |               | 25 (33.8)           |                             |                                                              |

Table 1. Patient’s clinical characteristics. *For adenoma patients only.

APC and MLH1 promoter methylation. The promoter methylation status of \textit{APC} and \textit{MLH1} genes was studied by MS-HRM.

There was no remarkable difference in the distribution of promoter methylation in the \textit{APC} gene (mean 9%); for in situ carcinoma, the mean promoter methylation in affected tissue was 8% and in adenomas it was 8.4% (9.1% for low grade and 6.8% for high grade dysplasia).

Interestingly, methylation of the \textit{APC} promoter was significantly associated with the presence of \textit{POLE} genetic variations (\textit{p} = 0.02, Fig. 1A; \textit{n} = 11).

The only one hypermethylated \textit{MLH1} promoter corresponded to the only sample with MSI-H status.

MSI status. MSI status of all adenomas and in situ carcinomas was tested. In our set, only 3 samples had MSI instability: 2 of them with MSI-L and one with MSI-H status. The MSI-H status was observed in an in situ carcinoma located in the right colon while MSI-L was noticed in two low grade dysplasia samples located in the left colon.

Mutation spectrum. In total, 96 adenomas and in situ carcinomas were analyzed for mutations in 11 genes (\textit{APC, BRAF, EGFR, NRAS, KRAS, PIK3CA, POLE, POLD1, SMAD4, PTEN, and TP53}). The mutation hotspots in \textit{KRAS, NRAS, BRAF, PIK3CA, EGFR, SMAD4} genes as well as exonuclease domain for \textit{POLE} and \textit{POLD1}
Figure 1. The mutated gene signature of colorectal adenomas and in situ carcinomas. (A) The APC promoter methylation distribution with POLE genetic variations, (B) The mutation distribution of APC gene between low-, high-grade adenomas and in situ carcinomas, (C) The mutation distribution of KRAS gene between low-, high-grade adenomas and in situ carcinomas, (D) The mutation distribution of TP53 gene between low-, high-grade adenomas and in situ carcinomas, (E) The mutation distribution of POLE gene between low-, high-grade adenomas and in situ carcinomas, (F) The Venn diagram of mutations of APC, TP53, and KRAS genes in in situ carcinomas, (G) The Venn diagram of mutations of APC, TP53, KRAS, and POLE genes in adenomas.
genes were studied. Concerning the \textit{APC}, \textit{PTEN} and \textit{TP53} genes, we have focused on the entire open reading frame (ORF).

Of all 96 samples, 21 (18 adenomas and 3 in situ carcinomas) of them did not bear any mutation in the studied genes. Out of the remaining 75 samples with mutation(s), 56 were adenomas and 19 carcinomas (Fig. 2). We did not observe any significant differences in mutation distribution between adenomas and in situ carcinomas.

\textbf{APC.} In the present study, we have focused on the entire ORF of the \textit{APC} gene as it harbors most of the mutations described in the \textit{APC} gene. In both the low- and high-grade adenoma samples (Fig. 1B), 36 deleterious (according to HGMD guidelines and pathogenic according to ACMG guidelines) mutations in 35 individuals were observed: 12 deletion (p.E1268fs, p.1275_1275del, p.D1279fs, p.T1283fs, p.1289_1291del, p.1302_1304del, p.1344_1350del, p.P1406fs, p.1431_1432del, p.T1469fs, p.P1479fs, and p.1561_1562del), 7 insertion (p.Q1226fs, p.S1316fs, p.V1359fs, p.P1391fs, p.T1469fs, p.G1481fs, and p.E1536fs), 15 nonsense (three times p.Q1273X, twice p.Q1276X, twice p.E1288X, p.S1297X, p.E1304X, p.E1335X, p.E1379X, p.Q1276X, p.Q1349X), and 2 missense (p.T1274M and p.E1299Q) mutations. While in carcinoma samples, 13 mutations in 12 patients were detected: 4 deletion (p.1284_1286del, p.1454_1455del, p.T1469fs, and p.E1542fs), 4 insertion (p.Q1226fs, p.M1365fs, p.S1377fs, and p.T1478fs), 4 nonsense (p.E1198X, p.K1292X, p.E1361X, and p.E1379X), and 1 missense (p.L1493I) mutations. Interestingly, one patient with low grade adenoma and one patient with carcinoma had two concurrent \textit{APC} deleterious mutations (2 insertions (p.G1481fs and p.E1536fs) in the ade-

---

**Figure 2.** The distribution of genetic alterations detected in low-grade, high-grade adenomas, and in situ carcinomas. Each row represents a patient, and each column represents a gene. Different mutation types are indicated by different colors. The bar chart on the top shows the total number of the given gene's mutations observed in the sample.
noma sample and 1 deletion (p.E1542fs) and 1 nonsense (p.E1379X) type of **APC** mutation in the carcinoma sample.

Interestingly, several identical **APC** mutations have been observed both in high-grade adenomas and in situ carcinomas, namely: deletion (p.T1469fs), insertion (p.Q1226fs) and nonsense (p.E1379X) mutations.

**KRAS.** For the **KRAS** gene, several missense mutations were successfully identified in 34 individuals [24 individuals with either low- or high-grade adenoma and 10 with in situ carcinoma, (Fig. 1C)] at codon 12: c.34G > C/A/T, c.35G > A/T/C, codon 13: c.38G > A/C/T, codon 61: c.182A > G, c.183A > C and 146: c.437C > T. Both adenomas and carcinomas were most frequently mutated at codon 12, position 35 (22 cases out of 34: in particular, 16 adenomas and 6 carcinomas, 73% and 27%, respectively) and a G > A transition was the most common nucleotide for both phenotypes (14 cases out of 34: in particular, 10 in adenomas and 4 in carcinomas, 71% and 29%, respectively). The presence of **KRAS** mutation was significantly associated with the presence of villous histology (p = 0.02).

**TP53.** Concerning the **TP53** gene, we have focused on the entire ORF. Nine patients with adenomas and 4 carcinoma patients bore mutations in the **TP53** gene (Fig. 1D). In the adenoma samples (either grade), 5 of them had missense (p.R43H, p.P45S, p.H47R, p.R49C, and p.R116Q) mutations and 4 had insertion (twice p.H46fs and twice p.C143fs). On the other hand, 4 mutations were identified in carcinoma samples: 2 missense (p.R43H and p.I122V) and 2 insertion (p.P59fs and p.V71fs). None of the analyzed patients had more than one **TP53** mutation. The p.R43H missense mutation was observed both in high-grade adenoma and in in situ carcinoma tissues.

**APC, KRAS and TP53 co-mutations.** Only 4 out of 96 samples had all 3 **APC, KRAS** and **TP53** genes mutated. Interestingly, 1 sample was a carcinoma, 2 samples were high grade dysplasia adenomas, and 1 sample a low grade dysplasia adenoma (Fig. 1EG). All above samples had an MSS status and were located in the right colon. The individual with low grade dysplasia later developed another adenoma in the bowel.

Simultaneously, when we compared the frequency of mutations in these three genes between both low- and high-grade adenomas and in situ carcinoma, the **APC** mutation frequency was lower in adenoma samples than in carcinoma samples (47.3% vs. 54.5%, p = 0.63). The **KRAS** mutation frequency was again lower in adenoma than in carcinoma samples (32.4% vs. 45.5%, 0.31), while the **TP53** mutations frequency was the lower in adenomas and the highest in carcinoma samples (12.2% vs. 18.2%, p = 0.27). These distribution differences among adenomas and in situ carcinomas were not significant. Further, when we stratified mutation frequency for low- and high-grade adenoma, the distribution was as follows: low-grade adenoma—high-grade adenomas- in situ carcinoma: **APC** gene 42.9–56.0–45.5%; **KRAS** gene 32.7–32.0–45.5%; **TP53** gene 8.2–20.0–18.2%.

**Other mutations.** Four **NRAS** mutations were found, two missense (twice c.181A (p.Q61K) in the right colon and c.38A (p.G13R)) mutations and insertion (c.430dupA:p.T144fs) in the left colon. The first two mentioned missense mutations have recently been described in adenomas7. The second mentioned **NRAS** mutation was observed in in situ carcinoma in our set of patients.

In the present study, **BRAF** mutation c.1799 T > A (p.V600E) was only observed in a single carcinoma sample that also displayed MSI-H status. Further, three **PIK3CA** mutations were detected, namely a hotspot missense mutation (c.1624G > A, p.E542K) in an in situ carcinoma and two deletions ((twice c.3114delT:p.Y1038fs), one in a low-grade and one in a high-grade dysplasia sample). **SMAD4** (c.941dupT:p.I314fs in low-grade dysplasia and c.353_354insA:p.A118fs in low-grade dysplasia) and **PTEN** (twice c.908delT:p.T303fs in low- and high-grade dysplasia) mutations were also recorded.

Additionally, mutations in **POLD1** and **POLE** genes were studied. For the **POLD1** gene, a c.1263 dupG:p.L421fs mutation was observed in low grade dysplasia. Interestingly, in 12 samples (11 adenomas and 1 in situ carcinoma, (Fig. 1E), **POLE** (c.T1166Cp.F389S) mutation was identified. However, no record about this particular mutation was published in COSMIC10, LOVD11 or HGMD12. For this reason, we rather assessed this genetic variant as variant of uncertain significance (VUS) rather than pathogenic mutation.

There was no significant overlap between these mutations identified in our set of samples.

**Follow up.** For 70 out of 96 patients included into the study, follow up data were available. Out of these, 21 developed a subsequent adenoma. Seventeen of them had previously adenoma (7 high-grade and 10 low-grade adenoma) and four in situ carcinoma (Table 2). The only common feature of these patients is that their first samples included in our study were all of an MSS status (apart one low grade adenoma that was MSI-L). Unfortunately, we were not able to obtain the following adenoma tissue for analysis. Apart from one low-grade and one high-grade adenoma, all of them had previously at least one mutation in the high-risk genes, such as **APC,**

### Table 2. Follow up of patients included into the study.

|        | Normal findings | Recurrence of adenoma | Occurrence of CRC |
|--------|-----------------|-----------------------|-------------------|
| Low grade adenomas | 39              | 10                    | 2                 |
| High grade adenomas | 18              | 7                     | 1                 |
| In situ carcinomas | 12              | 4                     | 6                 |
KRAS, or TP53, however no association was observed in relation of the presence of any singular mutation and occurrence of subsequent adenoma. Concerning the histology and subsequent adenoma, patients with mixed histology (tubule-villous) were less prone to develop further adenomas than those with villous or tubular histology alone (p = 0.02). Besides, patients with higher age also develop subsequent adenomas rather than younger patients (p = 0.04).

In all patients with in situ carcinoma, the tumor was surgically removed, and patients were regularly monitored by colonoscopy procedure.

Furthermore, out of 22 patients with in situ carcinoma, 4 developed adenoma (as stated above) and 6 even invasive carcinoma within few years after the first in situ carcinoma. The proportion of patients developing subsequent invasive carcinoma depended significantly on the type of their primary lesion (p = 0.009), being by far the highest for in situ carcinomas (27.3%) in comparison to low-grade and high-grade adenomas (4.1% and 4.0%, respectively).

Most of the patients with in situ carcinoma who underwent a clinical follow up had further developed CRC in about the same location as the previous findings quite soon or within two years.

For the patient with adenoma and subsequently developed CRC, the situation is a bit different. In a few years, the emerging CRC was located in a completely different colon segment than the previous adenoma. One patient with low grade adenoma developed liver metastases very quickly.

**Discussion**

The progression from adenoma into cancer can take as long as 20 years and is not usually affected by a single pathway5,11,14. This transition represents a multistep process that is characterized by chromosomal instability (CIN), MSI, and CIMP. Effects of all these pathways may combine and are responsible for genetic instability in an adenoma that underlies malignant transformation15.

Despite this, little is known about the mutation profiles of advanced adenomas and in situ carcinomas. It is not certain whether they share the same genetic background, or the driver mutations are more abundant in in situ carcinomas compared to adenomas. As these two forms of neoplasia are described as subsequent stages of carcinogenesis in the Vogelstein model, the cascade of low-grade—high-grade adenoma and in situ carcinoma represents a suitable model for the analysis of CRC development. Up to now, the research of the transformation of colon adenoma into cancer has mostly focused on association studies assessing the cancer risk or advanced colorectal carcinomas.

The aim of the present study was to use a well-defined series of adenomas and in situ carcinomas to perform a parallel investigation of the mutation status of 11 genes known to be involved in CRC, as hypothesized by Vogelstein. The studied genes are involved in several different signal transduction pathways.

According to the recent study by Lee-Six et al.16, mutations in APC, KRAS and TP53 genes are common in CRC (accounting for 56% of base-substitution and indel driver mutations) while being rare among unaffected colonic crypts. The authors suggested that mutations in these genes confer higher likelihoods of conversion of normal epithelium to adenoma and carcinoma. However, in our study, only 4 out of 96 individuals had all APC, KRAS and TP53 genes mutated concurrently suggesting that further alterations in mutational frequency and spectrum may occur along with CRC progression.

In our study, we did not observe any significant differences in the distribution frequency of mutations in APC, KRAS and TP53 genes between adenoma and in situ carcinomas. Without the stratification for low- and high-grade adenoma, the mutation frequencies were rather similar. The APC mutation frequency was moderately lower in adenoma samples (47.3%) than in carcinoma samples (54.5%). The KRAS mutation frequency was again lower in adenoma than in carcinoma samples (32.4% and 45.5%), as were the TP53 mutation frequency (12.2% and 20%). Only 4 out of 96 individuals had all APC, KRAS and TP53 genes mutated concurrently. Further, when we stratified mutation frequencies for low- and high-grade adenoma, the distribution frequencies of mutations in the APC and TP53 genes had an increasing tendency towards in situ carcinoma, while the frequency of mutations in the KRAS gene in the low- and high-grade adenoma was lower than that in in situ carcinoma. This may point to the fact that in situ carcinoma still carries the mutation profile of the adenoma and only during further progression the mutation frequencies change, or, alternatively vice versa that high-grade adenomas are already approaching in situ carcinomas with their mutation profile. Therefore, a more thorough examination and assessment of the risk of CRC in people with adenomas is needed. Nonetheless, the observed higher mutation frequency in in situ carcinomas resembles the generally accepted Fearon and Vogelstein model of carcinogenesis4. However, additional studies are warranted to track the dynamics of mutational in relation to the disease heterogeneity.

The rather higher KRAS mutation frequency than expected might be explained by the fact that the current study investigated KRAS mutations in codons 12, 13, 61 and 146 covering most of all reported mutations for KRAS in CRC, while earlier studies investigated mostly codon 12 and/or 13 of the KRAS gene only5,14.

In our study we have observed the presence of KRAS mutation associated with the presence of villous histology. Similar outcomes were obtained by Zauber et al.15. The authors hypothesized that non-mucinous and MSS CRC with wild-type KRAS gene may have had a mutation in the KRAS gene during their earlier stages, however the mutation was lost during further growth.

Over the last years, many studies have shown that germline mutations in the proofreading domains of POLD1 and POLE predispose to CRC and other malignancies20,21. These mutations arise early in oncogenesis and serve as gatekeeper mutations—confering a growth advantage to cellular subpopulations and driving tumor growth. Interestingly, in 12 samples (11 adenomas and 1 in situ carcinoma), POLE (c.T1166C:p.F389S) VUS was identified. However, no record about this identified VUS was published in public databases yet, this VUS was observed in oral squamous cell carcinoma22 and small cell lung cancer23 and thus it deserves further attention. Besides this, APC promoter methylation was significantly associated with the presence of POLE VUS. Although inactivating
framing not likely leads to a hypermutation profile, Poulos et al. observed the presence of coding mutation hotspots in POLE-mutant cancers at highly-methylated CpGs in the tumor-suppressor genes APC and TP53. This finding points to the links between methylation and mutations and DNA repair, and these mechanisms define a key part of the mutational background of cancer genomes.

While BRAF mutations are more common in MSI colorectal cancers, they are less prevalent in adenomas. In our study, we have observed BRAF mutation only in one in situ carcinoma sample that simultaneously possessed the MSI-H phenotype. BRAF and KRAS mutations are mutually exclusive, as was observed in our set of samples.

In the current study, three mutations were found in the PI3KCA and two mutations in the negative regulator of the PI3K- Akt pathway, the PTEN gene. According to the literature, the PIK3CA mutation frequency in CRC ranges between 10–30%. However, PIK3CA mutations are less common in colorectal adenomas, around 3%, indicating that mutations in PIK3CA would generally arise later during the adenoma-carcinoma transition. Although it may seem that our results are not in complete agreement with these observations, we have detected a hotspot mutation (c.1624G > A, p.E542K) in an in situ carcinoma and two deletion (c.3114delT:p.Y1038fs), one in a low-grade, and one in a high-grade dysplasia sample. However, we focused only on in situ carcinomas and not on invasive CRC and the mutation frequency rate in adenomas is in concordance with other studies, being around 3%.

A further aim of the study was to regularly follow up all patients included into study. Unfortunately, we were not able to follow many of the patients for various reasons: patients did not attend the regular check-ups, moved away, died for other than gastrointestinal reasons, etc. Follow-up data were recorded by clinicians to determine if patients had, a) clear colonoscopy findings, b) recurrence of adenoma, and c) occurrence of CRC. Clinical follow-up of 70 patients out of 96 was performed within this study. Several patients developed a subsequent adenoma or even CRC. The only common feature of these patients is that their first samples included in our study were all of an MSS status and all of them previously had at least one mutation in the high-risk genes, such as APC, KRAS, or TP53.

Concerning the histology and subsequent adenoma, patients with mixed histology (tubule-villous) were less prone to develop further adenomas than those with tubular or villous histology (p = 0.02). The fact that patients with higher age developed subsequent adenomas rather than younger patients might be a result of more regular colonoscopy controls advised among older people.

Besides, out of 22 patients with in situ carcinoma, 4 further developed adenoma and 6 even invasive carcinoma within few years after the first in situ carcinoma diagnosis. The occurrence of in situ carcinoma is in itself a clear evidence of a malignant reversal in the body and therefore these conclusions are not so unexpected.

As it was stated earlier, mutations identified in both adenomas and in situ carcinomas are likely to represent early driver events. However, mutations present predominantly in carcinomas may indicate later driver mutations involved in tumor progression. Genes mutated in adenomas and not mutated in cancer tissue can illustrate either random mutation events that are not as important for cancer onset or rare events that have not been identified in cancers. So how do we know that the adenoma is no longer embarking on transformation into cancer and, conversely, that the cancer does not carry even the historical “adenoma” mutations? Or can it be stated that once the adenoma tissue has accumulated all the necessary mutations, it will switch to carcinoma so quickly that we do not have much chance of catching it in this transitional phase?

Of course, it is still more likely to find those driver mutations in carcinomas and rare events in adenomas, but depending on how fast such a transformation takes place, the classification of genes as potential early and late driving events can help dissect pathways involved in both tumor initiation and progression.

The weaknesses of the present study include the still limited number of patients and incomplete clinical follow-up, partly responsible for the impossibility of comparing the mutational profile of initial lesion and following lesion. Also, the used method detected the presence of the mutation in the one region of the lesion, thus the results do not reflect possible heterogeneity of the non-invasive colorectal lesions. Furthermore, only adenoma tissue was analyzed in this study. Unfortunately, due to Ethical reason, the adjacent unaffected tissue was not collected and analyzed. For this reason, we cannot say with certainty whether we have identified exclusively somatic variants.

The early cancer biomarkers are strongly needed and are taking advantage of rapid progress in molecular biology. These biomarkers should be able to distinguish healthy people from patients with adenomas and subjects with early-stage CRC (stage Tis, I or II) with relative ease and low cost, and to be minimally invasive with aim to increase screening acceptability. With this respect, circulating nucleic acid-based biomarkers (or so-called “liquid biopsy”) are currently extensively studied in cancer research. Circulating cell-free DNA (cfDNA) is probably the most promising tool among all components of liquid biopsy.

Pathogenic mutations in the KRAS, BRAF, APC, and TP53 genes have been predominantly analyzed in the cfDNA isolated from CRC patients and less in patients with adenoma. The concordance of the mutations found in these genes in tumor tissue and plasmatic cfDNA was 100% (reviewed in29). Recently, the study by Cervena et al. proved the clinical relevance of APC and TP53 genes especially in the light of longitudinal monitoring of CRC patients.

However, the sensitivity of cfDNA based markers for early-stage disease is lower than for advanced stages. To our knowledge, this is problem that has not been overcome yet. Although in our intended studies we would like to address this problem and attempt to detect identified pathogenic mutations in cfDNA isolated from both plasma and stool of patients with adenoma and early cancer stages.
Conclusion

Our data confirms the Vogelstein's theory of gradual accumulation of mutations, especially in the driver genes, such as APC, TP53 and KRAS. The set of adenomas and in situ carcinomas only very rarely exhibited MSI-H phenotype and the role of mutations in POLE and PIK3CA genes in adenoma to carcinoma transition warrants further investigations.

Material and methods

Tissue samples. Fresh frozen tissue samples from adenomas and in situ carcinomas were collected consecutively at three different institutes during the planned colonoscopy (Thomayer University Hospital, University Hospital Kralovske Vinohrady, and Medicnas, all in Prague, Czech Republic). The study included individuals with adenomas of tubular, villous or tubulo-villous histology and individuals with in situ carcinomas who underwent colonoscopy examination as a part of CRC screening or for intestinal symptoms. There were no age, gender, and ethnicity restrictions. The exclusion criteria were proven hereditary CRC syndromes, inflammatory bowel disease (IBD), histology of hyperplastic polyps, and size smaller than 5 mm. Patients with any personal history of previous malignancy, or with colorectal cancer-associated well-defined inherited syndromes (including Lynch syndrome, familial adenomatous, and MUTYH-associated polyposis) were also excluded from the study.

The study was approved by Ethical committees of all institutions (Institute of Experimental Medicine, Prague, Czech Republic, IKEM and Thomayer hospital) and all individuals agreed with participation in the study and signed an informed consent in accordance with the World Medical Association Declaration of Helsinki.

Colorectal adenomas were histologically classified according to the revised Vienna classification32. Low-grade dysplasia was marked Category 3, while category 4.1 was assigned to high-grade dysplasia. Accordingly, category 4.2 was as assigned to carcinomas in situ. Adenomas with dysplasia (categories 3 and 4.1) or with carcinoma (other categories, such as 4.2) were analyzed separately.

All patients were monitored with a regular follow-up until December 31, 2019. Follow-up data were recorded by clinicians to determine if patients had normal findings on follow-up colonoscopy, recurrence of adenoma, or occurrence of CRC.

DNA and RNA isolation and quality control. Total DNA was isolated using AllPrep DNA/RNA Isolation kit according to the manufacturer's protocol (Qiagen, Germany). Quantity and purity of DNA was measured using Nanodrop. OD260/280 ratios of all samples ranged between 1.8 and 2.0. After the isolation, DNA was stored at -80 °C.

Bisulfite modification. 200 ng of DNA from each sample were treated with sodium bisulfite using the “EpiTect Bisulfite Kit” (Qiagen, Germany) according to the manufacturer's protocol.

MS- HRM. For the MS-HRM of the APC gene, methylation independent primers, based on Migheli et al33, were employed. Primer sequences for MLH1 gene were described earlier46. All analyses were run according to the following conditions: 1 cycle of 95 °C for 12 min, 60 cycles of 95 °C for 30 s, Ta for 30 s and 72 °C for 15 s; followed by an HRM step of 95 °C for 10 s and 50 °C for 1 min, 65 °C for 15 s, and continuous acquisition to 95 °C at one acquisition per 0.2 °C. PCR was performed in a final volume of 25 µl, containing 12.5 µl of master mix (Qiagen), 10 pmol of each primer and 1 µl (almost 10 ng) of bisulfite-modified DNA template. Each reaction was performed in triplicate. We analyzed 10% of the samples independently on separate occasions to verify the inter-assay variability and observed a good reproducibility.

Fully methylated and unmethylated DNA (EpiTectH methylated and unmethylated human control DNA, bisulfite converted, Qiagen, Germany) were mixed to obtain the following ratios of methylation: 0%, 12.5%, 25%, 50%, 75%, 100%. Standard curves with known methylation ratios were included in each assay and were used to deduce the methylation ratio of each tumor and reference sample.

MSI Status. MSI status was determined by molecular testing of five mononucleotide repeat markers (Bethesda consensus panel, BAT-25, BAT-26, NR-21, NR-24, and NR-27) that were run as a pentaplex, using fluorescently labeled primers and standard PCR as described in Kroupa et al.35. Fragment analysis was performed on ABI 3130 (Applied Biosystems). A comparison between the adenoma or in situ carcinoma and adjacent mucosa DNA short tandem repetition profiles were analyzed with GeneMapper v4.1 software (Applied Biosystems). When one or more markers were unstable, the sample was interpreted as MSI, all other samples were classified as microsatellite stable (MSS). Further, when one unstable marker was presented, the sample was indicated as MSI-Low (MSI-L), in the case that 2 or more markers were instable, the sample was marked as MSI-High (MSI-H).

Mutation analysis. DNA concentrations were measured prior to amplification, using the Qubit® dsDNA HS assay (Life Technologies) and diluted to a concentration of 3 ng/µl.

Mutations were determined using a custom multiplex PCR sequencing panel consisted of M13-tailed primer pairs, as described previously46. The custom primers cover mutational hotspots in the genes BRAF, EGFR, KRAS, NRAS, PIK3CA and SMAD4, the exonuclease domain of POLE and POLED1 and the entire coding sequence of APC, PTEN and TP53 (primer sequences available upon request).

PCR was performed with FastStart HiFi Enzyme Blend (Sigma-Aldrich, St. Louis, MO, USA) in two PCR pools with non-overlapping M13-tailed primers. PCR products per sample were combined and purified with
Statistical analysis. The occurrence of somatic mutations (independently) between groups was assessed by the Fisher’s exact test. Similarly, their possible associations with the localization, gender, or histology type and mutually with each other were determined by the Fisher’s exact test. Associations of mutations with age, Vienna classification and APC methylation were analyzed using the Mann–Whitney U test. The statistical analysis was performed using STATISTICA (version 11Cz; TIBCO Software Inc., Palo Alto, CA, USA), Matlab (version 2019b; The MathWorks, Inc., Natick, MA, USA), SISA (https://www.quantitativestats.com/sisa/statistics/fiveby2.htm) and jVenn (https://jvenn.toulouse.inra.fr/app/index.html). Confidence intervals of mutation frequencies were calculated according to Agresti and Coull. All reported p-values are two-tailed and the level of statistical significance was set at α = 0.05.

References
1. Siegel, R. L. et al. Cancer statistics, 2021. CA Cancer J. Clin. 71(1), 7–33 (2021).
2. Grady, W. M. & Markowitz, S. D. The molecular pathogenesis of colorectal cancer and its potential application to colorectal cancer screening. Dig. Dis. Sci. 60(3), 762–772 (2015).
3. Carethers, J. M. & Jung, B. H. Genetics and genetic biomarkers in sporadic colorectal cancer. Gastroenterology 149(5), 1177-1190 e3 (2015).
4. Fearon, E. R. & Vogelstein, B. A genetic model for colorectal tumorigenesis. Cell 61(5), 759–767 (1990).
5. Siskova, A. et al. Colorectal adenomas-genetics and searching for new molecular screening biomarkers. Int. J. Mol. Sci. 21(9), 3260. https://doi.org/10.3390/ijms21093260 (2020).
6. Beggs, A. D. et al. A study of genomic instability in early preneoplastic colonic lesions. Oncogene 32(46), 5333–5337 (2013).
7. Voorham, Q. J. et al. Comprehensive mutational analysis in colorectal flat adenomas. PLoS One 7(7), e41963 (2012).
8. Wolff, R. K. et al. Mutation analysis of adenomas and carcinomas of the colon: Early and late drivers. Genes Chromosom. Cancer 57(7), 366–376 (2018).
9. Liang, T. J. et al. APC hypermethylation for early diagnosis of colorectal cancer: A meta-analysis and literature review. Oncotarget 8(28), 46468–46479 (2017).
10. Tate, J. G. et al. COSMIC: The catalogue of somatic mutations in cancer. Nucleic Acids Res. 47(D1), D941–D947 (2019).
11. Fokkema, I. F. et al. LOVD v2.0: The next generation in gene variant databases. Hum. Mutat. 32(5), 557–63 (2011).
12. Stenson, P. D. et al. Human gene mutation database (HGMD): 2003 update. Hum. Mutat. 21(6), 577–581 (2003).
13. Loeve, F. et al. National polyph study data: evidence for regression of adenomas. Int. J. Cancer 111(4), 633–639 (2004).
14. Cross, W. et al. The evolutionary landscape of colorectal tumorigenesis. Nat. Ecol. Evol. 2(10), 1661–1672 (2018).
15. Muller, M. F., Ibrahim, A. E. & Arends, M. J. Molecular pathological classification of colorectal cancer. Virchows Arch. 469(2), 125–134 (2016).
16. Lee-Six, H. et al. The landscape of somatic mutation in normal colorectal epithelial cells. Nature 574(7779), 532–537 (2019).
17. Lewandowska, M. A., Jozwicki, W. & Zurawski, B. KRAS and BRAF mutation analysis in colorectal adenocarcinoma specimens with a low percentage of tumor cells. Mol. Diagn. Ther. 17(3), 193–203 (2013).
18. Wang, J. Y. et al. Molecular mechanisms underlying the tumorigenesis of colorectal adenomas: Correlation to activated K-ras oncogene. Oncol. Rep. 16(6), 1245–1252 (2006).
19. Zauber, A., Markowitz, S. D. & McFadden, D. M. KRAS gene mutations are more common in colorectal villous adenomas and in situ carcinomas than in carcinomas. Int. J. Epidemiol. Genet. 4(1), 1–10 (2013).
20. Rayner, E. et al. Polymerase proofreading domain mutations in cancer. Nat. Rev. Cancer 16(2), 71–81 (2016).
21. Palles, C. et al. Germline mutations affecting the proofreading domains of POLE and POLD1 predispose to adenomatous polyps and carcinomas. Nat. Genet. 45(2), 136–144 (2013).
22. Boot, A. et al. Characterization of colibactin-associated mutational signature in an Asian oral squamous cell carcinoma and in other mucosal tumor types. Genomi Res. 30(6), 803–813 (2020).
23. Augert, A. et al. Small cell lung cancer exhibits frequent inactivating mutations in the histone methyltransferase KMT2D/MLL2: CALGB 151111 (Alliance). J. Thorac. Oncol. 12(4), 704–713 (2017).
24. Poulos, R. C., Olivier, J. & Wong, J. W. The interaction between cytosine methylation and processes of DNA replication and repair shape the mutational landscape of cancer genomes. Nucleic Acids Res. 45(13), 7786–7795 (2017).
25. Chan, T. L. et al. BRAF and KRAS mutations in colorectal hyperplastic polyps and serrated adenomas. Cancer Res. 63(16), 4878–4881 (2003).
26. Yuen, S. T. et al. Similarity of the phenotypic patterns associated with BRAF and KRAS mutations in colorectal neoplasia. Cancer Res. 62(22), 6451–6455 (2002).
27. Velloso, S. et al. The prevalence of PIK3CA mutations in gastric and colon cancer. Eur. J. Cancer 41(11), 1649–1654 (2005).
28. Marcuillo, M. et al. Circulating biomarkers for early detection and clinical management of colorectal cancer. Mol. Aspects Med. 69, 107–122 (2019).
29. Cervena, K. et al. Mutational landscape of plasma cell-free DNA identifies molecular features associated with therapeutic response in patients with colon cancer. A pilot study. Mutagenesis 36(5), 358–368 (2021).
30. Cohen, J. D. et al. Detection and localization of surgically resectable cancers with a multi-analyte blood test. Science 359(6378), 926–930 (2018).
31. Marzouli, O. et al. Updates on clinical use of liquid biopsy in colorectal cancer screening, diagnosis, follow-up, and treatment guidance. Front Cell Dev Biol 9, 660924 (2021).
32. Schlemper, R. J. et al. The Vienna classification of gastrointestinal epithelial neoplasia. Gut 47(2), 251–255 (2000).
33. Migheli, F. et al. Comparison study of MS-HRM and pyrosequencing techniques for quantification of APC and CDKN2A gene methylation. PLoS One 8(1), e52501 (2013).
34. Vymetalkova, V. P. et al. Molecular characteristics of mismatch repair genes in sporadic colorectal tumors in Czech patients. BMC Med. Genet. 15, 17 (2014).
35. Kroupa, M. et al. Relationship of telomere length in colorectal cancer patients with cancer phenotype and patient prognosis. Br. J. Cancer 121(4), 344–350 (2019).
36. Schubert, S. A. et al. Evidence for genetic association between chromosome 1q loci and predisposition to colorectal neoplasia. Br. J. Cancer 117(6), 1215–1223 (2017).
37. Bardou, P. et al. JVENN: An interactive Venn diagram viewer. BMC Bioinform. 15, 293 (2014).
38. Agresti, A. & Coull, B. A. Approximate is Better than “exact” for interval estimation of binomial proportions. Am. Stat. 52, 119–126 (1998).

Author contributions:
Conceptualization, T.W., L.V., P.V., and V.V.; methodology, M.U., A.B., A.S., J.S., T.W., V.V.; formal analysis, M.U., P.H., A.B.; sample resources, J.J., M.K., D.T., S.S., Z.B., T.B., P.K., T.H., R.M.; data curation, T.W., P.V., V.V.; writing—original draft preparation, V.V., J.J.; writing—review and editing, J.J., M.U., A.B., P.H., A.S., J.S., M.K., D.T., S.S., Z.B., T.B., P.K., T.H., R.M., L.V., T.W., P.V., V.V.; supervision, T.W., P.V.; project administration, L.V.; funding acquisition, T.W., P.V., V.V., P.K., T.H.. All authors have read and agreed to the published version of the manuscript. We thank Ludovit Bielik for his excellent technical support.

Funding
This project was supported by the Czech Health Research council of the Ministry of Health of the Czech Republic (AZV NV18-03-00199), the Grant Agency of the Czech Republic (GACR 18-09709S). We are thankful to Charles University Research Centre program UNCE/MED/006 “University Centre of Clinical and Experimental Liver Surgery” and by project No. CZ.02.1.01/0.0/0.0/16_019/0000787 „Fighting INfectious Diseases “, awarded by the MEYS CR „, financed from EFRR. This publication is part of a project that has received funding from the European Union’s Horizon 2020 research and innovation program under grant agreement Nº856620. This article is based upon work from COST Action CA17118 (www.transcoloncan.eu), supported by COST (European Cooperation in Science and Technology). www.cost.eu.

Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to V.V.

Reprints and permissions information is available at www.nature.com/reprints.

Publisher’s note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons licence, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons licence, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons licence and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this licence, visit http://creativecommons.org/licenses/by/4.0/.

© The Author(s) 2022, corrected publication 2022