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***Basic Study***

**Gene mutations in stool from gastric and colorectal neoplasia patients by next generation sequencing**

Youssef O *et al*. Gene mutations in stool

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**Abstract**

***AIM***

To study cancer hotspot mutations by next generation sequencing (NGS) in stool DNA from patients with different gastrointestinal tract (GIT) neoplasms.

***METHODS***

Stool samples were collected from 87 Finnish patients diagnosed with various gastric and colorectal neoplasms, including benign tumors and from 14 healthy controls. DNA was isolated from stools the PSP® Spin Stool DNA Plus Kit. For each sample, 20 ng of DNA was used to construct sequencing libraries using Ion AmpliSeq Cancer Hotspot Panel v2 or Ion Ampliseq Colon and Lung Cancer panel v2. Sequencing was performed on Ion PGM. Torrent Suite Software v.5.2.2 was used for variant calling and data analysis.

***RESULTS***

NGS was successful in assaying 72 GIT samples and 13 healthy controls, a success rate of the assay being 78% for stomach neoplasia and 87% for colorectal tumors. In stool specimens from patients with gastric neoplasia, five hotspot mutations were found in *APC, CDKN2A,* and *EGFR* genes, in addition to seven novel mutations. From colorectal patients, 20 mutations were detected in *AKT1, APC, ERBB2, FBXW7, KIT, KRAS, NRAS, SMARCB1, SMO, STK11,* and *TP53.* Healthy controls did not exhibit any hotspot mutations except for two novel ones. *APC* and *TP53* were the most frequently mutated genes in colorectal neoplasms with five mutations, followed by *KRAS* with two mutations. *APC* was the most commonly mutated gene in stools of patients with premalignant/benign gastric lesions.

***CONCLUSION***

Our results show that in addition to colorectal neoplasms, mutations can also be assayed from stool specimens of patients with gastric neoplasms.

**Key words:** Stool DNA; next generation sequencing; Mutations; Gastric neoplasia; Colorectal neoplasia

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**Core tip:** Next generation sequencing (NGS) was successfully applied for detecting cancer gene mutations in stool DNA of patients with different gastrointestinal neoplasms. Using a gene panel, comprising of up to 50 cancer genes, it was found that mutations not only could be detected in stool DNA from colorectal cancer patients, but also in patients with stomach cancer and those with benign or premalignant lesions. No hotspot mutations were detected in healthy controls. Our results show that NGS could be useful in screening for neoplastic changes of the gastrointestinal tract.

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**INTRODUCTION**

Gastrointestinal tract (GIT) malignancies are diverse group of neoplasms with diverse epidemiology and incidences that affect different regions of GIT from the stomach to the large intestine. The value of somatic mutations in GIT malignancies is recognized; (1) as markers for early detection; (2) as markers that predict drug resistance, and 3) for follow up of cancer treatment[1-6]. In global terms, gastric carcinoma is the fifth most common cancer and the third most common cause of cancer related mortality. Adenocarcinoma is its most typical subtype and present in 90% of the all cases[7]. *TP53, PIK3CA, ARID1A* and cell adhesion pathway genes have been found to be most frequently mutated genes in gastric adenocarcinomas[8]. *CDH1* gene is described to be involved in the pathogenesis of diffuse gastric carcinoma[9]. In sporadic colorectal cancer *APC, TP53, KRAS, PI3CA, FBXW7, SMAD4* and *BRAF* are the most commonly mutated genes[10].

One of the major issues in GIT malignancies, specifically gastric carcinoma, is that they are usually detected at an advanced stage due to late diagnosis[11]. Moreover, recent studies have demonstrated the diversity of morphological (intestinal and diffuse subtypes) and molecular subtypes (mesenchymal-like type, microsatellite-unstable tumors type, and *TP53* tumor types) of gastric carcinoma, which contributes to the challenge of optimizing proper diagnosis and treatment[12]. The principal problem hindering early detection of gastric and colorectal neoplasia is the lack of symptoms or even when symptoms are present, they tend to be mild and non-specific which may delay subjecting the patient for endoscopic examination. Exfoliation of cells, whether premalignant or malignant, is continuously occurring from epithelial layer into the digestive lumen[13,14]; these display various genetic changes that have occurred in these cells, and can provide evidence of tumor pathogenesis[15]. Testing DNA abnormalities in stool specimens from GIT carcinoma patients represent a promising non-invasive approach for early cancer detection and for treatment follow-up. Multi-target stool DNA test (MSTD is currently being used for colorectal cancer screening[16].

We have previously shown that next generation sequencing (NGS) can be successfully applied for investigating mutations in stool DNA obtained from patients with colorectal cancer[13]. In the current study, we applied the NGS method to determine whether cancer mutations could also be detected in stool samples from patients with other GIT tumors including both diffuse and intestinal subtypes of gastric adenocarcinoma, benign gastric dysplasia, colorectal adenocarcinoma and adenoma, and colorectal leiomyoma.

**MATERIALS AND METHODS**

***Patients***

During the period from April 2015 to May 2017, 79 gastric cancer, 38 gastrointestinal stromal tumor (GIST), 669 colon cancer, and 271 primary rectal cancer patients were referred to three hospitals; Kirurgi, Meilahti and Jorvi in Finland. Three authors Kokkola A, Carpelan-Holmström M, Koskensalo S, collected stool samples from patients who were referred to them for surgery. Stool specimens were collected from 87 patients with stomach or colorectal neoplasia: 41 stomach neoplasia (18 intestinal type, 20 diffuse type, one neuroendocrine tumor, one benign gastric dysplasia and one hamartoma), 46 colorectal lesions (40 adenocarcinoma, four adenoma, one benign dysplasia and one colorectal leiomyoma), as well as from 14 healthy individuals (Table 1). A total number of 21 patients had received treatment before the time of sampling (10 patients with stomach neoplasia and 11 with colorectal lesions). Treatments were in the form of chemotherapy (17 patients), radiotherapy (one patient), or antibiotics for *Helicobacter pylori* infection (three patients).

Patients were diagnosed and treated in Meilahti Hospital in Helsinki. The study was approved by the Hospital District of Helsinki and Uusimaa (HUS) review board (Ethical permission number 351/13/03/02/2014). Written informed consent was obtained from all subjects.

***Stool Specimens***

Stool specimens were collected in special collection tubes provided in the extraction kit (PSP® Spin Stool DNA Plus Kit) (Stratec Biomedical, Berlin, Germany). These tubes are prefilled with 8 ml of stool DNA stabilizer to allow collection, transport, and storage of the samples without DNA degradation. One spoon of stool specimen (spoon provided with the collection tubes) was transferred to the tube and mixed thoroughly to obtain a stool homogenate. The samples were stored at -20oC until analysis for an average of seven days before extraction.

***DNA extraction***

Before starting the DNA isolation, each stool specimen tube was vortexed vigorously to ensure proper mixing of the contents with the stabilizer liquid provided in each collection tube. A volume of 1.4 ml of the stabilized stool specimens was transferred to 2 ml tubes. Then, DNA was extracted from each stool specimen using the PSP® Spin Stool DNA Plus Kit (Stratec Biomedical, Berlin, Germany) according to the manufacturer’s instructions. Extracted DNA was eluted in 50 µl of elution buffer, and then DNA was quantified by a Qubit® 2.0 Fluorometer (Life Technologies, California, United States) using the Qubit® dsDNA BR Assay kit. The extracted DNA was stored at -20 oC.

***NGS***

**Library preparation:** Twenty nanograms of stool DNA was used for preparing amplicon libraries using Ion AmpliSeq™ Library kit 2.0 (Life Technologies, California, United States) according to the manufacturer’s guidelines. Gene panels comprising of pool of primer mixes were used to amplify templates. The gene panels used were one the following:(1) Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies, California, United States) consisting of primer pool for 207 amplicons from an average of 2800 mutational hotspot regions in 50 genes, including KIT and PDGFRA mutations. The genes included in the panel are *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, JAK2, JAK3, IDH2, KDR, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NMP1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, and VHL.*(2) Ion Ampliseq Colon and Lung Cancer panel v2 (Life Technologies, California, United States), consisting of primer pool for 92 amplicons from 504 hotspot regions in 22 genes frequently mutated in colorectal cancer (CRC). The genes included in this panel are *AKT1, ALK*, *BRAF, CTNNB1*, *DDR2*, *EGFR*, *ERBB2*, *ERBB4*, *FBX7*, *FGFR1*, *FGFR2, FGFR3*, *KRAS*, *MAP2K1*, *MET*, *NOTCH1*, *NRAS*, *PIK3CA, PTEN*, *SMAD4, STK11*, and *TP53*.

All specimens from patients with gastric neoplasms and 19 specimens from patients with colorectal neoplasia were assayed using Ion AmpliSeq Cancer Hotspot Panel v2, while the remaining of specimens from colorectal neoplasia patients were studied Ion Ampliseq Colon and Lung Cancer panel v2. The major reason for using two different platforms was that Ion AmpliSeq Cancer Hotspot Panel v2 contains several genes that are commonly mutated in gastric neoplasia.

The amplified libraries were purified using Agencourt AMPure XP beads (Beckman Coulter Genomics, High Wycombe, United Kingdom). The concentration of the purified libraries was measured on the Qubit® 2.0 Fluorometer, using the Qubit® dsDNA HS Assay kit. The DNA libraries were stored at -20 oC till further use.

**Template preparation and sequencing:** The amplified and purified libraries were diluted to 100 pmol/L, and the templates were prepared and enriched using the Ion OneTouch™ 2 System (Life Technologies, Ca, United States), an automated emulsion PCR system. Finally, sequencing was carried out on the Ion Personal Genome Machine System (PGM™, Life Technologies, Ca, United States) using Ion 316™ chips and the Ion PGM™ Sequencing Hi-Q kit v2.

***Data analysis***

The Torrent Suite Software v.5.2.2 (Life Technologies) was used to assess run performance and data analysis, and Integrative Genomics Viewer (IGV v 2.2, Broad Institute) was used for visual inspection of the aligned reads.

Variants were further filtered based on Quality score (score of 15 or more) and mutant allele frequency (more than 3%). Only SNVs resulting in a non-synonymous amino acid change, or a premature stop codon, and all short indels resulting in either a frameshift or insertion/deletion of amino acids were selected. All SNVs were analyzed for previously reported hotspot mutations (somatic mutations reported in COSMIC database) and novel variations, i.e. new mutations detected by NGS but not reported in either COSMIC or dbSNP (build 150) databases.

**RESULTS**

***Success rate***

Successful DNA extraction was performed on 77/87 patient stool samples, while NGS assay was successfully carried out on 72 patient stool DNA samples. Five samples were removed from NGS assay due to poor DNA quality (too little or degraded DNA). Of the 14 controls, DNA could be isolated from 13 samples and all were successfully sequenced. The success rates of sequencing stool samples for stomach and colorectal neoplasia were rather similar (78% and 87% respectively) (Table 1).

***Hotspot (Cosmic) and novel mutations***

In patients’ stool samples, a total of 25 hotspot mutations were found (20 in patients with colorectal neoplasia and five in patents with stomach neoplasia), while nine novel mutations were detected (seven in patients with stomach neoplasia and two in control samples).

Thirteen control samples from healthy individuals did not reveal any hotspot mutations, but two novel mutations were observed in *ALK,* and *STK11* genes in two subjects.

***Mutations in patients with stomach neoplasms***

A total number of five hotspot mutations that have been reported earlier in COSMIC database were detected in *APC, CDKN2A,* and *EGFR* genes in stool specimens from three gastric adenocarcinoma patients, one neuroendocrine tumor, and one patient with gastric dysplasia (Table 2). Four samples from patients with adenocarcinoma (diffuse type) revealed a total of seven novel mutations that led to an amino acid change and which had not been reported previously in either the COSMIC or dbSNP databases. The detected novel mutations were found in seven genes that included *APC, CDH1, DDR2, HRAS, NRAS, PTEN,* and *SMARCB1* (Table 2)*.*

***Mutations in patients with colorectal tumors***

Twenty hotspot mutations in *AKT1, APC, ERBB2, FBXW7, KIT, KRAS, NRAS, SMARCB1, SMO, STK11,* and *TP53* were seen in nine patients with adenocarcinomas, two with benign adenoma, and one with leiomyoma. *APC* was the most frequently mutated gene with five mutations, followed by *TP53* (five mutations), and *KRAS* (2 mutations) (Table 2). One case of benign leiomyoma revealed a *TP53* mutation (R306\*) mutation, which has been reported as a germline mutation associated with the hereditary cancer predisposing syndrome. Additionally, this mutation has also been reported as a somatic pathogenic mutation in COSMIC in tumors of the colon and other parts of the digestive tract (Table 2)*.* No novel mutation were found in our study in stool samples from colorectal cases.

**DISCUSSION**

We are one of the first groups that applied NGS to detect mutations in DNA isolated from stool samples of colorectal carcinoma patients[13]. We have now applied NGS analysis on stool samples of not only malignant colorectal carcinoma, but also demonstrated that it is possible to detect mutations in stool specimens from patients with gastric neoplasms, and also from patients with benign colorectal tumors. Moreover, we observed mutations in stool from patients with early tumor stages, with no hotspot mutations in stools of healthy subjects. As in our previous study, we set the threshold for variant quality score at 15 and the mutant allele frequency cutoff at 3% and when using these threshold values, no cosmic hotspot mutations were found in the 13 control specimens.

***Mutations in patients with colorectal neoplasms***

The overall success rate of NGS for colorectal neoplasm patients was 87% which is similar to the 80% reported in our earlier study[13]. In our previous study, the patients were of Iranian origin, whereas in the current study, the patients were from Finland; nonetheless the mutation rate and the types were similar in both of these ethnic groups. In the current series, the most common mutated genes were *APC, TP53* and *KRAS* while in the earlier study, the top mutated genes were *TP53, KRAS, FBXW7, EGFR and SMAD4.*

The most recurrently occurring mutation in colorectal carcinoma cases was *APC* mutation (A1582P) that was found in three patients. In our series, four *TP53* mutations were seen in stool samples from colorectal cancer patients.

We detected *KRAS* codon 12 mutations (G12V, and G12D) in two specimens and *NRAS* codon 61 (Q61R) in one specimen from colorectal carcinoma patients. Similar to our present results, codon 12 mutations were the most common *KRAS* mutations found in our previous study on Iranian samples, although other *KRAS* mutations at codons 12, 13, 20, 63, 117, 146, and 43 were also found previously[13]. Additionally, recent study demonstrated the detection of *KRAS* G12D mutation in stool samples from patients with colorectal carcinoma by using droplet PCR[17]. Clinical data available from those patients where *KRAS* testing in tumor tissue was carried out correlated to *KRAS* mutation status in stool. In patient number 55, the presence of *KRAS* G12V mutation was confirmed in tumor tissue specimens with 20% mutant allele fraction. The same mutation (*KRAS* G12V) was detected in the stool DNA from the same patient with 13% mutant allele fraction. Moreover, tissue samples from patient number 23 revealed no *KRAS* or *NRAS* mutations, and the same negative findings for those two mutations were also observed in stool DNA specimen from this case.

Among the patients with benign colorectal tumors, *APC* mutations were most common and found in two samples with colorectal benign adenoma. Adenomas with *APC* mutations have been reported more likely to progress into large adenomas and invasive carcinomas[18,19]. Inactivation of the *APC* gene and the subsequent activation of Wnt signaling pathway fare key factors in the initiation of tumorigenesis of colorectal cancer[20,21]. The R505C mutation in *FBXW7* seen in a colorectal adenoma patient in present study was also reported in our previous study in a colorectal carcinoma patient[13].

*TP53* is another gene commonly mutated in colorectal cancer, and plays a crucial role in the adenoma to carcinoma transition during carcinogenesis, and may have an impact on cancer prognosis[22]. A patient with leiomyoma revealed a nonsense *TP53* mutation (R306\*) which has been reported as a somatic mutation in colon tumors and also considered as a germline mutation associated with hereditary cancer-predisposing syndrome, and Li-Fraumeni syndrome in colorectal cancer although not in gastric carcinoma[23]. In our case this *TP53* mutation is apparently somatic as the allele fraction was 5.5%. A meta-analysis of studies carried out on stool DNA testing has shown an overall sensitivity of 68% and 93% specificity in the diagnosis of advanced colorectal adenoma[24].

***Mutations in patients with stomach neoplasms***

As far as we aware, this is the first study to have utilized stool samples from patients with stomach neoplasia. Eight out of 32 patients’ samples (25%) with stomach neoplasia revealed 12 mutations (both hotspot cosmic and novel).

In gastric neoplasia, the *APC* gene mutations were those most frequently encountered. Four *APC* mutations were detected in patients with gastric neoplasia (three mutations in gastric carcinoma, and one in benign gastric dysplasia). *APC* is a tumor suppressor gene that has a key role in several molecular processes such as suppression of canonical Wnt signaling[25],and the presence of *APC* mutations have been demonstrated in gastric adenocarcinoma samples[26,27]. *APC* gene mutations have been reported in both intestinal and diffuse types of gastric carcinoma with a higher frequency in the intestinal subtype of the disease[28,29]. The adenoma to carcinoma transition pathway has a 20% *APC* mutation in the intestinal type of gastric carcinoma[30]. In our study, an *APC* A1582P mutation was seen in both the intestinal and diffuse types, and an *APC* D1570N mutation was seen in the diffuse type. Furthermore, we detected the same *APC* mutation (A1582P) in a stool specimen from a patient with benign gastric dysplasia. This is in concordance with an earlier study that identified the presence of *APC* mutations in tumorous tissue in cases with gastric adenomas or flat dysplasia, and also in benign cases associated with adenocarcinoma[5,31].

In diffuse type of gastric carcinoma *CDH1* is reported to be commonly mutated[32] and *CDH1* germline mutations have also been reported to play an important role in diffuse gastric carcinoma development[33]. *EGFR* mutations are also commonly encountered in the diffuse subtype[34-36] of gastric neoplasia, although their role is still controversial. We found E-cadherin gene (*CDH1*) V82A mutation in diffuse gastric carcinoma patient, and also found exon 19 *EGFR* (A750T) mutation in another case with diffuse gastric carcinoma.

Novel mutations were found in *NRAS* (K135R), *DDR2* (E523K) and in exon 7 of *PTEN* (E256). Codon 12 or 13 *NRAS* mutations in tumor tissues have been reported to be associated with a poor prognosis in metastatic stomach carcinoma[37,38], whereas *DDR2* expression in gastric tumor tissues has been described to be associated with an increased risk of peritoneal dissemination[39]. Despite the low frequency of *PTEN* mutations in gastric malignant tumors, they tend to be associated with poorly differentiated gastric carcinoma, TNM staging and resistance effect to chemotherapy[40,41]. Interestingly, the novel *PTEN* (E256G) mutation seen in our study was found in a gastric cancer case with an advanced tumor stage (T4bNxM1).

In conclusion, our results demonstrate that NGS technology can be applied for detection of gene mutations in stool specimens from not only colorectal cancer patients but also from patients with stomach neoplasms, as well as those with benign tumors of the gastrointestinal tract.

**Article Highlights**

***Research background***

Stool DNA sample is a simple, non-invasive source for studying genetic markers of diagnostic/prognostic or predictive significance in colorectal cancer. The significance of stool DNA testing is however not well known for stomach cancers and for benign tumors. Current assays screen only individual or few mutations only that do not cover all important cancer mutations. Amplicon based NGS could thus provide a sensitive method for DNA testing from stool samples in GIT malignancies.

***Research motivation***

The main challenge in stool DNA based genetic testing is that only a small proportion of stool DNA is of human origin thus requiring a very sensitive test. We therefore hypothesized that diagnostic value of stool based DNA testing could be enhanced by applying sensitive amplicon –based NGS to stool DNA. With the application of NGS we could screen all important mutations in 50 genes from a small amount of input DNA in a single test.

***Research objectives***

The objective of the study was to apply NGS for screening hotspot mutations in commonly mutated genes in gastrointestinal malignancies from stool DNA. The aim was also to see if mutations could be detected in patients with gastric cancer and in patients with early neoplasms in addition to those with colorectal cancer.

***Research methods***

Mutation detection was by performed by amplicon based next generation sequencing using Ion AmpliSeq Cancer Hotspot Panel v2 and Ion Ampliseq Colon and Lung Cancer panel v2. Template preparation was done using the Ion OneTouch™ 2 System and sequencing performed on Ion PGM (Thermofisher Scientific).

Sequencing data analysis and variant calling was performed by using the Torrent Suite Software v.5.2.2 with variant caller plugin. All SNVs were analyzed for previously reported hotspot mutations (reported in COSMIC database) and novel variations, i.e not reported in either COSMIC or dbSNP.

***Research results***

Hotspot mutations in stool DNA was found in *APC, CDKN2A, EGFR* in patients with stomach neoplasms and in *AKT1, APC, ERBB2, FBXW7, KIT, KRAS, NRAS, SMARCB1, SMO, STK11, TP53* in patients with colorectal neoplasia. *APC* was the most commonly mutated gene in stools of patients with premalignant/benign GIT lesions.

***Research conclusions***

This study demonstrates that NGS based mutation screening can be successfully applied to stool DNA from patients with GIT neoplasms. In addition to mutation detection in stool DNA from colorectal cancer patients, mutations can also be detected from gastric cancer patients, as well as from patients with premalignant or benign neoplasms.

Mutation testing from stool DNA is mainly carried out for individual gene mutations by PCR based methods for colorectal cancer screening. Since the amount of DNA of human origin is very low in stool, it was hypothesized that amplicon-based NGS could be highly sensitive and suitable for studying large number of mutations that could greatly enhance the diagnostic value of stool DNA testing. The methods used in this study requires low input of DNA , can amplify around 200 targeted regions of important cancer genes, and together with high sensitivity of NGS, provides a great advantage over prevailing methods for mutation detection from stool DNA.

This study showed that mutations can also be detected in stool DNA from patients with stomach neoplasms. Detection of mutations in stool DNA of patients with premalignant neoplasm and also in patients with stage I and II of tumor, demonstrates its application for early detection of GIT neoplasms.

The results of this study could have implication in future NGS based stool DNA diagnostic tests that could be useful for screening of GIT malignancies and for detection at the premalignant stage. It could also act as a guide in targeted therapy regimen, and in easier follow-up of the treatment.

***Research perspectives***

Genetic mutations can be detected by amplicon–based NGS in stool DNA from patients with GIT tumors other than colorectal cancer also. Moreover, early neoplastic changes in gastrointestinal tract can also be detected in stool DNA. These results open up possibilities of development of NGS based stool DNA test. Further testing of this method on a larger number of samples from patients with different GIT malignancies, premalignant lesions and healthy individuals is needed to fully assess it applicability in cancer diagnostics.

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**REFERENCES**

1 **De Roock W**, Claes B, Bernasconi D, De Schutter J, Biesmans B, Fountzilas G, Kalogeras KT, Kotoula V, Papamichael D, Laurent-Puig P, Penault-Llorca F, Rougier P, Vincenzi B, Santini D, Tonini G, Cappuzzo F, Frattini M, Molinari F, Saletti P, De Dosso S, Martini M, Bardelli A, Siena S, Sartore-Bianchi A, Tabernero J, Macarulla T, Di Fiore F, Gangloff AO, Ciardiello F, Pfeiffer P, Qvortrup C, Hansen TP, Van Cutsem E, Piessevaux H, Lambrechts D, Delorenzi M, Tejpar S. Effects of KRAS, BRAF, NRAS, and PIK3CA mutations on the efficacy of cetuximab plus chemotherapy in chemotherapy-refractory metastatic colorectal cancer: a retrospective consortium analysis. *Lancet Oncol* 2010; **11**: 753-762 [PMID: 20619739 DOI: 10.1016/S1470-2045(10)70130-3]

2 **Miranda C**, Nucifora M, Molinari F, Conca E, Anania MC, Bordoni A, Saletti P, Mazzucchelli L, Pilotti S, Pierotti MA, Tamborini E, Greco A, Frattini M. KRAS and BRAF mutations predict primary resistance to imatinib in gastrointestinal stromal tumors. *Clin Cancer Res* 2012; **18**: 1769-1776 [PMID: 22282465 DOI: 10.1158/1078-0432.CCR-11-2230]

3 **Antonescu CR**, Romeo S, Zhang L, Nafa K, Hornick JL, Nielsen GP, Mino-Kenudson M, Huang HY, Mosquera JM, Dei Tos PA, Fletcher CD. Dedifferentiation in gastrointestinal stromal tumor to an anaplastic KIT-negative phenotype: a diagnostic pitfall: morphologic and molecular characterization of 8 cases occurring either de novo or after imatinib therapy. *Am J Surg Pathol* 2013; **37**: 385-392 [PMID: 23348204 DOI: 10.1097/PAS.0b013e31826c1761]

4 **Joensuu H**, Rutkowski P, Nishida T, Steigen SE, Brabec P, Plank L, Nilsson B, Braconi C, Bordoni A, Magnusson MK, Sufliarsky J, Federico M, Jonasson JG, Hostein I, Bringuier PP, Emile JF. KIT and PDGFRA mutations and the risk of GI stromal tumor recurrence. *J Clin Oncol* 2015; **33**: 634-642 [PMID: 25605837 DOI: 10.1200/JCO.2014.57.4970]

5 **Lee JH**, Abraham SC, Kim HS, Nam JH, Choi C, Lee MC, Park CS, Juhng SW, Rashid A, Hamilton SR, Wu TT. Inverse relationship between APC gene mutation in gastric adenomas and development of adenocarcinoma. *Am J Pathol* 2002; **161**: 611-618 [PMID: 12163385 DOI: 10.1016/S0002-9440(10)64216-2]

6 **Fenoglio-Preiser CM**, Wang J, Stemmermann GN, Noffsinger A. TP53 and gastric carcinoma: a review. *Hum Mutat* 2003; **21**: 258-270 [PMID: 12619111 DOI: 10.1002/humu.10180]

7 **Carneiro F**. World Cancer Report 2014. In: Stewart BW and Wild CP. Cancer by organ site: stomach cancer. Lyon, France, IARC, 2014: 383-391

8 **Zang ZJ**, Cutcutache I, Poon SL, Zhang SL, McPherson JR, Tao J, Rajasegaran V, Heng HL, Deng N, Gan A, Lim KH, Ong CK, Huang D, Chin SY, Tan IB, Ng CC, Yu W, Wu Y, Lee M, Wu J, Poh D, Wan WK, Rha SY, So J, Salto-Tellez M, Yeoh KG, Wong WK, Zhu YJ, Futreal PA, Pang B, Ruan Y, Hillmer AM, Bertrand D, Nagarajan N, Rozen S, Teh BT, Tan P. Exome sequencing of gastric adenocarcinoma identifies recurrent somatic mutations in cell adhesion and chromatin remodeling genes. *Nat Genet* 2012; **44**: 570-574 [PMID: 22484628 DOI: 10.1038/ng.2246]

9 **Guilford P**, Hopkins J, Harraway J, McLeod M, McLeod N, Harawira P, Taite H, Scoular R, Miller A, Reeve AE. E-cadherin germline mutations in familial gastric cancer. *Nature* 1998; **392**: 402-405 [PMID: 9537325 DOI: 10.1038/32918]

10 **Staudacher JJ**, Yazici C, Bul V, Zeidan J, Khalid A, Xia Y, Krett N, Jung B. Increased Frequency of KRAS Mutations in African Americans Compared with Caucasians in Sporadic Colorectal Cancer. *Clin Transl Gastroenterol* 2017; **8**: e124 [PMID: 29048416 DOI: 10.1038/ctg.2017.48]

11 **Gore RM**, Mehta UK, Berlin JW, Rao V, Newmark GM. Upper gastrointestinal tumours: diagnosis and staging. *Cancer Imaging* 2006; **6**: 213-217 [PMID: 17208679 DOI: 10.1102/1470-7330.2006.0032]

12 **Cristescu R**, Lee J, Nebozhyn M, Kim KM, Ting JC, Wong SS, Liu J, Yue YG, Wang J, Yu K, Ye XS, Do IG, Liu S, Gong L, Fu J, Jin JG, Choi MG, Sohn TS, Lee JH, Bae JM, Kim ST, Park SH, Sohn I, Jung SH, Tan P, Chen R, Hardwick J, Kang WK, Ayers M, Hongyue D, Reinhard C, Loboda A, Kim S, Aggarwal A. Molecular analysis of gastric cancer identifies subtypes associated with distinct clinical outcomes. *Nat Med* 2015; **21**: 449-456 [PMID: 25894828 DOI: 10.1038/nm.3850]

13 **Armengol G**, Sarhadi VK, Ghanbari R, Doghaei-Moghaddam M, Ansari R, Sotoudeh M, Puolakkainen P, Kokkola A, Malekzadeh R, Knuutila S. Driver Gene Mutations in Stools of Colorectal Carcinoma Patients Detected by Targeted Next-Generation Sequencing. *J Mol Diagn* 2016; **18**: 471-479 [PMID: 27155048 DOI: 10.1016/j.jmoldx.2016.01.008]

14 **Youssef O,** Sarhadi VK, Lehtimäki L, Tikkanen M, Kokkola A, Puolakkainen P, Armengol, G, Knuutila, S. Mutations by next generation sequencing in stool DNA from colorectal carcinoma patients – a literature review and our experience with this methodology. *J Anal Oncol* 2016; **5**: 24-32 [DOI: 10.6000/1927-7229.2016.05.01.3]

15 **Bosch LJ**, Carvalho B, Fijneman RJ, Jimenez CR, Pinedo HM, van Engeland M, Meijer GA. Molecular tests for colorectal cancer screening. *Clin Colorectal Cancer* 2011; **10**: 8-23 [PMID: 21609931 DOI: 10.3816/CCC.2011.n.002]

16 **Brenner H**, Chen H. Fecal occult blood versus DNA testing: indirect comparison in a colorectal cancer screening population. *Clin Epidemiol* 2017; **9**: 377-384 [PMID: 28761377 DOI: 10.2147/CLEP.S136565]

17 **Olmedillas-López S,** Lévano-Linares DC, Alexandre CLA, Vega-Clemente L, Sánchez EL, Villagrasa A, Ruíz-Tovar J, García-Arranz M, García-Olmo D. Detection of KRAS G12D in colorectal cancer stool by droplet digital PCR. *World J Gastroenterol* 2017; **23**: 7087-7097 [PMID: 29093617 DOI: 10.3748/wjg.v23.i39.7087]

18 **Samowitz WS**, Powers MD, Spirio LN, Nollet F, van Roy F, Slattery ML. Beta-catenin mutations are more frequent in small colorectal adenomas than in larger adenomas and invasive carcinomas. *Cancer Res* 1999; **59**: 1442-1444 [PMID: 10197610]

19 **Lamlum H**, Papadopoulou A, Ilyas M, Rowan A, Gillet C, Hanby A, Talbot I, Bodmer W, Tomlinson I. APC mutations are sufficient for the growth of early colorectal adenomas. *Proc Natl Acad Sci U S A* 2000; **97**: 2225-2228 [PMID: 10681434 DOI: 10.1073/pnas.040564697]

20 **Fodde R**. The APC gene in colorectal cancer. *Eur J Cancer* 2002; **38**: 867-871 [PMID: 11978510 DOI: 10.1016/S0959-8049(02)00040-0]

21 **Rowan AJ**, Lamlum H, Ilyas M, Wheeler J, Straub J, Papadopoulou A, Bicknell D, Bodmer WF, Tomlinson IP. APC mutations in sporadic colorectal tumors: A mutational "hotspot" and interdependence of the "two hits". *Proc Natl Acad Sci U S A* 2000; **97**: 3352-3357 [PMID: 10737795 DOI: 10.1073/pnas.97.7.3352]

22 **Li XL**, Zhou J, Chen ZR, Chng WJ. P53 mutations in colorectal cancer - molecular pathogenesis and pharmacological reactivation. *World J Gastroenterol* 2015; **21**: 84-93 [PMID: 25574081 DOI: 10.3748/wjg.v21.i1.84]

23 **Yurgelun MB**, Masciari S, Joshi VA, Mercado RC, Lindor NM, Gallinger S, Hopper JL, Jenkins MA, Buchanan DD, Newcomb PA, Potter JD, Haile RW, Kucherlapati R, Syngal S; Colon Cancer Family Registry. Germline TP53 Mutations in Patients With Early-Onset Colorectal Cancer in the Colon Cancer Family Registry. *JAMA Oncol* 2015; **1**: 214-221 [PMID: 26086041 DOI: 10.1001/jamaoncol.2015.0197]

24 **Yang H**, Xia BQ, Jiang B, Wang G, Yang YP, Chen H, Li BS, Xu AG, Huang YB, Wang XY. Diagnostic value of stool DNA testing for multiple markers of colorectal cancer and advanced adenoma: a meta-analysis. *Can J Gastroenterol* 2013; **27**: 467-475 [PMID: 23936877 DOI: 10.1155/2013/258030]

25 **Aoki K**, Taketo MM. Adenomatous polyposis coli (APC): a multi-functional tumor suppressor gene. *J Cell Sci* 2007; **120**: 3327-3335 [PMID: 17881494 DOI: 10.1242/jcs.03485]

26 **Nakatsuru S**, Yanagisawa A, Ichii S, Tahara E, Kato Y, Nakamura Y, Horii A. Somatic mutation of the APC gene in gastric cancer: frequent mutations in very well differentiated adenocarcinoma and signet-ring cell carcinoma. *Hum Mol Genet* 1992; **1**: 559-563 [PMID: 1338691 DOI: 10.1093/hmg/1.8.559]

27 **Horii A**, Nakatsuru S, Miyoshi Y, Ichii S, Nagase H, Kato Y, Yanagisawa A, Nakamura Y. The APC gene, responsible for familial adenomatous polyposis, is mutated in human gastric cancer. *Cancer Res* 1992; **52**: 3231-3233 [PMID: 1317264]

28 **Fang DC**, Luo YH, Yang SM, Li XA, Ling XL, Fang L. Mutation analysis of APC gene in gastric cancer with microsatellite instability. *World J Gastroenterol* 2002; **8**: 787-791 [PMID: 12378616 DOI: 10.3748/wjg.v8.i5.787]

29 **Ghatak S**, Chakraborty P, Sarkar SR, Chowdhury B, Bhaumik A, Kumar NS. Novel APC gene mutations associated with protein alteration in diffuse type gastric cancer. *BMC Med Genet* 2017; **18**: 61 [PMID: 28576136 DOI: 10.1186/s12881-017-0427-2]

30 **Tahara E**. Genetic pathways of two types of gastric cancer. *IARC Sci Publ* 2004; : 327-349 [PMID: 15055305]

31 **Tamura G**, Maesawa C, Suzuki Y, Tamada H, Satoh M, Ogasawara S, Kashiwaba M, Satodate R. Mutations of the APC gene occur during early stages of gastric adenoma development. *Cancer Res* 1994; **54**: 1149-1151 [PMID: 8118796]

32 **Lee YS**, Cho YS, Lee GK, Lee S, Kim YW, Jho S, Kim HM, Hong SH, Hwang JA, Kim SY, Hong D, Choi IJ, Kim BC, Kim BC, Kim CH, Choi H, Kim Y, Kim KW, Kong G, Kim HL, Bhak J, Lee SH, Lee JS. Genomic profile analysis of diffuse-type gastric cancers. *Genome Biol* 2014; **15**: R55 [PMID: 24690483 DOI: 10.1186/gb-2014-15-4-r55]

33 **Liu X**, Chu KM. E-cadherin and gastric cancer: cause, consequence, and applications. *Biomed Res Int* 2014; **2014**: 637308 [PMID: 25184143 DOI: 10.1155/2014/637308]

34 **Moutinho C**, Mateus AR, Milanezi F, Carneiro F, Seruca R, Suriano G. Epidermal growth factor receptor structural alterations in gastric cancer. *BMC Cancer* 2008; **8**: 10 [PMID: 18199332 DOI: 10.1186/1471-2407-8-10]

35 **Atmaca A**, Werner D, Pauligk C, Steinmetz K, Wirtz R, Altmannsberger HM, Jäger E, Al-Batran SE. The prognostic impact of epidermal growth factor receptor in patients with metastatic gastric cancer. *BMC Cancer* 2012; **12**: 524 [PMID: 23153332 DOI: 10.1186/1471-2407-12-524]

36 **Liu Z**, Liu L, Li M, Wang Z, Feng L, Zhang Q, Cheng S, Lu S. Epidermal growth factor receptor mutation in gastric cancer. *Pathology* 2011; **43**: 234-238 [PMID: 21436633 DOI: 10.1097/PAT.0b013e328344e61b]

37 **Takahashi N**, Yamada Y, Taniguchi H, Fukahori M, Sasaki Y, Shoji H, Honma Y, Iwasa S, Takashima A, Kato K, Hamaguchi T, Shimada Y. Clinicopathological features and prognostic roles of KRAS, BRAF, PIK3CA and NRAS mutations in advanced gastric cancer. *BMC Res Notes* 2014; **7**: 271 [PMID: 24774510 DOI: 10.1186/1756-0500-7-271]

38 **Fukahori M**. Analysis of gene mutations in KRAS, NRAS, BRAF, and PIK3CA in patients who received systemic chemotherapy with metastatic gastric cancer. *J Clin Oncol* 2013; **31**: 27 [DOI: 10.1200/jco.2013.31.4]

39 **Kurashige J**, Hasegawa T, Niida A, Sugimachi K, Deng N, Mima K, Uchi R, Sawada G, Takahashi Y, Eguchi H, Inomata M, Kitano S, Fukagawa T, Sasako M, Sasaki H, Sasaki S, Mori M, Yanagihara K, Baba H, Miyano S, Tan P, Mimori K. Integrated Molecular Profiling of Human Gastric Cancer Identifies DDR2 as a Potential Regulator of Peritoneal Dissemination. *Sci Rep* 2016; **6**: 22371 [PMID: 26934957 DOI: 10.1038/srep22371]

40 **Wen YG**, Wang Q, Zhou CZ, Qiu GQ, Peng ZH, Tang HM. Mutation analysis of tumor suppressor gene PTEN in patients with gastric carcinomas and its impact on PI3K/AKT pathway. *Oncol Rep* 2010; **24**: 89-95 [PMID: 20514448 DOI: org/10.3892/or\_00000832]

41 **Xu WT**, Yang Z, Lu NH. Roles of PTEN (Phosphatase and Tensin Homolog) in gastric cancer development and progression. *Asian Pac J Cancer Prev* 2014; **15**: 17-24 [PMID: 24528021 DOI: 10.7314/APJCP.2014.15.1.17]

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**Table 1 Summary of stool samples collected and analyzed *n* (%)**

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Tumor location** | **Total collected samples** | **Successful DNA extraction** | **Successful sequencing** | **Number of cases with only known mutations** | **Number of cases with****mutations (all)** |
| Stomach | 41 | 35 | 32 (78) | 5 (15.6) | 8 (25) |
| CarcinomaIntestinal typeDiffuse type | 381820 | 321715 | 291514 | 312 | 615 |
| BenignHamartomaDysplasia | 311 | 311 | 311 | 201 | 201 |
| NET | 1 | 1 | 1 | 1 | 1 |
| Colorectal  | 46 | 42 | 40 (87) | 12 (30) | 12 (30) |
| Carcinoma | 40 | 37 | 35 | 9 | 9 |
| BenignAdenomaDysplasiaLeiomyoma  | 6411 | 5311 | 5311 | 3201 | 3201 |
| Healthy controls | 14 | 13 | 13 (92.9) | 0 | 2 (15) |
| Total | 101 | 90 | 85 | 17 | 22 |

NET: Neuroendocrine tumor.

**Table 2 Cosmic and novel mutations detected by ion torrent sequencing along with mutation type and mutant allele frequency**

|  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- |
| **Histopathology** | **Cases ID** | **Age (yr)** | **Sex** | **TNM Staging** | **Gene** | **Mutation type** | **Cosmic No.** |
| Stomach |  |  |  |  |  |  |  |
| AC (D) | 3 | 66 | F | T2N0 | *SMARCB1* | p.T72K | Novel |
| AC (D) | 14 | 80 | F | T4aNxM1 | *APC* | p.D1570N | Novel |
|  |  |  |  |  | *CDH1* | p.V82A | Novel |
|  |  |  |  |  | *HRAS* | p.V44M | Novel |
|  |  |  |  |  | *NRAS* | p.K135R | Novel |
| AC (D) | 39 | 65 | F | T1aN0M0 | *EGFR* | p.A750T | COSM1651572 |
|  |  |  |  |  | *DDR2* | p.E523K | Novel |
| AC (D) | 43 | 43 | M | T4bNxM1 | *PTEN* | p.E256G | Novel |
| AC (D) | 77 | 78 | M | TxNxM1 | *APC* | p.A1582P | COSM4170230 |
| AC (I) | 100 | 69 | M | T3N3M1 | *APC* | p.A1582P | COSM4170230 |
| NET | 11 | 67 | M | 5% PR | *CDKN2A* | p.V126I | COSM13778 |
| Dysplasia | 78 | 77 | M | T0N0M0 | *APC* | p.A1582P | COSM4170230 |
| Colorectal |  |  |  |  |  |  |  |
| AC | 20 | 70 | M | T3N0M0 | *KIT* | p.N564S | COSM30732 |
| AC | 12 | 71 | F | T3N1bM0 | *APC* | p.A1582P | COSM4170230 |
|  |  |  |  |  | *TP53* | p.P72A | COSM3738520 |
| AC | 21 | 79 | M | T2N0 | *APC* | p.A1582P | COSM4170230 |
| AC | 22 | 64 | F | T2N0 | *STK11* | p.F354L | COSM21360, COSM4169323 |
|  |  |  |  |  | *SMO* | p.N202S | COSM5979442 |
| AC | 23 | 53 | M | TxNxM1 | *APC* | p.A1582P | COSM4170230 |
| AC | 31 | 64 | M | T2N0 | *TP53* | p.Y205D | COSM43844 |
| AC | 55 | 69 | M | T3N0M0 | *KRAS* | p.G12V | COSM520 |
|  |  |  |  |  | *TP53* | p.T172fs | COSM44371 |
| AC | 68 | 63 | M | T3N0M0 | *TP53* | p.Y163H | COSM43846 |
| AC | 28 | 77 | F | T1N0 | *KRAS* | p.G12D | COSM521 |
|  |  |  |  |  | *AKT1* | p.E17K | COSM34142, COSM33765 |
| Adenoma | 24 | 81 | M |  | *APC* | p.E1295\* | COSM18961 |
| Adenoma | 19 | 64 | F |  | *NRAS* | p.Q61R | COSM584, COSM28048 |
|  |  |  |  |  | *FBXW7* | p.R505C | COSM22975, COSM33844 |
|  |  |  |  |  | *APC* | p.S1465fs | COSM18873, COSM19688 COSM19332, COSM18931, COSM13864 |
|  |  |  |  |  | *ERBB2* | p.V842I | COSM14065, COSM1666633 |
|  |  |  |  |  | *SMARCB1* | p.R377C | COSM3972885 |
| Leiomyoma | 94 | 69 | F |  | *TP53* | p.R306\* | COSM10663, COSM145026 |
| Healthy controls |  |  |  |  |  |  |  |
|  | C 2 | F | 27 |  | *ALK* | p.L1190P | Novel |
|  | C 8 | F | 33 |  | *STK11* | p.Y36H | Novel |

AC: Adenocarcinoma; (D): diffuse type; (I): Intestinal type; PR: proliferative rate; NET: Neuroendocrine tumor.