

The article type: Original Article

A clinical trial of somatic and germline analyses for healthy longevity in a postoperative cancer patient

DUAL GENOMIC ANALYSES FOR HEALTHY LONGEVITY IN CANCER

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ST-2018-0737-CO.R1

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Keywords: single nucleotide polymorphism, somatic mutation, disease risk, quality of life,
healthy longevity

Abbreviations

QOL Quality of life

DNA	Deoxyribonucleic acid
SNP	Single-nucleotide polymorphism
WES	Whole-exome sequencing
UICC	Union for international cancer control
KRAS	Kirsten rat sarcoma viral oncogene homolog
CT	Computed tomography
XELOX	Xeloda(capecitabine) plus oxaliplatin
SLC22A1	Solute carrier family 22 member 1
CYP2C9	Cytochrome P450 2C9
RR	Risk rate
RNA	Ribonucleic acid
UTR	Untranslated region
COSMIC	Catalogue of somatic mutations in cancer
KEGG	Kyoto Encyclopedia of Genes and Genomes
VAFs	Variant allele fractions
FEV1	forced expiratory volume in one second
FVC	forced volume capacity

MAPK	Mitogen-activated protein kinase
ErbB	Erythroblastic leukaemia viral oncogene homolog
Jak-STAT	Janus kinase-signal transducer and activator of transcription
mTOR	Mammalian target of rapamycin
Wnt	Wingless/integrated
VEGF	Vascular endothelial growth factor
PPAR	Peroxisome proliferator-activated receptor
ABI	Ankle brachial index
BRAF	V-raf murine sarcoma viral oncogene homolog B1
PIK3CA	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha
SMAD4	SMAD family member 4
FBXW7	F-box and WD-40 domain protein 7
CCDC120	Coiled-coil domain containing 120
MICAL	Microtubule-associated monooxygenase, calponin and LIM domain containing
MUC17	Mucin 17
SETDB1	SET domain bifurcated 1

CRC	Colorectal cancer
ARMS2	Age-Related Maculopathy Susceptibility 2
OSBPL10	Oxysterol binding protein-related protein 10
CAVI	Cardio ankle vascular index
PI3K	Phosphoinositide-3-kinase
EGFR	Epithelial growth factor receptor
MEK	MAPK/ERK kinase
ERK	Extracellular signal regulated kinase
CDK	Cyclin-dependent kinase

Abstract

Purpose: Recent developments in molecular-targeted therapies have improved the clinical outcome of cancer patients; however, the issue of adverse effects due to treatments has often gone unconsidered. We herein report the results of a clinical trial of dual genomic analyses for healthy longevity in a postoperative cancer patient.

Methods: We performed dual genomic analyses for a representative 79-year-old rectal cancer patient who relapsed with liver metastasis. First, we determined single-nucleotide polymorphisms according to the constitution and disease risk in the genomic DNA from the patient's saliva by referring to the data of 10,000 Japanese patients obtained from Yahoo Japan Corporation. Second, we conducted whole-exome sequencing to detect druggable mutations in the primary tumour.

Results: Forty of 59 determinable characters related to the constitution were consistent with the clinical phenotype. Several diseases classified as 'high risk' diseases actually occurred during the patient's clinical course. Of the 129 significant mutations, we identified somatic mutations in BRAF, PIK3CA, and SMAD4 as targets.

Conclusion: The dual genomic examination will improve the follow-up observation system to support primary care doctors in the social community for taking care of postoperative cancer

patients.

Introduction

Despite recent technological innovations, cancer is still a fatal disease; it is one of the major causes of human death and gradually diminishes the patient's quality of life (QOL), regardless of the success or failure of treatment. The following two factors determine the fate of patients: recurrence with no druggable target genes, and unpredictable disease complications after operation. Even if curative surgery has been conducted, it is impossible to predict the onset of critical diseases, such as dementia, or various complications associated with being bedridden for a long duration.

Recent technological innovations in anticancer drugs, including combination chemotherapy and molecular-targeted therapy, have helped improve the progression-free survival of cancer patients. However, the number of cancer patients with no treatment options left is increasing, as most therapeutic strategies have already been attempted. Furthermore, with the extension of the life expectancy of patients, the number of "cancer refugees" whose QOL needs to be preserved has increased, representing a serious global issue. To address these issues, we have proposed the following two strategies: 1) predicting adverse events earlier in order to improve the QOL and 2) personalising anticancer therapy.

We have also focused on establishing a social community not only for cancer patients

but also for primary care doctors who are taking care of these patients. The Japanese government has gradually implemented the functional differentiation of beds in hospitals; as a result, primary care doctors, who participate in community-based healthcare, play a major role in postoperative follow-up. Given that long follow-up periods are expected in order to care for postoperative cancer patients, technological support will be needed in order to easily observe patients for a long period of time and to predict the onset of diseases as accurately as possible.

Recently, next-generation sequencing has enabled us to analyse driver mutations as therapeutic targets. The single-nucleotide polymorphism (SNP) array was developed to detect germline SNPs and analyse the genetic background, allowing for the administration of precision medicine based on individual cancer patients' genomic mutations. We therefore started a patient care system using SNPs to predict and prevent disease complications, in cooperation with Yahoo Japan Corporation. Yahoo Japan Corporation stores a huge data library of SNPs and statistical information on about 10,000 Japanese people for whom the onset of illness could not be predicted. This company has also established a technique for non-invasively extracting DNA from cells in saliva. Using the information obtained from somatic mutation analyses of cancer and individual SNPs that is stored in the secure Yahoo cloud, we can perform our analyses comprehensively and share the data with other medical institutions.

In this way, we constructed an area integration-type medical system that facilitates precision medicine and promotes healthy longevity in society by preventing complications that affect the QOL. In addition, accumulating the data of many cases will help further increase the accuracy of our analysis system.

We herein report a representative case of rectal cancer in our system. This patient with rectal cancer recurrence was the first included in our study. Through this first case, we examined how useful our dual genomic analysis system was at present. We found that the adverse events that occurred during the postoperative follow-up period were predictable, showing that we can prepare for cancer recurrence by analysing cancer somatic mutations in order to select the most appropriate anticancer drugs. Based on these results, we would like to discuss the future aspects of our current system.

Patient and methods

Clinical features of the case

We summarised the course of the present case in the left column of Fig. 1. A 79-year-old man was diagnosed with resectable rectal cancer and underwent laparoscopic low anterior resection with D3 lymphadenectomy. Minor anastomotic leakage occurred on postoperative day 2. Accordingly, he underwent conservative treatment, including fasting, total parental nutrition, and drainage, for over two weeks, and he was discharged on postoperative day 56. A histopathology analysis revealed a UICC Stage II (pT3N0M0, and R0) with a KRAS mutation, and accordingly, no adjuvant therapy was given. Follow-up computed tomography (CT) six months after surgery revealed liver metastasis in segment 4. Therefore, we conducted metastasectomy after a biweekly capecitabine regimen in combination with oxaliplatin (XELOX) plus bevacizumab therapy. According to the Common Terminology Criteria for Adverse Events version 4.0, Grade 1 peripheral neuropathy and grade 3 hypertension were observed. Consequently, a complete pathological response was demonstrated by the histopathological examination, and the case was subsequently observed with no additional treatment. Seven months after metastasectomy, follow-up CT revealed a partial splenic infarction not associated with cancer or surgery. He joined our study when the first course of

chemotherapy was started.

Confronting postoperative issues for this case

We focused on the following clinical questions (Fig. 1, right column). 1) Concerning the somatic mutations of the primary cancer: 1-1) What was the driver mutation in this case? 1-2) What was an eligible anticancer drug for this case? 2) Concerning the germline SNPs: 2-1) What risk originally existed for the patient? 2-2) What adverse event was the most probable after the administration of the anticancer drugs? 2-3) What SNPs influenced the partial splenic infarction?

To answer these questions, we performed an SNP array analysis with the patient's saliva (salivary SNP array) and whole-exome sequencing (WES) using DNA extracted from samples of the primary cancer tissue and the normal colon tissue.

The SNP array analysis of salivary samples

We collected 2 ml of the patient's saliva, and Genequest Inc. (5-29-11, Shiba, Minato-ku, Tokyo 180-0014, Japan), a consociate of Yahoo Japan Corporation, performed the SNP array analysis of the salivary DNA using their DNA chip. We evaluated the results of the SNP array analysis

according to constitution and disease risk by referring to the data of 10,000 Japanese patients that were in the possession of Yahoo Japan Corporation.

- Constitution: We classified the constitution-related characters determined by the SNP array analysis under the following four types: ‘physical characteristics’, ‘response to food and foreign object’, ‘blood components’, and ‘customs and personality’. First, Yahoo Japan Corporation presented their interpretations regarding the characters associated with SNPs to us based on previous studies. Second, if the SNP had been reported by ≥ 1 studies targeted at ≥ 750 people, we regarded the characters of the SNP as a target of our evaluation. We then evaluated whether or not these characters were applicable to the present patient (Table 1). Numerical characters, such as the results of the blood test, were evaluated using the Japanese mean or the interquartile range of reference in our hospital. The following three situations were excluded as indeterminable: i) unable to decide whether or not a character was applicable to the patient because of a vague criterion for evaluation, ii) more than two SNPs indicated different interpretations, and iii) unable to evaluate the characters by a general medical examination. We also evaluated the SNPs associated with the molecules involved in the anticancer drug metabolism (referred to <http://www.genome.jp/kegg/drug>), including

solute carrier family member 1 (SLC22A1), which works as a drug transporter of oxaliplatin, and cytochrome P450 2C9 (CYP2C9), which works as a metabolic enzyme of capecitabine.

- Disease risk: We evaluated the risk rate (RR) of the genotype with regard to the Japanese average risk after the disease onset. We defined RR values of ≤ 0.79 as ‘low risk’, 0.80–1.19 as ‘average risk’, and ≥ 1.2 as ‘high risk’. If two or more SNPs were related to a disease, the highest RR was defined as the RR of the disease. As with the constitution, SNPs that were the subject of studies targeting ≥ 750 people were regarded as evaluation parameters.

Somatic mutation analyses

DNA was extracted from fresh-frozen samples of the tumour and the normal colon mucosa using the All Prep DNA/RNA Mini Kit (Qiagen, Hiden, Germany). Whole-exome capture was performed with The SureSelect XT Human All Exon V5 capture library for all samples. The sequence data were processed through an in-house pipeline (<http://genomon.hgc.jp/exome/>).

Mutation calling was performed with the following parameters: (I) Mapping Quality score ≥ 20 , (II) Base Quality score ≥ 15 , (III) Both the tumour and normal depths ≥ 8 , (IV) Variant

reads in tumours ≥ 4 , (V) Variant allele fractions (VAFs) in tumour samples ≥ 0.05 , (VI) VAFs in normal samples ≤ 0.1 , and (VII) EB call P value- $\log_{10} \geq 4$.

Mutations were then eliminated using the following parameters: (a) Fisher P value- $\log_{10} \leq 1.3$, (b) MIS rate in normal samples ≥ 0.01 , (c) Variant pair numbers in normal samples > 1 , (d) Mutation listed in SNP131 but not listed in COSMIC, and (e) Functional category of the mutational gene, including 'synonymous' or 'intronic' or 'intergenic' or 'upstream' or 'downstream' or 'UTR' or 'ncRNA intronic'. From these mutation calls, we extracted the mutated gene name, which accorded with the gene name associated with 15 signalling pathways, using KEGG gene sets (<http://software.broadinstitute.org/gsea/msigdb/index.jsp>). The categories of each pathway were as follows: 'Cell cycle', 'Apoptosis', 'MAPK signalling pathway', 'Pathways in cancer', 'Epithelial cell signalling in *Helicobacter pylori* infection', 'ErbB signalling pathway', 'Jak-STAT signalling pathway', 'Mismatch repair', 'mTOR signalling pathway', 'Notch signalling pathway', 'p53 signalling pathway', 'Wnt signalling pathway', 'VEGF signalling pathway', 'Toll-like receptor signalling pathway', and 'PPAR signalling pathway'. Subsequently, we extracted the names of genes significantly mutated in colorectal cancer or other cancers [1].

Results

Constitution-related SNPs

Of the 145 evaluation targets extracted by salivary SNP array, 86 were indeterminable because they met the (i) to (iii) conditions. Of the 59 determinable characters, 40 (67.8%), such as the ‘ankle brachial index’ (ABI), ‘alcohol flush reaction’, and ‘platelet level’, were applicable to the real state of the patient, while the remaining 19 (32.2%) were not (Table 1).

SNPs related to drug metabolism

With regards to SNPs related to CYP2C9, the genotypes of rs1057910 and rs1799853 were common, and that of rs2860975 was heterozygous; however, the TG genotype of rs2860975 was frequently seen in Asians (Table 2). The genotype of the SNP related to SLC22A1 was common (www.ncbi.nlm.nih.gov/projects/SNP/). Bevacizumab was an indeterminable drug because it had no known metabolic enzyme or transporter.

Disease risk

The present patient was defined as having a ‘high risk’ for 10 diseases, an ‘average risk’ for 38 diseases, and a ‘low risk’ for 9 diseases (Table 3). Of all the diseases among the evaluation

targets, the patient had ‘peripheral arterial disease’ and ‘age-related macular degeneration’, which were classified as ‘high risk’ diseases, as well as ‘colon cancer’, which was classified as an ‘average risk’ disease. The patient did not develop any ‘low risk’ diseases.

Somatic mutations in this case

For this case, 383 mutations were called, and 254 were excluded based on the previously mentioned criteria (a) to (e): (a) $P \geq 0.05$; (b), (c), and (d) the mutation represents a prominent mutation in normal tissue and is not greatly related to cancer; and (e) the mutation will not greatly affect the gene expression. Of 129 candidate mutations, several that are known to be notably mutated in cancers, such as BRAF/PIK3CA/SMAD4/FBXW7 in colorectal cancer, CCDC120 in clear cell renal carcinoma, MICAL in endometrial cancer, MUC17 in glioblastoma multiforme, and SETDB1 in lymphatic leukaemia and oesophageal adenocarcinoma, were filtered [1]. Among these eight genes, BRAF, PIK3CA, and SMAD4 were related to signalling pathways (Fig. 2). The details of these mutant genes are as follows: BRAF (N581S), PIK3CA(K111E), PIK3CA(H1047R), and SMAD4(R361C).

Discussion

Risk prediction with a salivary SNP array analysis classified colorectal cancer as an average-risk disease for the present patient. This serves as a reminder that colorectal cancer carcinogenesis is determined not only by one's genetic background but also by lifestyle and diet. Furthermore, this patient also suffered from age-related macular degeneration, which was classified as a high-risk disease. This patient had four SNPs associated with age-related macular degeneration, and rs10490924 (Age-Related Maculopathy Susceptibility 2, ARMS2) had the highest RR value (2.89). Among these four SNPs, ARMS2 is known to be associated with all subtypes of age-related macular degeneration [2]. These findings suggest that we should not neglect any positive SNP findings.

It is noteworthy that an asymptomatic partial splenic infarction also occurred in this patient. Our SNP analysis provided a clue suggesting a risk of partial splenic infarction through findings concerning 'peripheral arterial disease'. The spleen receives a segmental arterial blood supply, and branches of the splenic artery have little collateral circulation. Therefore, obstruction or injury of an arterial branch can result in an infarction [3]. We concluded that surgery had not been the direct cause of the splenic infarction in this case because CT performed on postoperative day 7 after metastasectomy did not reveal a splenic infarction (Fig.

3b). Furthermore, a preoperative examination showed scattered calcification in some arteries, with particularly prominent calcification at the splenic artery (Fig. 3a). A salivary SNP analysis revealed that this patient had the risk allele rs1902341 located at the oxysterol binding protein-related protein 10 (OSBPL10) gene, and this SNP has been reported to be strongly related to peripheral arterial disease [4]. The function of OSBPL10 is unclear at present; however, we speculate that this protein influences lipid metabolism and causes arteriosclerosis [5]. In addition, the patient had a slightly low ABI of the right leg (0.94, less than the first quartile of the reference range in our hospital), and a tendency toward a high cardio-ankle vascular index (CAVI) in both legs (right: 8.7, left: 9.9), indicating the existence of arteriosclerosis. The SNP related to peripheral arteries likely caused these vascular changes. The accumulation of further clinical data will help clarify the SNPs related to various diseases and facilitate the selection of suitable medical treatments according to the expected patient risk.

XELOX plus bevacizumab therapy was performed to treat the liver metastasis in the present case. Each anticancer drug used in this case carries a risk of inducing certain adverse effect, such as peripheral neuropathy, cytopenia, and hypertension. We believe that the SNPs related to metabolic enzymes or drug transporters affected the incidence of these adverse events. Therefore, we focused on SNPs related to CYP2C9 as a metabolic enzyme of capecitabine and

SLC22A1 as a drug transporter of oxaliplatin. Given that the SNPs related to both CYP2C9 and SLC22A1 are a common genotype in Asians (Table 2). These result showed that a risk of inducing certain adverse effect in the present case was typical level. The adverse events that actually occurred were peripheral neuropathy grade 1 and hypertension grade 3. We concluded that the hypertension was related to the administration of bevacizumab, and the background arteriosclerosis might have accelerated the symptoms. SNP analyses performed in advance will enable the suitable and safe administration of chemotherapy.

We suspected that a mutation related to downstream signal transduction was an important factor involved in the carcinogenesis and cancer progression in this patient. We therefore performed a pathway analysis and detected 10 mutations. The most frequently detected mutations were in BRAF, PIK3CA, and SMAD4, which have been reported to be driver mutations of colorectal cancer in databases such as MutSig [1] and ActiveDriveDB [6]. The mutant of BRAF was BRAF^{N581S}. The N581S mutation is located in the kinase domain and contributes to intermediate kinase activation [7]. For this reason, we expect a BRAF inhibitor to be more effective agent in cases of N581S than in cases of wild-type BRAF. PIK3CA^{H1047R} is a hotspot mutation in the catalytic subunit p110 α of phosphoinositide-3-kinase (PI3K) and activates the lipid kinase activity more than wild-type PIK3CA. In the

present case, we detected a mutation in the PIK3CA kinase domain, which consequently renders the disease refractory to anti-epithelial growth factor receptor antibody therapies (anti-EGFR therapies), including cetuximab [8, 9, 10]. Therefore, in cases of PIK3CA^{H1047R}, an mTOR inhibitor is likely to be more effective than anti-EGFR therapy. However, preoperative genetic analysis of the cancer tissue in the present case revealed a KRAS mutation in codon 12, which is considered to confer resistance to anti EGFR therapy, but our mutation calling did not detect this mutation. Heterogeneity was presumed to have caused this discrepancy. Even if the KRAS status is regarded as wild, BRAF, MEK, and mTOR inhibitors are expected to be more effective in this case than anti-EGFR therapy because of BRAF^{N581S} and PIK3CA^{H1047R}, as mentioned previously (Fig. 4). The effectiveness of vascular endothelial growth factor (VEGF) inhibitors, such as bevacizumab, is also promising in this case because the activation of the PI3K/AKT/mTOR pathway in cancer increases VEGF secretion through hypoxia-inducible factor 1 and activates angiogenesis [11]. SMAD family member 4 (SMAD4) is a molecule that forms a complex with another SMAD and controls signal transduction. The mutation of R361 located in the C-terminal Mad homology domain-2 of SMAD4 is a hotspot mutation. This mutant is unable to form a complex with SMAD2 or SMAD3, leading to a loss of its function [12]. As a result, this mutant cannot suppress cyclin-dependent kinase (CDK) activity, and the

cell cycle is promoted. Therefore, we regarded CDK inhibitors as candidate therapeutic agents (Fig. 4). In the present study, based on the WES data, we concluded that mutations of BRAF, PIK3CA, and SMAD4 were driver mutations and considered eligible therapeutic strategies against future recurrence in this case by referencing previous studies. However, we should bear in mind two important matters when determining targeted driver genes: differences based on race and the issue of heterogeneity.

Of note, the lack of an appropriate control was a limitation associated with the present study; however, the marked difference between the true positive rate (68%) and the false positive rate (32%) in Table 1a may strongly encourage us to apply the current assay in the clinical setting. We therefore expect that this proposed approach will improve the frailty rate, for instance, in future postoperative observations. We intend to accumulate more data on cancer mutations in Japanese patients on order to resolve any issues related to race and integrate these data with clinical information. Establishing precision methods by detecting driver mutations and preventing adverse effects using integrated data, including those from SNP analyses, will help improve the therapeutic index and facilitate the selection of appropriate therapeutic agents.

Notes

Compliance with ethical standards

The study design was approved by the institutional review boards and ethics committees of Kyushu University Hospital (Kyushu University Hospital Institutional Review Board: Protocol Number713–00). All methods were performed in accordance with the relevant guidelines and regulations. We have obtained written informed consent from all of the patients in this study.

Acknowledgements

This study was funded by A-STEP (Adaptable and Seamless Technology Transfer Program through Target-driven R & D). The SNP analysis of the salivary sample was performed with the full cooperation of Yahoo Japan Corporation.

Conflict of interest

This study was conducted in collaboration with Yahoo Japan Corporation; however, we received no financial support from them.

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Figure Legends

Fig. 1. The clinical course. The patient experienced various complications during the perioperative period. Because liver metastasis was found, he underwent 4 three-week cycles of capecitabine (3000 mg/kg body weight) administered orally on days 1 to 14, intravenous oxaliplatin (130 mg/m²) on day 1, intravenous bevacizumab (7.5 mg/kg) on day 1, and 1 three-week cycle of capecitabine (3000 mg/kg body weight) administered orally on days 1 to 14 before metastasectomy. He joined our study when the first course of the chemotherapy was started. The clinical questions of note are presented in the right column. We examined whether or not dual genomic analyses enabled us to solve these questions.

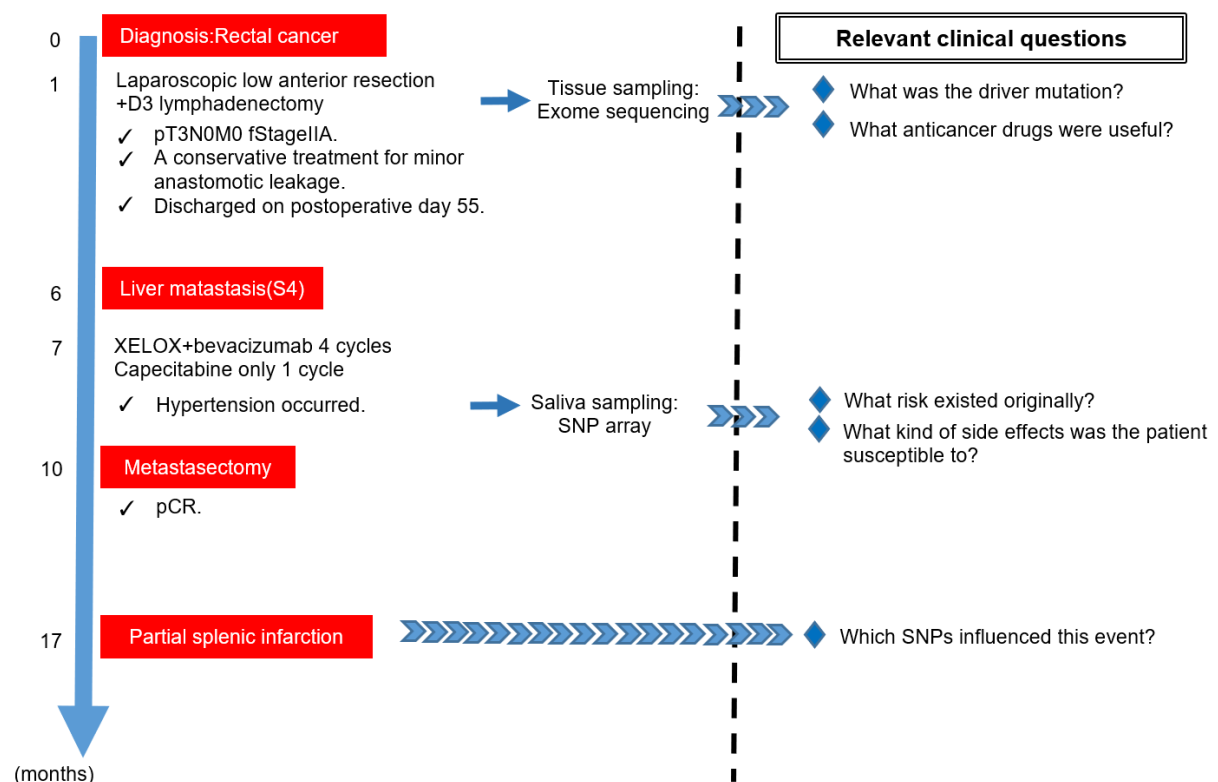


Fig. 2. Extracting genes associated with various pathways from 15 categories. Ten categories were associated with this case, and 10 of 129 mutations were related to some signalling pathways. Eight of the 129 mutations shown in the red dotted circle were classified as significantly mutated genes in solid or hematologic cancer. *SMAD4*, *PIK3CA*, and *BRAF* met both criteria.

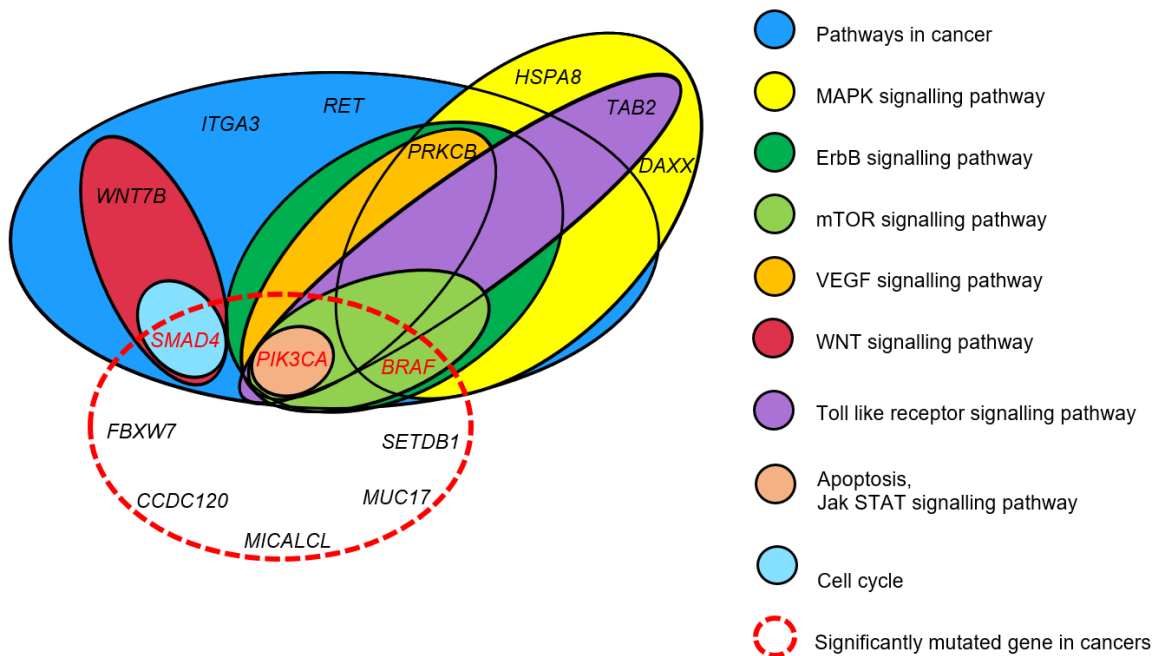


Fig. 3. The course of CT findings concerning splenic infarction. a) Severe calcifications were observed in the splenic artery before rectal cancer surgery; b) no abnormalities were observed in the spleen on postoperative day 7 after metastasectomy; c) partial splenic infarction was observed seven months after metastasectomy.



a) Calcification of splenic artery



b) Postoperative day 7 after resection of metastasis



c) Partial splenic infarction

Fig. 4. The pathways associated with *PIK3CA*, *BRAF*, and *SMAD4*. We assumed that inhibiting proteins coded by these genes or their main downstream molecules was an effective therapeutic strategy.

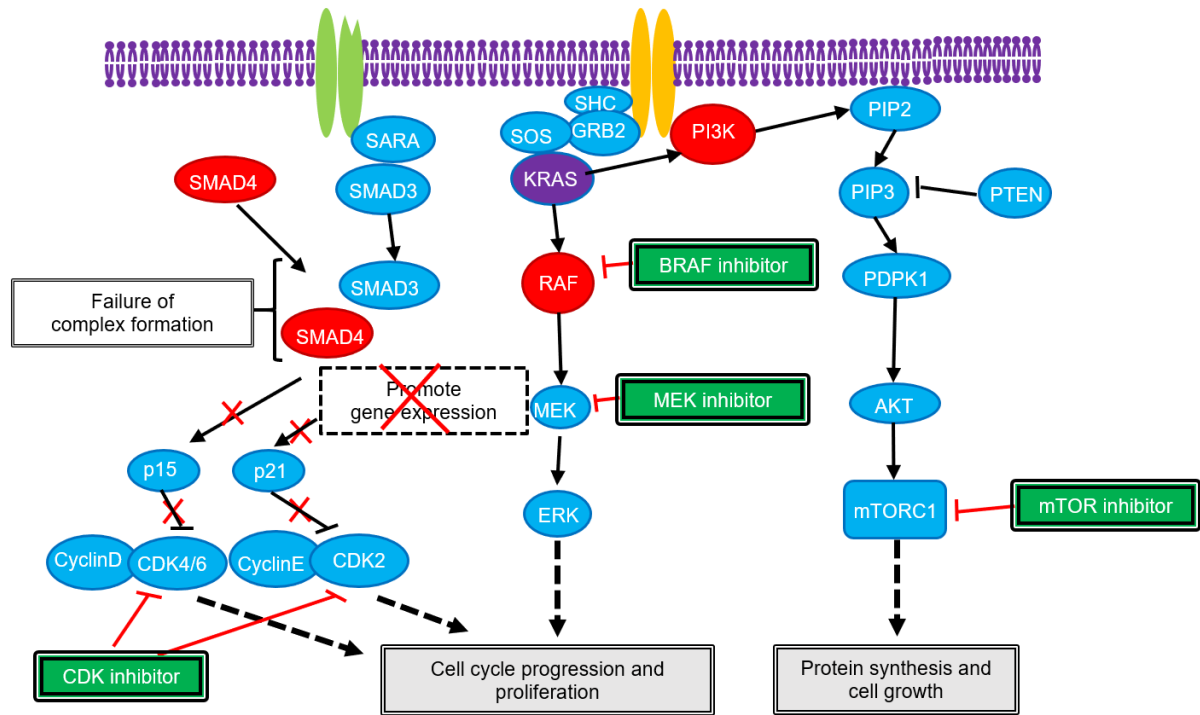


Table 1. SNPs related to constitution indicated by the salivary SNP array analysis.

We evaluated 145 items and excluded 86. Forty of the remaining 59 items were detected in this patient. The true positive rate was 68%, and the false positive rate was 32%.

	classification	fit	not fit		
SNP List	physical characteristics	hair curl	hair colour	FEV1/FVC ratio	heart rate
		eye colour	iris pattern	PR interval	QRS interval
		type of ear wax	height of nose ridge	QT interval	
		ease of cavity development	length of vertebrae		
		length of second and third digit	forced expiratory volume		
		evening pulse pressure	change in blood pressure upon standing		
		ankle brachial index	left ventricular wall thickness		
	reponse to food and foreign objects	codependence on alcohol and nicotine	alcohol flush reaction	alcohol intake	
		nicotine dependence	lactose tolerance		
	characteristics of blood components	CEA level	white blood cell count	CA19-9 level	monocyte count
		neutrophil count	eosinophil count	hematocrit level	uric acid level
		basophil count	hemoglobin level	γ-GTP level	iron level
		platelet count	mean platelet volume	serum albumin level	non-albumin blood protein level
		calcium level	serum total protein level	A/G ratio	triglyceride level
		bilirubin level	fasting blood sugar level	total cholesterol level	prothrombin time
blood urea nitrogen level		serum creatinine level	activated partial thromboplastin time		
characteristics of customs and personality	CRP level	creatinine kinase level			
	exercise habits	bedtime			
	sleep quality	perseverance			
	curiosity				
ratio		67.8%(40/59)	32.2%(19/59)		

Table 2. SNPs related to anticancer drugs indicated by the salivary SNP array analysis.

The incidence of adverse events due to these anticancer drugs was considered to be typical.

chemo drug	Enzyme or drug transporter	SNP	Genotype	Assessment
capecitabine	CYP2C9	rs1057910	AA	common
		rs1799853	CC	common
		rs2860975	TG	heterozygous mutant common in Asians
oxaliplatin	SLC22A1	rs662138	CC	common
bevacizumab	none	none	none	none

Table 3. The risks for disease development indicated by the salivary SNP array analysis.

We defined low risk (≤ 0.79), high risk (≥ 1.2), and average risk (0.80–1.19). Two high-risk

diseases (peripheral arterial disease and age-related macular degeneration) and one average-risk disease (colon cancer) actually occurred in this patient. He developed no low-risk diseases.

risk	high risk	average risk		low risk
Disease List	narcolepsy	chronic myelogenous leukaemia	atrial fibrillation	lumbar disc herniation
	testicular cancer	normal tension glaucoma	colon cancer	heart attack
	excessive myopia	gout	insomnia	prostate cancer
	chronic hepatitis B	chronic periodontitis	hepatitis B virus derived hepatocellular carcinoma	essential tremor
	astigmatism	susceptibility to human papillomavirus (HPV)	adenocarcinoma of the lung	atopic eczema
	peripheral arterial disease	nasopharyngeal carcinoma	chronic widespread pain	squamous cell carcinoma of the oesophagus
	restless legs syndrome	staphylococcus aureus infection	Barrett's oesophagus	gallbladder cancer
	migraine	duodenal ulcer	adolescent idopathic scoliosis	emphysema
	age-related macular degeneration	pancreatic cancer	syndrome of dengue fever	lung cancer
	type II diabetes	Graves' disease	iron deficiency anemia	
		severe acne	cerebral aneurysm	
		osteoarthritis	chronic hepatitis C	
		basal cell carcinoma	squamous cell carcinoma of the lung	
		gastric ulcer	renal cell carcinoma	
		hypothyroidism	asthma	
		sick sinus syndrome	melanoma	
		keratoconus	coronary heart disease	
	risk of calcification of the aortic valve	non-obstructive azoospermia		
	rheumatoid arthritis	glaucoma		
number	10	38		9
onset	peripheral arterial disease, age-related macular degeneration	colon cancer		