Original articles

EVALUATION OF THE KRAS MUTATIONS IN COLORECTAL AND PANCREATIC CANCER PATIENTS

Samatkyzy D.[1], Rakhimova S.[1], Begimbetova D.[2], Kukanova A.[4], Fazyl F.[2], Stefanov I.[5], Pirozhenko O.[5], Gabdulkayum A.[1], Kozhamkulov U.[1], Makishev A.[4], Tuleutayev M.[5], Akilzhanova A.[1,*], Sarbassov D.[3]

[1]Laboratory of genomic and personalized medicine, Private Institution National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan.

[2]Laboratory of translational medicine and Life Sciences Technologies, Private Institution National Laboratory Astana, Nazarbayev University, Astana, Kazakhstan.

[3]School of Sciences and Humanities, Nazarbayev University, Astana, Kazakhstan.

[4]NJSC "Astana Medical University", Astana, Kazakhstan.

[5]"Multidisciplinary Medical Center", Astana, Kazakhstan.

**akilzhanova@nu.edu.kz*

ABSTRACT

The RAS gene is the most mutated and studied oncogene in human cancer. The presence of a KRAS mutation causes heightened activation of RAS effectors, resulting in the development of tumor tissue. The effect of KRAS mutations on a patient's prognosis and survival with adenocarcinoma and other types of cancer stimulate a lot of scientific research for the study their molecular mechanisms. To further develop a protocol for the treatment of mutant KRAS cases in Kazakhstan we study KRAS mutations status in Kazakhstani colorectal and pancreatic cancer patients. In this article, we review the literature on the role of KRAS mutation testing for management of patients and demonstrate preliminary data on KRAS mutation status in Kazakhstani patients with colorectal and pancreatic cancer.

Keywords: adenocarcinoma, RAS, KRAS, Sanger sequencing, missense mutation, codon.

INTRODUCTION

Cancer diseases develop in the presence of many gene mutations, resulting in an increase in cell proliferation [1-3]. Numerous studies have been conducted on the development of benign into malignant lesions, particularly in colorectal cancer (CRC) and pancreatic cancer (PC) [4].

It is known that an obstacle to more effective treatment of cancer is its severe course with rapid metastasis. General risk factors for cancer development are: family predisposition, environment and eating habits [5, 6].

KRAS encodes a GTPase-binding protein and plays a main role in the activation of the epidermal growth factor receptor (EGFR). The study of CRC gives us a great opportunity to obtain more information for further research on carcinogenesis and the main mechanisms involved in tumor development. Furthermore, mutations in genes play an important role in the regulation of epithelial development and cell differentiation (Figure 1). In other words, the CRC model allows collecting fundamental data on the progress of one or more tumor variants and their further transition to metastatic cancer [7]. Along with this and based on the fact that both heredity and environment contribute to the development of this type of cancer, these tumors permit the study of both somatic genetic alterations and environmental and dietary changes [8].

In approximately 75% of the cases, CRC progresses through the chromosomal instability pathway and these tumors can harbor mutations of different genes, such as APC, KRAS, and TP53 [9]. The KRAS gene is located on the short arm of chromosome 12 (Figure 2) [41].

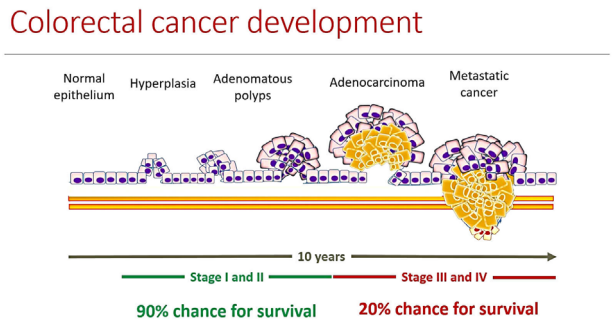

Figure 1 – Colorectal cancer development [43].

In this article, we will focus on studying the KRAS mutation. The KRAS belongs to the RAS family of genes. The RAS family is a group of proteins that have the ability to bind guanosine triphosphate and diphosphate nucleotides. Additionally, RAS family is also known as small GTPases. These enzymes hydrolyze GTP and form GDP. This process affects cellular organization and signal transmission (Figure 3). Nowadays, 160 RAS proteins are known and determined in human body [10]. Researchers' interest in RAS proteins has grown as a result of mounting evidence that these proteins play key regulatory roles in a number of cancer types.

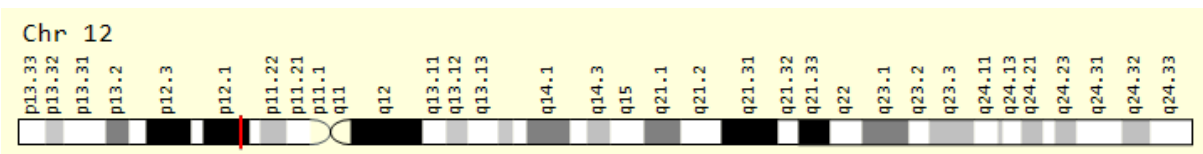

Figure 2 – Location of the KRAS gene on chromosome 12 [41].

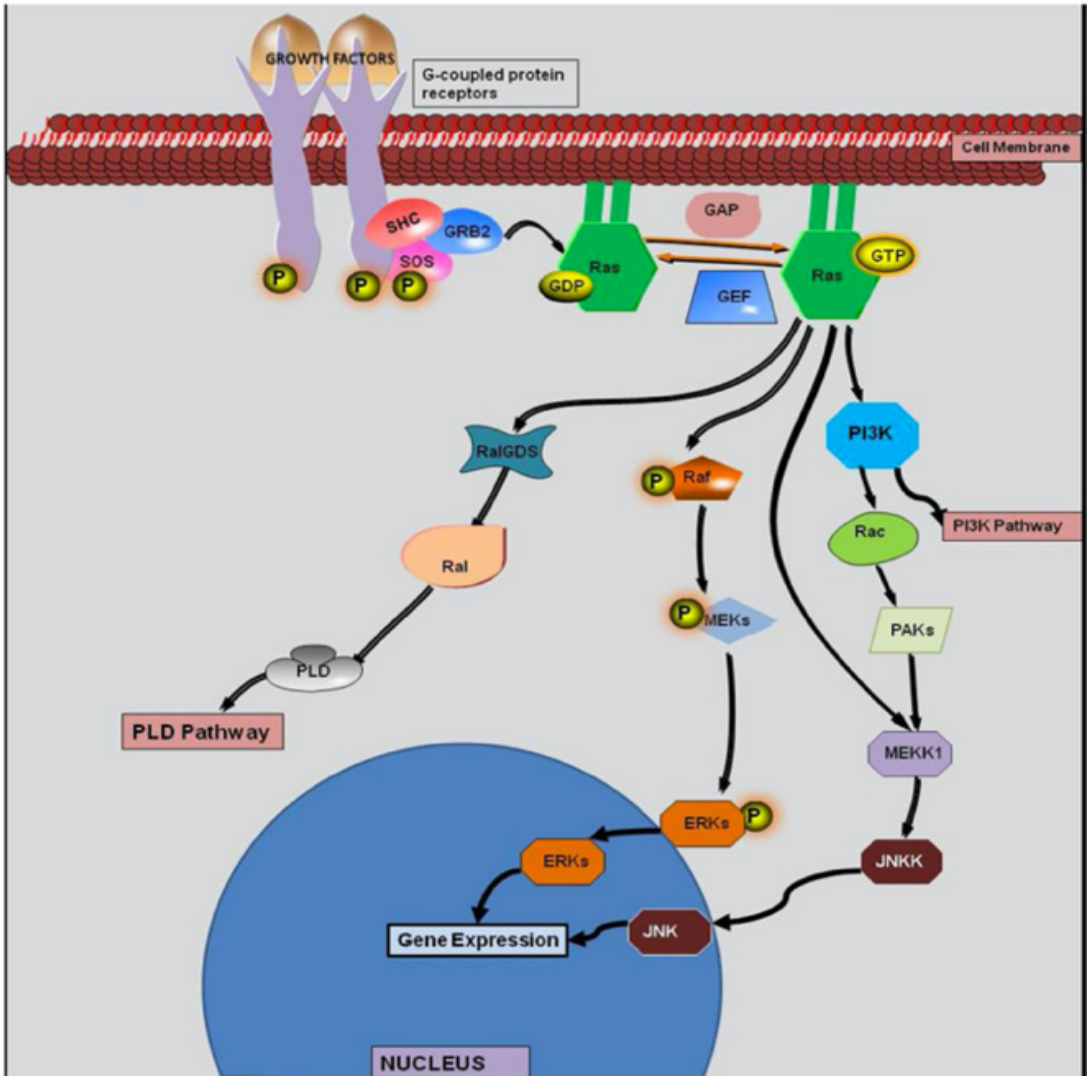

Figure 3 – The *Ras* Activation Cascade [44].

KRAS is the most prevalent isoform of RAS gene's in humans. It is positive in 30%–50% of cases with CRC, 80% of instances with pancreatic adenocarcinoma, 55% of cases with thyroid cancer, and 35% of cases with lung cancer [13-17]. KRAS was first discovered in a human lung cancer cell in 1982, and it has subsequently been demonstrated that 35% to 50% of all non-small cell lung cancers harbor this mutation [18-19].

Significance of KRAS mutations in tumor progress.

KRAS controls key signal transduction pathways in cells, including the PI3K-Akt, RAS-RAF-MAPK, and RAS-GEF signaling pathways that are connected to cell proliferation and cytokine production [19–21]. Consider the RAS-RAF-MAPK signaling cascade as an example. This pathway is the best known in cell biology. Activation occurs when the signal binds to the protein tyrosine kinase receptor. Upon binding of the EGFR and the platelet-derived growth factor receptor (PDGFR), kinase activation and transphosphorylation occur. This activation leads to the activation of the Grb2 protein, which binds guanine nucleotide exchange factors (GEF). GEF interacts with RAS proteins, further causing changes and the exchange of GDP for GTP, which contributes to the maintenance of an active form of KRAS [22].

Overall, when KRAS is mutated, it causes distribution of GTP hydrolysis. Thus, KRAS accumulates in an active state which leads to continuous activation of signaling pathways. Then it causes proliferation of cells (Figure 4) [23]. The constitutive active KRAS causes aberrant and uncontrollable cell growth and cell transformation, promotes cancer metastasis, and also increases resistance to chemotherapy and EGFR targeted therapy in many cancer types [24,25].

In CRC, KRAS mutations lead to abnormal activation of the RAS/ RAF/MEK/ERK signaling pathway. It is reported that KRAS mutations upregulates IGF-1R expression via a novel MEK-SP1-DNMT1-miR-137 pathway in CRC cells to promote the liver metastasis [26]. KRAS mutations also determine migration patterns and effectiveness through RhoA and Rac1 signals [27]. Studies have demonstrated that CRC HCT116 cells carrying KRAS mutants form a structure without normal cell polarity and lumen apoptosis, implying KRAS mutations are involved in the destruction of cell polarity and inhibition of apoptosis [28]. KRAS mutation also affects the tumor microenvironment. One study has demonstrated that KRAS mutation status is associated with the differentially expressed IL-17, IL-22, and IL-23 levels in the tumor microenvironment [29].

Many studies have shown that the KRAS oncogene stimulates the development of cancer in the pancreas and the prognosis is unfavorable. The mutation detection rate is 85%, and most of them appear to be SNP mutations. These mutations

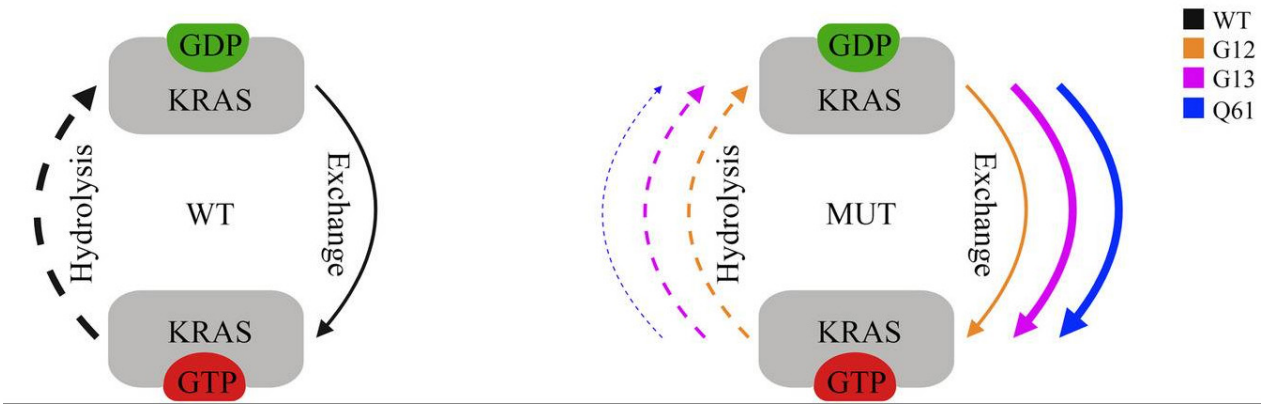

Figure 4 – KRAS activity regulation [23].

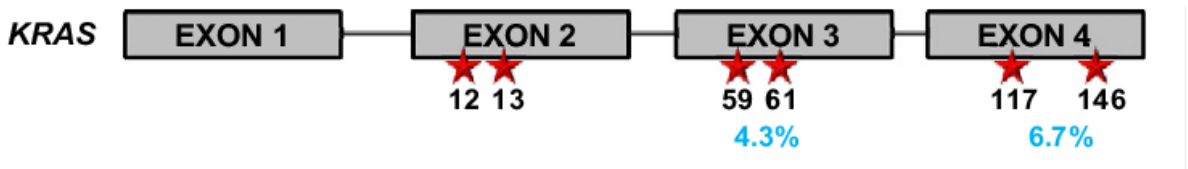

Figure 5 – Most common mutations in KRAS [45].

occur in three hotspot residues G12, G13 and Q61. The mutation detection rate is 85%, and most of them appear to be SNP mutations (Figure 5). Of these, the most common mutation is at codon 12 (17% of all KRAS mutations) [40].

With respect to CRC, approximately 40% of cases carry activating missense mutations in KRAS (Fig. 5). The most common of these are at codons 12 and 13 (80% are G12D, G12V, G12C, G12A and G13D), codon 61 (5% are Q61H, Q61L and Q61R) and 146 (2% are A146T and A146V) [42].

Purpose of the study: To review the literature on the role of KRAS mutation testing for management of cancer and to identify of KRAS mutations in Kazakhstani CRC and PC patients.

MATERIALS AND METHODS

DNA samples

Study protocols were approved by the IREC of Nazarbayev University #03-2021 from 21.04.2021. This study included primary data from 65 samples with CRC and PC patients from 2 oncological clinics: "Multidisciplinary medical center", Oncology center, Astana, and "National scientific center for oncology and transplantation", Astana, Kazakhstan.

Each patient provided written informed consent to participate in the study and for genetic testing. Clinical and demographic information and histological conclusions were taken from the medical records. Diagnosis of CRC and PC was histologically confirmed. Totally 63 patients with CRC (n=52) and PC (n=11) were recruited into the study during 2021-2022 year: 39 males and 24 females, mean age was 68 years (range 41–86 years). Blood samples, resected tissue samples flash frozen in liquid nitrogen after resection and FFPE samples were obtained from each patient.

Preliminary data include analysis of KRAS mutations of 12 cases. DNA was extracted from 2 types of samples for each patient: 7 samples of frozen tumor tissue and 9 paraffin embedded tissue (FFPE), of which 4 samples are represented by two materials: FFPE and frozen tumor tissue samples (Table 1). Sample Type 1 - Sections of pathological material were prepared and fixed on paraffin-embedded (FFPE) slides (004, 006, 008, 010, 012, 013, 014, 015, 069). Sample Type 2 – Frozen tumor tissue samples (003, 004, 009, 010, 013, 015, 024)

Among the 12 samples were 4 female and 8 male. Of these, there were 8 patients with moderately differentiated adenocarcinoma, 1 patient with poorly differentiated adenocarcinoma, and in 3 samples adenocarcinoma metastases (Table 1).

DNA extraction

FFPE sample gDNA was extracted using the ReliaPrep FFPE gDNA Miniprep System (Promega). All other total gDNA was extracted from tissue samples by using the Gentra Puregene Blood (QIAGEN) according to the manufacturer's instructions. Qualitative and quantitative evaluation of the obtained gDNA was carried out using the NanoDrop (Thermo Scientific) and Qubit (Invitrogen) platforms.

PCR and Sequence PCR

Two pairs of PCR primers were used to amplify exon 2 (primer pair A/B and primer pair C/D) and exon 3 (primer pair E/F and primer pair G/H). Primer pair C/D and primer pair G/H were designed to amplify an amplicon shorter than 180 bp. The primer sequence is shown in table 2. Each amplicon was sequenced in both forward and reverse directions using M13 primers. PCR was conducted in 50 μL final volume according to the KRAS Variant Identification protocol (Applied Biosystems) [31]. Sequencing reactions were carried out using the BigDye Terminator v.3.1 Cycle Sequencing Kit and were performed on a 3730xL Genetic Analyzer capillary electrophoresis system (Applied Biosystems).

Sequences were aligned to a reference sequence using SeqAssem (Applied Biosystems) and variants assessed by visual inspection.

RESULTS

In total, we identified 31 mutations in the KRAS gene, exons 2,3. We observed that each patient carry more than one mutation. The results of sequencing exons 2 and 3 of the

Table 1 – Sample characteristics.

N	ID number	Gender/age	Diagnosis	Materials	Histopathology
1	003	Male/55	PC, IV stage. metastases to the liver, lung.	Flash frozen tumor tissue samples	Moderately differentiated ductal adenocarcinoma (G-II) of the pancreas with metastasis in the liver tissue.
2	004	Female/60	Cancer of the rectum, I st. Condition after neoadjuvant chemoradiotherapy	FFPE and flash frozen tumor tissue samples	Adenocarcinoma of the colon with therapeutic pathomorphosis, with ulceration with foci of tumor growth in the submucosal and muscular layers.
3	006	Male/72	Cancer of the splenic angle of the large intestine, IIb stage.	FFPE samples	Moderately differentiated adenocarcinoma of the colon (GII) with germination of the entire thickness of the intestinal wall.
4	008	Male/71	Cancer of the rectosigmoid colon. Abdominal carcinomatosis. Metastasis to the liver, left adrenal gland, greater omentum.	FFPE samples	Metastasis of adenocarcinoma to the omentum.
5	009	Male/68	Cancer of the descending colon, III b stage.	Flash frozen tumor tissue samples	Moderately differentiated adenocarcinoma of the colon (G-II) with germination of the entire thickness of the intestinal wall with metastases in 2 of the 6 examined lymph nodes.
6	010	Male/64	Cancer of the rectosigmoid colon, IIB stage.	FFPE and flash frozen tumor tissue samples	Moderately differentiated adenocarcinoma of the colon (G-II) with germination of the entire thickness of the intestinal wall.
7	012	Female/80	Cancer of the sigmoid colon, IIb stage. Subcompensated stenosis.	FFPE samples	Moderately differentiated adenocarcinoma of the large intestine with invasion into all layers.
8	013	Female/64	Cancer of the lower ampulla of the rectum, IIa stage. Subcompensated stenosis.	FFPE and flash frozen tumor tissue samples	Moderately differentiated adenocarcinoma of the lower ampulla of the rectum with invasion into the muscular membrane, skin of the perianal region.
9	014	Male/76	Cancer of the sigmoid colon, IV stage. With decay. Stricture of the lower third of the left ureter. Hydrouretoronephrosis on the left.	FFPE samples	Poorly differentiated mucinous adenocarcinoma of the sigmoid colon, invading all layers of the wall, with perifocal inflammation.
10	015	Male/65	Cancer of the rectum, IIIB stage. Complicated by tumor stenosis in the stage of subcompensation.	FFPE and flash frozen tumor tissue samples	Moderately differentiated adenocarcinoma of the large intestine (GII) with germination of the entire thickness of the intestinal wall with metastases in 4 of the 18 examined lymph nodes.
11	024	Male/67	Cancer of the rectosigmoid colon, IIb stage. Chronic obstructive colonic obstruction in the stage of decompensation.	Flash frozen tumor tissue samples	Moderately differentiated adenocarcinoma of the large intestine (G-II) with germination of the entire thickness of the intestinal wall into the per intestinal fatty tissue.
12	069	Female/60	Cancer of the splenic flexure of the colon, IV stage. With germination in the stomach, diaphragm, pancreas. Multiple metastatic liver disease.	FFPE samples	Metastasis of adenocarcinoma of the colon to the greater omentum.

Table 2 – Primers

Ex2	Primer A	5' tgtaaaacgacggccagtTATTTGATAGTGTATTAACCTTATGTGTG 3'
	Primer B	5' caggaaacagctatgaccGAAACCTTTATCTGTATCAAAGAATG 3'
Ex3	Primer E	5' tgtaaaacgacggccagtAGGTGCACTGTAATAATCCAGA 3'
	Primer F	5' caggaaacagctatgaccCTATAATTACTCCTTAATGTCAGCTTATT 3'
M13	5' tgtaaacgacggccagt 3'	
M13	5' caggaaacagctatgacc 3'	

Table 4 – Nucleotide changes in exon 2 and 3 of the KRAS gene from human intestinal adenocarcinoma samples.

Case ID	Specimen	Base Change	Type	Effect	Aa Change
004	**004t**_KRAS_3ex_F_H08	191a>M	Sub	missense	Y64S
	004t_KRAS_3ex_F_H08	193a>M	Sub	missense	S65R
010	**010t**_KRAS_2ex_R_C06	23t>M	Sub	missense	V8[E,A]
	010t_KRAS_2ex_R_C06	26t>C	Sub	missense	V9A
	010t_KRAS_2ex_R_C06	27t>M	Sub	missense	V9A
	010t_KRAS_2ex_R_C06	28g>A	Sub	missense	G10R
	010t_KRAS_2ex_R_C06	28−29insM	Ins	frameshift insertion	–
	010t_KRAS_2ex_R_C06	31g>A	Sub	missense	A11T
	010t_KRAS_2ex_R_C06	32c>M	Sub	missense	A11D
	010t_KRAS_2ex_R_C06	66g>K	Sub	missense	Q22H
	010t_KRAS_2ex_R_C06	67c>Y	Sub	missense	L23L
012	**012FFPE**_KRAS_3ex_R_G10	256a>W	Sub	missense	N86Y
013	**013t**_KRAS_2ex_R_B06	41delt	Del	frameshift deletion	V14fs
	013t_KRAS_2ex_R_B06	51delt	Del	frameshift deletion	S17fs
	013t_KRAS_2ex_R_B06	52g>S	Sub	missense	A18P
	013t_KRAS_2ex_R_B06	57g>A	Sub	silent	L19L
014	**014FFPE**_KRAS_3ex_R_A11	212a>W	Sub	missense	Y71F
	014FFPE_KRAS_3ex_R_A11	216g>R	Sub	missense	M72I
015	**015t**_KRAS_2exF_A03	42a>W	Sub	silent	V14V
	015t_KRAS_2exF_A03	43g>K	Sub	missense	G15C
	015t_KRAS_2exF_A03	93a>W	Sub	missense	E31D
	015t_KRAS_2exF_A03	95a>W	Sub	missense	Y32F
	015t_KRAS_2exR _A06	65a>W	Sub	missense	Q22L
	015t_KRAS_2exR _A06	66g>K	Sub	missense	Q22H
	015t_KRAS_2exR _A06	67c>Y	Sub	silent	L23L
	015t_KRAS_3ex_R_A12	132−133insC	Ins	frameshift insertion	–
	015t_KRAS_3ex_R_A12	173c>M	Sub	silent	T58K
069	**069FFPE**_KRAS_3ex_R_F10	234delt	Del	frameshift deletion	L79fs
	069FFPE_KRAS_3ex_R_F10	252a>M	Sub	missense	I84I
	069FFPE_KRAS_3ex_R_F10	253a>W	Sub	missense	N85Y
	069FFPE_KRAS_3ex_R_F10	262a>W	Sub	missense	K88X

KRAS gene are presented in table 4. Analysis have revealed 7 mutations of the KRAS gene among 12 samples in exons 2 and 3. Detected mutations indicated only in patients with CRC, no mutations identified in patient with PC.

According to the data presented in Table 4, nucleotide substitutions, deletions, and silent mutations were identified in patients with moderately differentiated adenocarcinoma. Additionally, insertions were found in exons 2, 3 of the KRAS

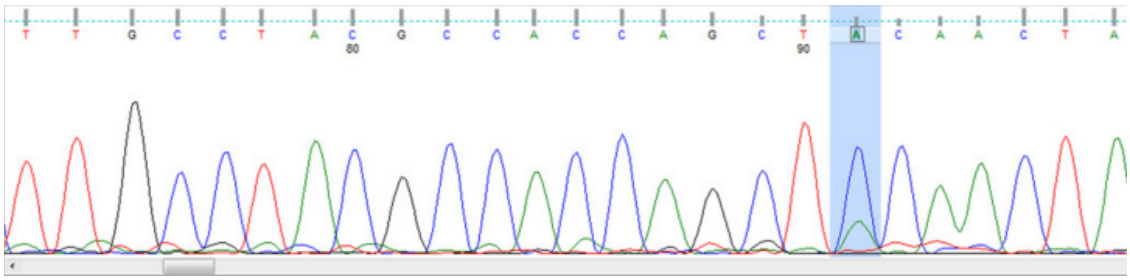

Figure 6 – Electropherogram of KRAS codon 10 mutation (c. 28G>A; p.G10R).

gene in two patients with metastases.

Among all identified mutations in exons 2 and 3 of the KRAS gene, missense mutations are the most common (70%). Identified missense mutations of exon 2 – c.43G>T (G15C), c.95A>T (Y32F), c.65A>T (Q22L), c.66G>T (Q22H), c.52G>C (p.A18T), c.26T>C (p.V9A), c.27T>C (p.V9A), c.28G>A (p.G10R), c.32C>A (p.A11D), c.31G> A (p.A11T), c.67C>T (p.L23L) are the most well-known and pathogenic in CRC. Several studies have shown that c.31G>A mutation leads to impaired purine processing [35,37,39]. Sequencing result of the 010 sample is represented in Figure 6.

The mutation in codon 32 (c.95A>T, p.Y32F) is show increased effectiveness of treatment with Scr and SHP2 inhibitors for sample 015. Another mutation variant c.23T>C in codon 8 indicate to highly resistant for treatment with sotarasib and adagrasib [38].

Also, found missense mutations of exon 3 of the KRAS gene in codons 71, 72, 88 and 64 are significant and have a severe course, leading to an unfavorable pathogenic prognosis [36, 37]. The exon 2 missense mutation c.93A>T (p.E31D) in the sample 015 has been described as a mutation that may contribute to resistance to anti-EGFR therapy [34]. Among identified missense mutations of exon 3: c.252A>C (p.I84I), c.253A>T (p.N85Y), c.256A>T (p.N86X), c.193A>C (p.S65R) are understudied and unknown variants.

We have found three variants deletion in 013, 069 samples and two frameshift insertion variants in samples 010 and 015 is not full researching and unknown variants. Other nucleotide substitutions that we identified and listed by the observed frequency included: three silent mutations of exon 2 of the KRAS gene - c.42A>T (p.V14V); c.67C>T (p.L23L); c.57G>A (p.L19L).

We checked our variants to the Mastermind, Cosmic, and SNPedia databases and their characteristics were described.

Of 12 patients with colorectal and pancreatic adenocarcinoma, 7 (58%) patients had a mutation in the KRAS gene. There were altogether 31 mutations in total, of which 70% are missense mutations, 17% frameshift mutations (deletions, insertions), and 13% silent mutations (Figure 7). Analyzing, we observe three samples 010, 013 and 015 with a lot of find mutations and including several variants mutation of the KRAS gene in exon 2,3. This mutations characterized his cancers form and helps for choose of patient management tactics.

In the present study we have demonstrated the constant and relatively high frequency of KRAS mutations in the 2 and 3 exons. It has been reported that the most common KRAS mutations are missense mutations representing 70% of all mutations reported and this was also confirmed with our study [32].

DISCUSSION

There are numerous investigations that are done on the KRAS gene in different countries. Taking into account known mutations of the KRAS gene it can be noted that their frequency and range of mutations depend on the studied populations. According to the literature, mutations in codons 12, 13, 61 of the gene KRAS are identified as pathogenic in CRC and PC [30, 33, 39]. Published studies provide important information to narrow down the number of KRAS mutations that can be used as pathogenic biomarkers. Moreover, results obtained by our research shows the presence of other mutations in exons 2,3 of the KRAS gene that require additional validation.

New optimized Sanger protocol was used to obtain reliable results. The use of a large cohort of patients to detect KRAS mutations will help to make a complete analysis of oncogenic mutations in CRC and PC. Going forward, we will expand the sample of patients with CRC and PC using our Sanger sequencing protocol. Getting reliable results on new KRAS variants in our population will allow implement genetic testing on KRAS mutations in to routine clinical practice in Kazakhstan.

CONCLUSION

In this review, we saw that mutations of oncogene KRAS is very important in the development, spread as well as in diagnostics and therapy of colorectal adenocarcinoma. It is very important to further develop research on this type of cancer, as through deeper study we can uncover and understand its mechanisms, processes, and interrelationships in depth in order to find better and more specific alternatives for therapeutic targets. Research is currently ongoing and further analysis of KRAS mutations will allow us to understand the role of KRAS mutations in the development of cancer in Kazakhstani patients, determine the genetic profile of mutations and correctly prescribe therapy.

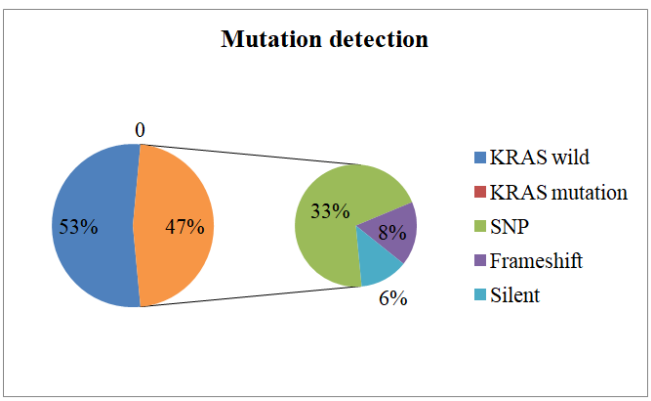

Figure 7 – The diagram demonstrates the number of identified mutations and their characteristics.

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КОЛОРЕКТАЛДЫ ЖӘНЕ ҰЙҚЫ БЕЗІНІҢ ҚАТЕРЛІ ІСІГІ БАР НАУҚАСТАРДАҒЫ KRAS МУТАЦИЯЛАРЫН БАҒАЛАУ

Саматқызы Д.[1], Рахимова С.Е.[1], Бегимбетова Д.А.[2], Куканова А.М[4]., Штефанов И.И.[5], Пироженко О.Л.[5], Ғабдулкаюм А.К.[1], Кожамкулов У.А.[1], Макишев А.К.[4], Төлеутаев М.Е.[5], Акилжанова А.Р.[1,*], Сарбасов Д.Ж.[3]

[1]Геномдық және Дербес Медицина Зертханасы, National Laboratory Astana жеке мекемесі, Назарбаев Университет, Астана, Қазақстан.

[2]Трансляциялық медицина және өмір туралы ғылымдар технологиялар зертханасы, National Laboratory Astana жеке мекемесі, Назарбаев Университет, Астана, Қазақстан.

[3]Жаратылыстану, әлеуметтік және гуманитарлық ғылымдар мектебі, Назарбаев Университеті, Астана, Қазақстан.

[4]"Астана медицина университеті" КЕАҚ, Астана, Қазақстан.

[5]"Көпсалалы медицина орталығы" МКК, Астана, Қазақстан.

**akilzhanova@nu.edu.kz*

ТҰЖЫРЫМ

RAS гені адам қатерлі ісігіндегі ең мутацияланған және зерттелген онкоген болып табылады. KRAS мутациясының болуы RAS эффекторларының белсенділенуінің жоғарылауын тудырады, нәтижесінде ісік тінінің дамуына әкеледі. KRAS мутацияларының аденокарцинома және басқа да қатерлі ісік түрлерімен науқастың болжамы мен өмір сүруіне әсері олардың молекулалық механизмдерін зерттеу үшін көптеген ғылыми зерттеулерді ынталандырады. Қазақстандағы мутантты KRAS жағдайларын емдеу хаттамасын одан әрі әзірлеу үшін біз Қазақстандық колоректальды ісігі мен ұйқы безі ісігі бар науқастардағы KRAS мутациясының жай-күйін зерттейміз. Бұл мақалада біз науқастарды басқару үшін KRAS мутациясының сынауының рөлі туралы әдебиеттерді қарастырамыз және колоректальды және ұйқы безінің қатерлі ісігі бар Қазақстандық науқастардағы KRAS мутациясының деректерін көрсетеміз.

Түйін сөздер: аденокарцинома, RAS, KRAS, Сэнгер секвенирлеу, миссенс мутация, кодон.

ОЦЕНКА МУТАЦИЙ KRAS У БОЛЬНЫХ КОЛОРЕКТАЛЬНЫМ РАКОМ И РАКОМ ПОДЖЕЛУДОЧНОЙ ЖЕЛЕЗЫ

Саматкызы Д.[1], Рахимова С.Е.[1], Бегимбетова Д.А.[2], Куканова А.М[4]., Штефанов И.И.[5], Пироженко О.Л.[5], Габдулкаюм А.К.[1], Кожамкулов У.А.[1], Макишев А.К.[4], Тулеутаев М.Е.[5], Акильжанова А.Р.[1,*], Сарбасов Д.Ж.[3]

[1]Лаборатория геномной и персонализированной медицины, Частное учреждение National Laboratory Astana, Назарбаев Университет, Астана, Казахстан.

[2]Лаборатория трансляционной медицины и технологий наук о жизни, Частное учреждение National Laboratory Astana, Назарбаев Университет, Астана, Казахстан.

[3]Школа естественных и гуманитарных наук, Назарбаев Университет, Астана, Казахстан.

[4]НАО "Медицинский университет Астана", Астана, Казахстан.

[5]"Многопрофильный медицинский цент", Астана, Казахстан.

**akilzhanova@nu.edu.kz*

АБСТРАКТ

Ген RAS является наиболее мутировавшим и изученным онкогеном рака человека. Наличие мутации KRAS вызывает повышенную активацию эффекторов RAS, что приводит к развитию опухолевой ткани. Влияние мутаций KRAS на прогноз и выживаемость пациентов с аденокарциномой и другими видами рака стимулирует множество научных исследований по изучению их молекулярных механизмов. Для дальнейшей разработки протокола лечения мутантных случаев KRAS в Казахстане мы изучаем статус мутаций KRAS у Казахстанских пациентов с колоректальным раком и раком поджелудочной железы. В этой статье мы делаем обзор литературы о роли тестирования мутации KRAS для ведения пациентов и демонстрируем предварительные данные о статусе мутации KRAS у Казахстанских пациентов с колоректальным раком и раком поджелудочной железы.

Ключевые слова: аденокарцинома, RAS, KRAS, секвенирование по Сэнгеру, миссенс-мутация, кодон.