DETECTION OF KRAS MUTATIONS AMONG LOCAL PANCREATIC CANCER PATIENTS

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ABSTRACT

Background

Pancreatic cancer poses a significant oncological challenge in the field of human health due to its aggressive behaviour and restricted scope of available treatment choices. Its multifaceted origin combined with genetic, environmental, and lifestyle factors, makes identification and management of this condition difficult.

Objectives

This study aims to investigate major KRAS gene mutations among local pancreatic cancer patients and further unveil underlying molecular mechanisms involved in the pathogenesis of this malignancy.

Materials and Methods

Blood and solid tissue samples were collected from patients diagnosed with pancreatic cancer after obtaining appropriate ethical approval. Genomic DNA was extracted, quality and quantity were evaluated prior to conducting gene mutation analysis using allele-specific PCR and specific primers for the detection of KRAS gene mutations in human tissues.

Results

Our data indicates that localization of tumors in the head of the pancreas was the most common among our study population; 34.5% of the patients were overweight according to body mass index; 21.9% had a history of other cancers, while 37.5% had a family history of pancreatic cancer. The KRAS mutation analysis detected 7 KRAS mutations in pancreatic tumor tissue: KRASG12C, KRASG12V, KRASG12D, KRASG13D, KRASG12R, KRASG12S, KRASG12A. The KRASG12C had the highest mutation rate (81.3%), followed by KRASG12V (73.5.4%) in pancreatic cancer patients.

Conclusion

Our findings highlight the importance of further investigating KRASG12C and KRASG12V mutations in the KRAS gene which may have implications for finding better diagnostic and targeted treatment strategies.

Keywords: *KRAS, gene, mutation, pancreatic cancer.*

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INTRODUCTION

Pancreatic cancer persists as a formidable adversary in the field of oncology, marked by its aggressiveness and resistance to therapeutic interventions [1-3]. This malignancy, renowned for its dismal prognosis and global health impact, presents a daunting challenge of paramount significance [4, 5]. Pancreatic cancer's onset can be influenced by various factors such as excess body weight, the presence of type 2 diabetes, family history and the consumption of tobacco products [6, 7]. Recent years have witnessed a striking surge in our understanding of the genetic underpinnings that govern the unknowable pathogenesis of pancreatic cancer [8, 9].

Approximately 5–10% of pancreatic cancer cases are believed to be the result of inherited risk factors [10]. Various familial cancer syndromes have been connected to an elevated likelihood of developing pancreatic cancer. One such syndrome is Peutz-Jeghers syndrome, which arises from a mutation in the serine/threonine kinase 11 (STK11) tumor suppressor gene and leads to a 35% increased risk of pancreatic cancer [11, 12]. Similarly, hereditary breast-ovarian cancer syndrome, primarily associated with mutations in Breast Cancer gene 1 (BRCA1) or Breast Cancer gene 2 (BRCA2), has been linked to an increased susceptibility to pancreatic cancer [13-15]. Mutations in Cyclin Dependent Kinase Inhibitor 2A (CDKN2A), associated with mole melanoma, was also linked with a 17% increased risk of pancreatic cancer [13, 16]. Germline mutations in genes crucial for the DNA damage response (e.g., ATM) and DNA repair (e.g., MLH1, MSH2, MSH6 and PALB2) are also correlated with an elevated risk of developing pancreatic cancer [17, 18].

Genetic mutations are detectable in nearly all cases of pancreatic cancers, including various alterations such as point mutations, insertions, deletions, amplifications, translocations, fusions, and inversions. Among the multitude of mutated genes, KRAS, TP53, CDKN2A, SMAD4, CDKN2B and ARID1A are consistently among the most frequently observed in pancreatic cancer cases [19, 20]. These genetic mutations can lead to functional changes in proteins, contributing to uncontrolled cell proliferation, enhanced cell mobility, reduced autophagy, and compromised DNA repair mechanisms [21, 22]. Kirsten rat sarcoma viral oncogene (KRAS) stands out as the most frequently mutated gene in pancreatic cancer residing on human chromosome 12 and encoding a small GTP enzyme responsible for downstream signal transduction from growth factor receptors [23-26]. Its pivotal roles span a wide range of cellular processes, including the regulation of cell growth, proliferation, differentiation, and apoptosis [27]. Mutations in the KRAS gene are identified in over 90% of pancreatic cancer cases and are considered the most common genetic alterations associated with carcinogenesis [28]. The primary mutation observed in KRAS leads to substitution of glycine (G) with aspartic acid (D) at the 12th position of the KRAS protein [28, 29]. Despite significant progress, the field faces numerous challenges, including an incomplete understanding of the functional consequences of many identified mutations, the difficulty of targeting specific mutations with therapeutic agents, and the intricate interplay between genetic determinants and environmental factors [30, 31].

The therapeutic options for pancreatic cancer are notably restricted and hindered by the inherent complexity of the disease at the genomic, epigenetic, and metabolic levels [32]. This complexity manifests as the activation of multiple pathways and intricate crosstalk among them. Surgical resection is a viable option for fewer than 20% of pancreatic cancer patients [33]. A broader perspective encompassing various genetic aberrations is crucial for a comprehensive understanding of pancreatic cancer's intricate biology and to overcome the challenges associated with translating these genetic insights into effective diagnostic and therapeutic strategies. This study aims to investigate mutations in KRAS gene among local pancreatic cancer patients in order to further unveil the genetics of this deadly malignancy which might lead to the development of targeted molecular therapeutic and diagnostic strategies.

MATERIAL AND METHODS

Samples

The study was carried out in the Advanced Molecular Biology Laboratory, College of Science, University of Sulaimani; Molecular Laboratory, Hawkari Nishtiman and PharmaGene Laboratory in Sulaimani, Iraq. Patients with different stages and grades of pancreatic cancer who were admitted to Hiwa Cancer Hospital from 10 April 2022 to 15 July 2022 were included in this study. Blood samples were collected in EDTA tubes to prevent blood clotting. Whole blood samples were centrifuged at 3500 g for 5 min. to obtain the plasma. Plasma was immediately aliquoted into sterile 1.5-mL Eppendorf tubes and stored at -20 or - 80 °C until use. The solid tissue samples were histopathology paraffin blocks (FFPE) obtained from Histopathology Department

of Shorsh Hospital. The process of FFPE sampling and data collection was performed from March 2022 to July 2022. FFPE samples collected between 2018-2022 were also used for this study. Appropriate ethical approval was obtained prior to sample collection from each patient. The study was approved by the Research Ethics Committee of the Biology Department, College of the Science, University of Sulaimani.

Extraction of Genomic DNA

Genomic DNA was extracted from plasma and solid tissue samples using QIAamp ® DNA Blood Mini Kit and QIAamp® DNA FFPE Tissue Kit respectively, following the manufacturer's instructions.

Determination of DNA concentration and purity

The quality and quantity of isolated DNA was determined by spectrophotometer (NanoDrop Lite, Thermo Fisher Scientific, USA) and measured by the ratio of DNA optical density (A260) and protein optical density (A280). The assumption was that samples with A260 A280 ratios of 1.7 - 2.1 were pure and free of contaminants.

Real-time Polymerase Chain Reaction

Two general approaches for assay design were employed for KRAS mutation testing. An allele-specific probe or (ARMS PCR) and a post-PCR melting-curve analysis. In allele-specific real-time PCR, the assay uses ARMS PCR technology for DNA amplification combined with Taqman probes for the detection. It includes mutation-specific reactions for codons 12 and 13 of exon 2 of the KRAS oncogene, and a wild-type control for exon 4.

KRAS Mutation Screening

The KRAS mutation analysis real-time assay was based on allele-specific PCR. Mutation-specific amplification was achieved by mutation-specific primers that distinguished matches and mismatches at the 3'-end of a PCR primer. The detection of the amplification product was done by using fluorescent hydrolysis probes. Each probe contained a fluorophore (FAM or VIC) at the 5'-terminus and a quencher at the 3'- terminus. Probes complementary to the KRAS gene was labelled with the FAM fluorophore, while the probe complementary to the endogenous control gene was tagged with the VIC fluorophore. Signal from the VIC tagged probe indicated that there was sufficient amplifiable DNA template in the reaction, while a signal from the FAM fluorophore indicated the presence of a mutation. The Oncoscreen KRAS Mutation Screening Kit is intended for the detection of seven hot spot somatic mutation of KRAS exon 2 (listed in Table 1). The PCR cycling conditions has been described in Table 2.

Statistical analysis

Statistical analyses were performed using descriptive statistics (Mean, Standard Deviation, Minimum, and Maximum) and frequency distribution tables to describe the variables. Forward Stepwise Regression was used to determine the candidate predictors or variables that entered the logistic regression model. The SPSS (Version 26) and JMP-Pro (Version 16) were used as statistical software tools to analyse our data.

Table 1. List of mutations detected by Oncoscreen KRAS mutation screening kit.

Mutation	Nucleotide Change	Cosmic ID	Exon
GLY12ALA (G12A)	GGT>GCT	522	2
GLY12ASP (G12D)	GGT>GAT	521	2
GLY12AG (G12R)	GGT>CGT	518	2
GLY12CYS (G12C)	GGT>TGT	516	2
GLY12SER (G12S)	GGT>AGT	517	2
GLY12VAL (G12V)	GGT>GTT	520	2
GLY13ASP (G13D)	GGC>GAC	532	2

Table 2. PCR cycling conditions for used for detection of KRAS mutations.

Parameter	Temperature	Time	Number of cycles	Data collection
Polymerase Activation	95 °C	10 minutes	1	No
Denature	95 °C	15 seconds	50	No
Annealing & Extension	60°C	60 minutes	50	Yes

RESULTS

Clinical characteristics

Our data indicates that the mean age of pancreatic cancer patients from which plasma samples were collected was 59.9 years, while for the solid tissue samples was 59.79 years. Furthermore, the mean Tumor size (T) for the plasma samples was 4.22 cm, and in the solid tissue samples was 4.909 cm, (Table 3). The rate of pancreatic cancer was higher in males (56.3%) compared to females (43.7%) in plasma samples, but equal in the solid tissue samples (Figure 1).

In most of the cases, the tumors were located in the head of the pancreas (50% of patients that plasma samples were obtained from and 53% of the patients that solid tissue samples were obtained from). The frequency of tumor localization in other parts of pancreas were as follow: the body (18.7% plasma samples; 2.9% solid tissue samples); the tail (6.3% plasma samples; 20.6% solid tissue samples); the body & tail at the same time (9.4% plasma samples; 6% solid tissue samples); tail & neck at the same time (6.3% plasma samples; 3% solid tissue samples); the bile duct & ampulla of vater (9% solid tissue samples); the head & body of pancreas at the same time (3% solid tissue samples) (Figure 2). Our data indicates that in both types of sample populations, the majority of cases (78.1%) were patients diagnosed with pancreatic ductal adenocarcinoma (78.1%), followed by pancreatic neuroendocrine tumor (15.6%). Only 6.3% of the cases were diagnosed with other type of pancreatic cancer, including pancreatic adenocarcinoma (Figure 3). Our results indicate that 34.3% of the plasma sample and 14.7% of the solid tissue sample populations were in the highest stage of the cancer which is stage four. The frequency of other pancreatic cancer stages distribution was as follow: stage three (31.3% plasma samples; 5.9% solid tissue samples); stage two (28.1% plasma samples; 47.1% solid tissue samples); stage one (6.3% plasma; 32.4% solid tissue sample populations) (Figure 4). The distribution of grades of pancreatic cancer development among patient's population were as follow: grade 1 (16% plasma sample; 48.5% solid tissue samples); grade 2 (62% plasma sample; 36.4% solid tissue samples); grade 3 (22% plasma; 15.1% solid tissue samples) (Figure 5). Our data from plasma sample population reveal that most of the patients (53.1%) had never smoked before. In contrast, smaller ratio of patients (31.3%) were active smokers, while 15.6% of the patients had quit smoking (Figure 6). The body mass index (BMI) of the patients indicate that most of the patients (37.5%) have their BMI in the normal range, followed by the overweight patients (34.5%) and obese group (28.1%) (Figure 7). The data shows that none of the patients included in the study were consuming alcohol. Meanwhile, 34.4% of the patients had Chronic diabetes mellitus, and 46.9% had chronic pancreatitis; while 21.9% had a history of other cancer types, and 37.55% had a family history of pancreatic cancer (Figure 8).

Detection of common mutations in KRAS gene in local pancreatic cancer patients

The KRAS mutation analysis was carried out using allele-specific PCR and mutation specific primers. The reference gene was positive for each sample, with a Ct value in the 25–30 range indicating sufficient DNA quantity for evaluation (Figure 9). Calculated sample ΔCt values as the difference between the mutation target Ct and control target Ct from the same sample and compared with predetermined cut-off values detected 7 mutations in the KRAS gene (Figure 10).

Our results show that in the plasma samples the highest mutation rate was the KRASG12C among the pancreatic cancer patients (81.3%), followed by KRASG12V (59.4%), then KRASG12D (15.6%) and KRASG13D (9.4%). KRASG12R and KRASG12S were equally present among the pancreatic cancer patients (6.3%). No mutation in the KRASG12A was detected (Figure 10). In the solid tissue samples the highest detected mutation was KRASG12V (73.5.4%), followed by the KRASG12R and KRASG12D (50%), then KRASG13D (41.2%), and KRASG12C (44.1%). The lowest mutation rate detected was KRASG12S (11.8%) (Figure 10).

Table 3. Age and tumor size in plasma and tissue samples from pancreatic cancer patients.

Groups	Plasma samples			Tissue samples		
	No.	Mean	Standard Deviation	No.	Mean	Standard Deviation
Age (year)	32	59.59	11.983	34	59.79	17.313
Tumor size (T), cm	32	4.22	3.8056	34	4.909	3.4802

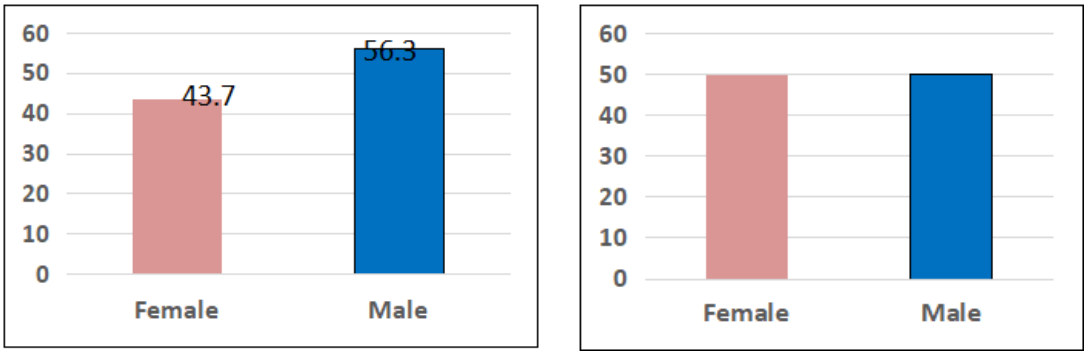


Figure 1. Gender distribution among pancreatic cancer population.
(a) Frequency and percentage of gender distribution among pancreatic cancer population in which plasma samples were collected. (b) Frequency and percentage of gender distribution among pancreatic cancer population in which solid tissue samples were collected.

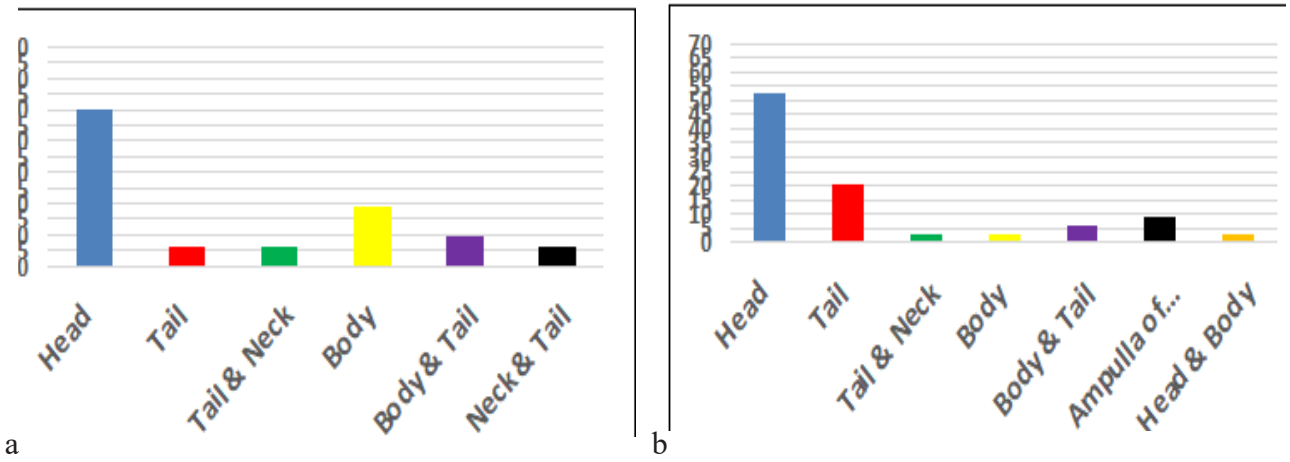


Figure 2. Frequency of tumor location on pancreas.
The Frequency distribution of tumor location on pancreas: (a) in plasma samples, (b) in solid tissue samples.

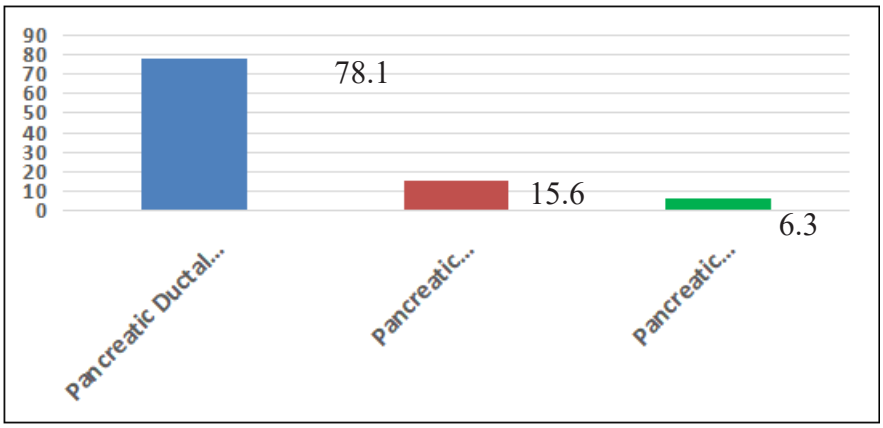


Figure 3. Frequency of pancreatic cancer types.
The pancreatic cancer types were divided in to pancreatic ductal adenocarcinoma (78.1%), pancreatic neuroendocrine tumor (15.6%) and pancreatic Adenocarcinoma (6.3%).

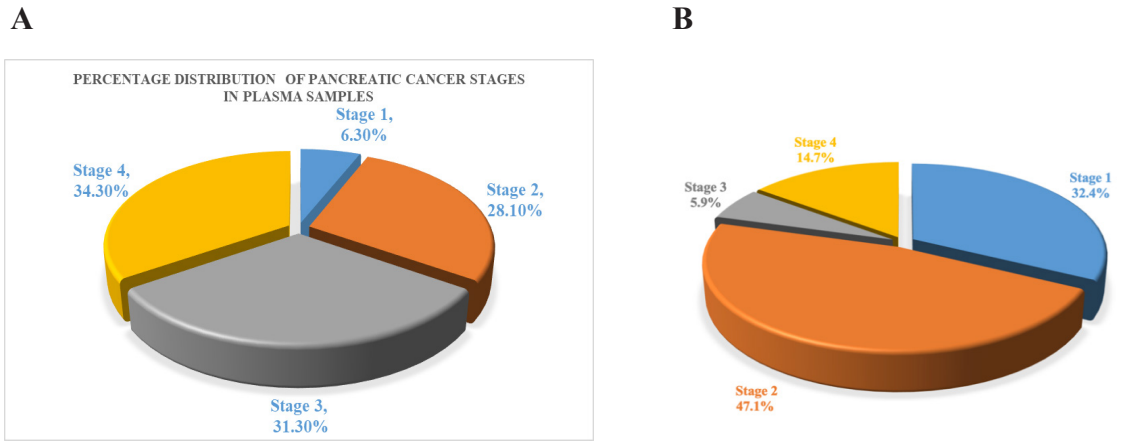


Figure 4. Percentage & frequency distribution of pancreatic cancer stages.
The percentage & frequency distribution of pancreatic cancer stages in (a) plasma and (b) solid tissue sample populations.

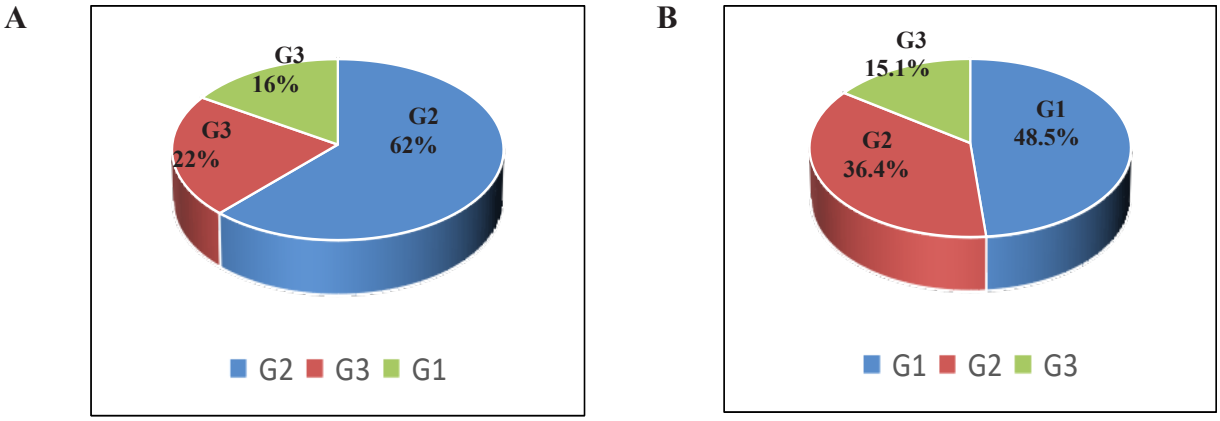


Figure 5. Percentage distribution of pancreatic cancer grades.
The Frequency distribution of pancreatic cancer grades: (a) in plasma samples, (b) in solid tissue samples.

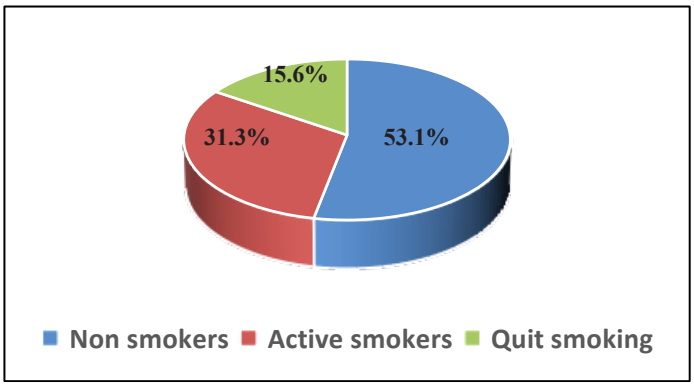


Figure 6. Percentage of smokers among pancreatic cancer patients.
The Frequency distribution of smoking habit among the pancreatic cancer patients.

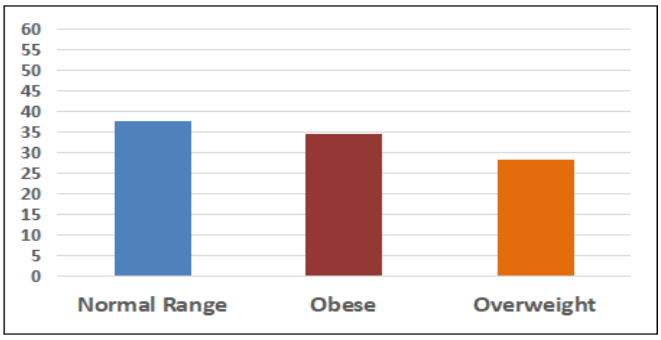


Figure 7. Percentage & frequency distribution of Body Mass Index.

The Frequency of BMI distribution among the pancreatic cancer patients, divided in to normal range, overweight and obese categories.

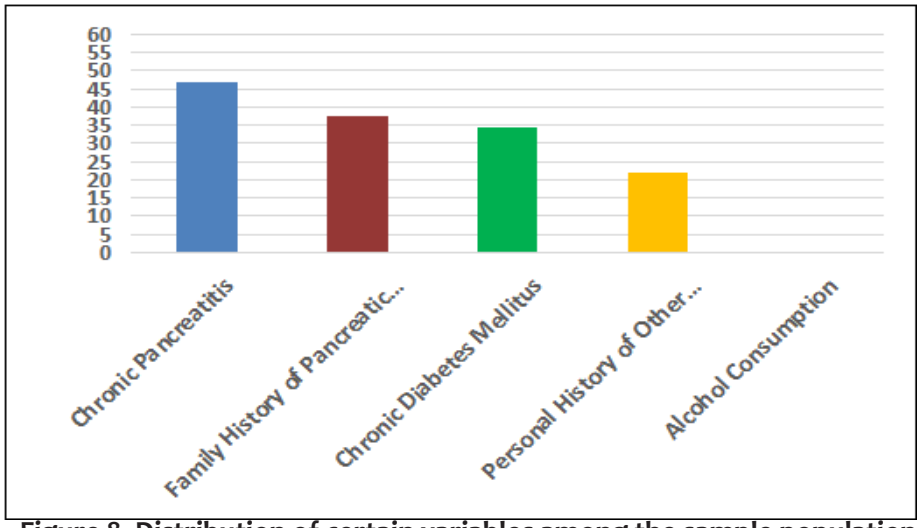
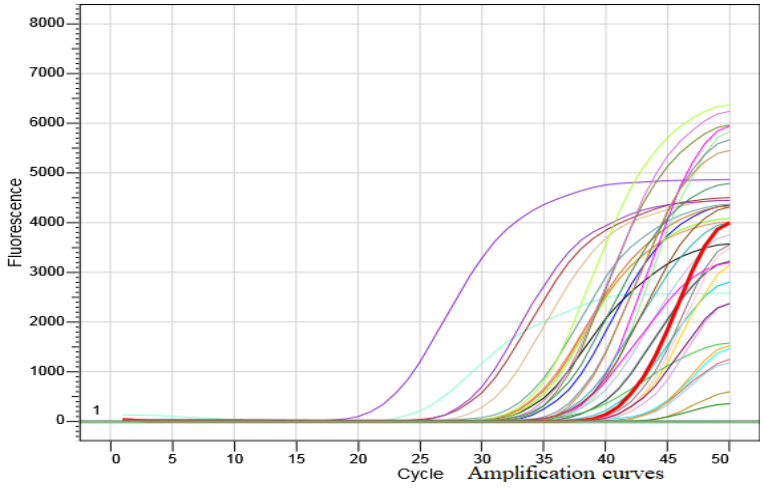


Figure 8. Distribution of certain variables among the sample population.

The percentage of alcohols consumption, chronic diabetic mellitus, chronic pancreatitis, personal history of other cancer, and family history of pancreatic cancer among the pancreatic cancer patients.

(a)



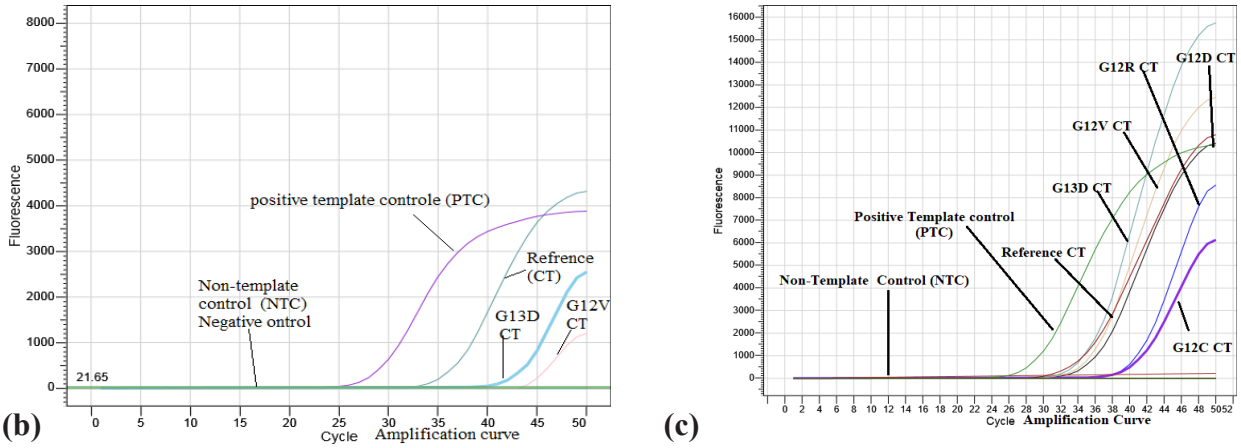


Figure 9. The amplification plots for allele-specific polymerase chain reactions.
(a) Amplification plots for combined samples run showing sharp single peak for each sample. (b) An example of amplification plots for plasma sample no. 2 showing KRAS mutations. (c) An example of amplification plots for solid tissue sample no. 1 showing KRAS mutations.

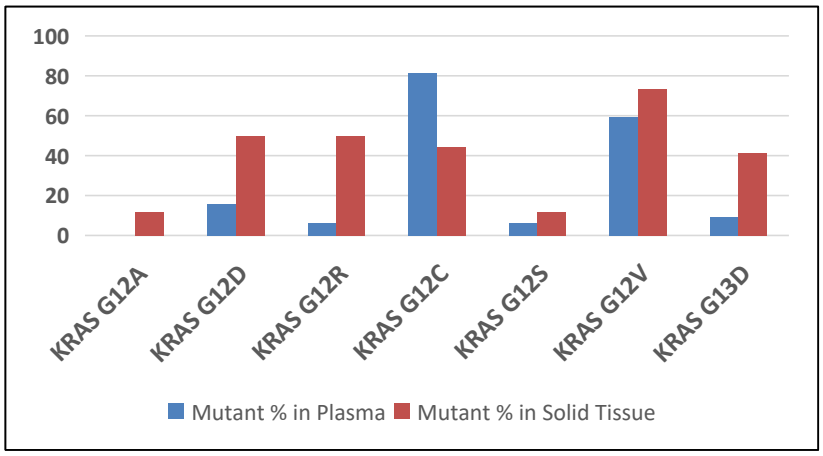


Figure 10. Frequency of mutant types & wild type of KRAS in pancreatic cancer patients.
The percentage of mutant types & wild type of KRAS in plasma and solid tissue samples of pancreatic cancer patients.

DISCUSSION

Pancreatic cancer stands as a significant contributor to cancer-related fatalities, characterized by an overall prognosis that has shown little improvement over several decades [19, 29, 34]. Presently, the challenge of prevention or early detection at a treatable stage remains formidable as symptoms seldom manifest in patients, and the tumor lack sensitive and specific markers to facilitate timely identification [31, 35]. Most cancers, including pancreatic, are very genetically complex [36].Many cancers develop because of gene mutations, which either render proteins in cells defective or totally delete them [37]. Pancreatic cancers exhibit a limited number of common genetic mutations, and none of them are presently amenable to pharmaceutical intervention [38].

While significant advancements have been made in understanding the genomics and signalling pathways of pancreatic cancer development, these efforts are impeded by challenges related to tumor heterogeneity, the limited availability of druggable targets, tumor evolution, early detection difficulties, and complex pathway interactions [39]. Therefore, studying gene mutations is essential for understanding the pancreatic cancer's underlying molecular mechanisms and

advancing our ability to detect the cancer at earlier stages.

Our data reveals that pancreatic cancer had a non-significantly higher occurrence in males (56.3%) compared to females (43.7%) when analysing plasma samples, but the rates were equal in solid tissue samples of our study population. The most common tumor location in the pancreas was in the head, according to our study. The results demonstrated that 34.5% of the patients were classified as overweight based on their body mass index (BMI). Additionally, 21.9% of the patients had a history of other types of cancer, and 37.5% had a family history of pancreatic cancer.

When we conducted KRAS mutation analysis using allele-specific PCR and mutation-specific primers, seven mutations in the KRAS gene have been identified within the pancreatic tumor tissue. These mutations included KRASG12C, KRASG12V, KRASG12D, KRASG13D, KRASG12R, KRASG12S, and KRASG12A. Among the pancreatic cancer patients, KRASG12C had the highest mutation rate in plasma samples, with a prevalence of 81.3%. Conversely, in solid tissue samples, the highest mutation rate was KRASG12V, which accounted for 73.5%.

The detection of seven distinct mutations in the KRAS gene associated with pancreatic cancer in Iraq sheds light on the genetic diversity of this deadly disease within a specific population. The identification of KRAS gene mutations in this study aligns with global trends where KRAS mutations are among the most common genetic alterations in pancreatic cancer [40, 41].

Four extensive research investigations into pancreatic cancer revealed presence of KRAS mutations in 85.8% of the examined pancreatic tumor samples [42, 43]. The majority of these mutations were characterized as missense mutations, which predominantly cluster in three key hotspot residues: G12, G13, and Q61 [40, 43]. Notably, the most common variants within these KRAS mutations are KRASG12D, accounting for 39.2% of all occurrences, and KRASG12V, representing 32.5% of the total mutations, which is in line with the results of our current study. In a research investigation, the KRASG12C mutation was observed to have a significant occurrence rate, and discovered that Sotorasib exhibits anti-cancer effects in individuals diagnosed with advanced pancreatic cancer bearing the KRASG12C mutation [44].

Lately, novel covalent inhibitors directed at the KRASG12C oncoprotein have emerged. These inhibitors have demonstrated encouraging efficacy in the early stages of clinical trials involving individuals with KRASG12C mutant pancreatic cancer [40]. In a research study, KRAS mutations were detected in 72% of the tumor samples, and 44% of these tumors exhibited the KRASG12D mutation, 20% had the KRASG12V mutation, and 10% displayed the KRASG12C mutation [45]. Multiple studies have independently identified the presence of the KRASG12V mutation in individuals diagnosed with pancreatic cancer [23, 24]. The distribution of KRAS mutations within the Iraqi population appears to follow certain patterns, with KRASG12V and KRASG12C being the most prevalent mutations, consistent with global reports [25, 46]. Understanding the distribution of specific mutations is essential as different KRAS mutations may have distinct functional consequences. For example, KRASG12D has been associated with increased aggressiveness and resistance to therapy, while KRASG13D mutations have shown differences in responsiveness to specific treatments [47, 48].

The identification of these seven mutations has important clinical implications. Firstly, it can guide the development of targeted therapies that aim to inhibit the activity of mutant KRAS proteins. As therapies targeting KRAS mutations are actively being investigated, understanding the prevalence of specific mutations can help tailor treatment strategies for patients. The presence of certain KRAS mutations may serve as predictive biomarkers for treatment response and prognosis [49].

CONCLUSION

The detection of KRASG12C and KRASG12V as the most common mutated alleles in the KRAS gene associated with pancreatic cancer in local population highlights the genetic diversity of this disease in a specific population. These findings highlight the importance of carrying out larger-scale investigations of KRASG12C and KRASG12V mutations in the KRAS gene which may have implications for the development of molecular diagnostic and targeted therapeutic strategies.

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