SYSTEMATIC REVIEW

The Role of *K-Ras* and *P53* in Biliary Tract Carcinoma

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Abstract

Objective: To focus mainly on the role of proto-oncogene Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (K-Ras) and tumour-suppressor gene p53 which are among the most commonly mutated genes in biliary tract carcinomas.

Methods: The systematic review comprised research articles published between 2002 and 2019 on PubMed and Google Scholar databases which were searched using the terms 'TP53', 'K-Ras', 'mutation', 'biliary tract carcinoma', 'cholangiocarcinoma', and 'murine model'. Repetitions, duplicates and irrelevant articles were excluded. No data was retrieved from posters, presentations and symposiums, and experiments involving bile aspirations were also excluded.

Results: Of the 72 articles reviewed, 11(15.3%) were included. Of them, 3(27.3%) studies, conducted in China, Japan and Taiwan, reported a positive correlation between K-Ras mutation and biliary tract carcinoma. Only 1(9%) study, conducted in China, showed the sole correlation between p53 inactivation and biliary tract carcinoma. Also, 4(36.4%) studies, conducted in China, Japan and Europe, showed a positive association of both K-Ras mutation and p53 inactivation with biliary tract carcinoma.

Conclusion: K-Ras and p53 mutation both contribute to biliary tract carcinoma. K-Ras mutation, however, has a much higher frequency compared to p53 inactivation in such cancers.

Keywords: P53, K-Ras, Mutation, Biliary tract carcinoma, Cholangiocarcinoma, Murine models. (JPMA 71: 2378; 2021)

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Introduction

Biliary tract cancers (BTCs) are among the most aggressive and highly fatal, malignant tumours[1] with cholangiocarcinoma forming the second most common form of hepatobiliary cancer.[2,3] Hepatobiliary carcinomas include gall bladder, intrahepatic, perihilar and distal biliary tree cancers, with the majority being classified as Klatskin tumours, which form at the site where the right hepatic duct joins with the left hepatic duct in the liver.[1] Cholangiocarcinoma, carcinoma of the gall bladder specifically, is divided into four types: tubular adenocarcinomas, papillary adenocarcinomas, mucoid carcinomas, and undifferentiated carcinomas.[4] These invasive carcinomas mainly arise from the epithelium lining of gall bladder and the bile ducts. The most important among them are the biliary intraepithelial neoplasia (BilIN).

BilIN is further categorised into three types: BilIN1 corresponding to low-grade dysplasia, BilIN2 corresponding to high-grade dysplasia, and BilIN3 corresponding to non-invasive or in situ carcinoma.[5] BilIN follows a developmental sequence with a stepwise progression through BilIN1, BilIN2 and BilIN3 to invasive intraepithelial cholangiocarcinoma.

BTCs have poor prognosis with five-year survival rates being 30-50% for resectable tumours and <5% for unresect¬able cases.[6] Old age, obesity, bile duct inflammation, presence of biliary cysts, infection with the liver fluke parasite, diabetes and pre-existing medical conditions, such as primary sclerosing cholangitis and chronic ulcerative colitis, predispose an individual to such cancers. However, a number of molecular alterations have also been identified to be involved in the pathogenesis of hepatobiliary carcinomas. Allelic loss of p53 and bcl-2 and oncogenic activation of the proto-oncogenes such as Kirsten rat sarcoma (K-Ras), master regulators of cell cycle entry and proliferative metabolism are c-myc, c-neu, c-erb-B2, c-met, receptor tyrosine kinases, cyclooxygenase-2 (COX-2) and human aspartyl (asparaginyl) beta-hydroxylase fall in this category. Mutations in K-Ras and p53 genes are found to be the most common genetic alterations in BTC patients.

K-Ras gene, encoded by the K-Ras viral gene homolog,[7] acts through the K-Ras / Mitogen-activated protein kinases (MAPK) cell signalling pathway.[8] On a molecular level, several germline point mutations have been identified in the K-Ras gene that result in the alteration of both structure and function of the corresponding protein with each protein having different molecular pathways. K-Ras mutations account for the pathogenesis of half of human cancers, including BTCs. Single amino acid substitutions are

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commonly found in codon 12 Glycine12Serine (G12S) genotype/ exon 1) and codon 13 in exon 1 of the K-Ras gene, while mutations have also been detected of a lower frequency in codons 61, 63, 117, 119 and 146.[7] The oncogenic mutation in codon 12 results in the single amino acid substitution (Glycine➠aspartic acid/ valine/ arginine) and activates the Guanosine triphosphate/ Guanosine diphosphate (GTP/GDP) binding protein, which upon activation initiates a pathway that terminates in the unregulated proliferation of cells.[9] K-Ras is also known to upregulate Glusocse tranporter 1 (GLUT-1) transporter in cells, thereby contributing to the Warburg effect, or unlimited proliferation of cells with energy derived from anaerobic glycolysis in preference to aerobic glycolysis due to increased uptake of glucose, in cancer cells.[10]

Tumour protein (TP53) gene is a tumour suppressor gene located at 17p 13.3.[9] It plays a central role in the pathogenesis of BTCs too, accounting for 30% of all gall bladder carcinomas. P53 controls the gap 1 (G1/S) phase and growth 2 (G2/M) phase of the cell cycle[11] allowing cycle cell arrest and deoxyribonuclease acid (DNA) repair to occur. Inactivation of p53 gene due to a mutation from exon 5 through exon 8 results in unregulated cell proliferation without DNA repair, which, in turn, results in tumour formation.[12]

The current systematic review was planned focussing mainly on the role of proto-oncogene K-Ras and tumour-suppressor gene p53 which are among the most commonly mutated genes in BTCs.

Methods

The systematic review comprised research articles published between 2002 and 2019 on PubMed, Nature, Wiley Online Library and Google Scholar databases which were searched using the terms 'TP53', 'K-Ras', 'mutation', 'biliary tract carcinoma', 'cholangiocarcinoma', and 'murine model'. Repetitions, duplicates and irrelevant articles were excluded. No data was retrieved from posters, presentations and symposiums, and experiments involving bile aspirations were also excluded.

Results

Of the 72 articles reviewed, 11(15.3%) were included (Figure). On an eight-item appraisal scale, 2(18.2%) studies scored a perfect 8 (Table-1), Of the 11 studies, 3(27.3%) studies, conducted in China, Japan and Taiwan, reported a positive correlation between K-Ras mutation and BTC; 1(9%) study, conducted in China, showed the sole correlation between p53 inactivation and BTC; and 4(36.4%) studies, conducted in China, Japan and Europe, showed a positive association of both K-Ras mutation and p53 inactivation with BTC (Table-2).

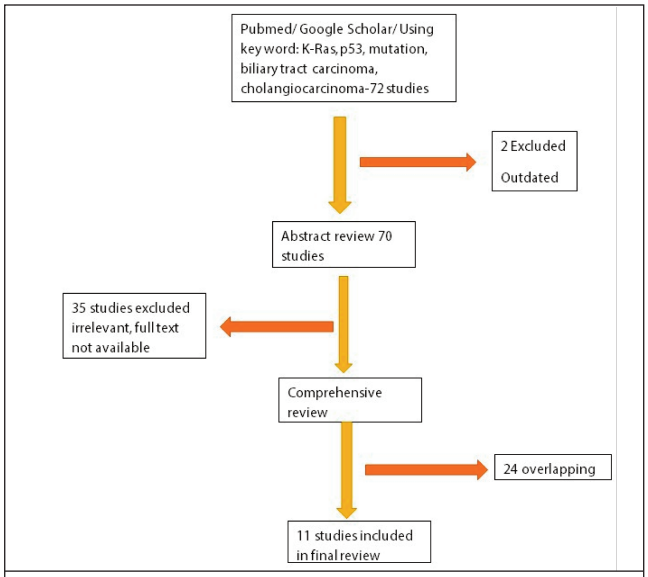

Figure: Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) flow-chart of literature search.

Role of K-Ras mutation in BTC: Different types of carcinomas are reported to show different frequencies of K-Ras mutation. For example, the frequency of K-Ras mutation is much higher in biliary intraepithelial neoplasia compared to intrahepatic cholangiocarcinoma (IHCC). In a study conducted on 30 Japanese patients at the Kanazawa University Hospital, K-Ras mutations was detected in 31.5% of the specimens of IHCC and 98.8% of BilIN[5] showing a difference in frequency with the difference in the type of carcinoma.

Several studies that report a positive correlation between K-Ras mutation and BTC incidence were mainly from Taiwan, China and Japan. One study done in Taiwan in June 2017 showed that 24.2% of the total 182 biliary tract cholangiocarcinoma patients were identified positive for K-Ras mutation. K-Ras point mutations were detected in 7.6% of IHCC patients, 13.3% of common bile duct cancer patients, and 3.3% of gallbladder carcinoma patients, indicating the potential role of K-Ras mutation in the pathogenesis of hepatobiliary carcinomas.[13]

In another group from the Taiwanese population, 16.8% of the 137 BTC patients were positive for K-Ras mutation. Paraffin-embedded specimens resected between 1995 and 2004 were identified with K-Ras mutation, with 22 of these located at codon 12 and only one in codon 13.[14]

In a retrospective analysis of IHCC specimens from 50 Japanese patients who underwent hepatectomy between May 2009 and August 2016, 32% showed mutations in K-Ras. Patients with K-Ras mutations exhibited a shorter overall survival (OS) compared to those with wild-type K-Ras.[15] In the same study, the association of K-Ras mutation

Table-1: Appraisal score of the selected articles.

Article reference number	**1.** Was the study question or objective clearly stated?	**2.** Was the study population clearly and fully described, including a case definition?	**3.** Were the cases consecutive?	**4.** Were the subjects comparable?	**5.** Were the outcome measures clearly defined, valid, reliable, and implemented consistently across all study participants?	**6.** Was the length of follow-up adequate?	**7.** Were the statistical methods well-described?	**8.** Were the results well-described?	Appraisal scores
(18)	√	√	√	√	√	√	√	√	8
(9)	√	√	√	√	√	√	√	√	8
(4)	√	√	√	√	√	NR	√	√	7
(13)	√	√	√	√	√	NR	√	√	7
(15)	√	√	X	√	√	√	√	√	7
(16)	√	√	√	√	√	NR	X	√	6
(6)	√	√	√	X	√	NR	√	√	6
(14)	√	√	√	X	√	NR	√	√	6
(12)	X	√	√	X	√	NR	√	√	6
(5)	√	√	√	X	√	NR	√	√	6
(17)	√	√	NR	√	NR	NR	√	√	5

√ = 1, X = 0, NR= not relevant

with increased GLUT-1 expression in tumour was also identified.[15]

From the Chinese population, K-Ras mutation was detected in 38.2% of the 34 patients diagnosed with cholangiocarcinoma in 2011.[16]

Role of p53 inactivation in BTC: A study conducted on a sample of Chinese population showed a positive correlation between p53 mutation and BTCs. From a sample of 36 frozen specimens resected between April 2000 and May 2005, p53 gene mutations were found in 61.1% of the specimens.[4]

Combined role of p53 and K-Ras mutations in BTC: Other studies report a positive relationship of both K-Ras and p53 mutation/overexpression with BTC. In a Japanese cohort of 86 patients with hepato-pancreaticobiliary disease who were treated between March 1994 and May 1998, 4 patients reported to have p53 mutation (3 in exon 7 and 1 in exon 8), and 2 patients were identified positive for K-Ras mutation in codon 12.[9]

In a retrospective analysis of 63 Japanese patients diagnosed with BTC between January 2005 and December 2011, K-Ras mutation was identified in 14% patients, of whom 2 patients had gall bladder carcinoma and 7 had extrahepatic cholangiocarcinoma.[6] Of these, the Glycine 12 Aspartic acid (G12D) mutation was identified in 5 BTC patients, Glycine12Serine (G12S) in 1 BTC patient and Glycine12Valine (G12V) in 3 patients. In the same study, TP53 overexpression was also identified in 48% patients.[6]

A study conducted in 2014, on a European cohort of 45 patients diagnosed with intraductal papillary neoplasm of the bile duct, reported p53 overexpression and K-Ras mutation to be the early genetic markers in the prognosis of the disease.[12] P53 overexpression and K-Ras mutation have, in fact, been identified to be the early genetic markers in the prognosis of all types of BTCs.

K-Ras mutations were detected in exons 2 and 3 in 36% of all intraductal papillary neoplasms of the bile duct and in 14% of all the cholangiocarcinomas.[12] Intraductal papillary neoplasms of the bile duct and cholangiocarcinomas have a similar molecular pathogenesis with respect to most of the gene expression, with the exception of p53 inactivation. While p53 inactivation marks an early event in the carcinogenesis of intraepithelial neoplasm of the bile duct, it occurs as a late molecular event in cholangiocarcinoma.[12] Patients with intraductal papillary neoplasm of the bile duct also showed significantly better OS than those with cholangiocarcinomas.[12]

The combined role of K-Ras mutation and p53 inactivation has also been studied on murine models in the United States in 2012. Mice with conditionally activated allele for K-Ras were cross-bred with those with conditional knock-out allele for p53 to evaluate the combined effect of K-Ras mutation and p53 inactivation on their off-springs. The results were then compared with mice engineered with K-Ras mutation only. On analysis, p53 was implicated in the

Table-2: Study populations, findings and objectives.

Article Reference Number	Study population	Findings	Objectives
(4)	A total 36 patients enrolled in this study. Study started from April 2000 to May 2005 in China.	p53 gene mutations were found in 22(61.1%) patients. Patients 19(52.8%)) were positive for P53 protein expression. There were significant differences in extent of differentiation and invasion between the positive and negative expression of P53 protein. The alterations of the p53 gene evaluated by DNA sequence analysis is relatively accurate. Expression of P53 protein could not act as an independent index to estimate the prognosis of cholangiocarcinoma.	To characterize the tumour suppressor gene p53 mutations and study the correlation of p53 gene mutation and the expression of P53 protein in cholangiocarcinoma.
(5)	The 27 patients included females 12 (44.4%) and males 15 (55.6%), with a median age of years 69 (range, 44–82 years). The study started from June 1997 and August 2013 in Japan.	This result may suggest that the activation of the PIK3CA-protein kinase B signalling pathway, in addition to the abrogation of p53, SMAD4 and RAS mitogen activated protein kinase may have a crucial role in the carcinogenesis of Japanese BTC. These findings may be useful for the development of personalized therapies for BTC.	The present study analysed somatic mutations of 50 cancer associated genes in 27 Japanese BTC cells, including: 11 EBDC, 14 GBC and 2 AVC.
(6)	63 Japanese patients with BTC. Started from January 2005 and December 2011	Our data suggest that KRAS mutation is a poor prognosis predictive biomarker for the survival in BTC patients	The aim of this study was to identify the unique molecular characteristics of biliary tract cancer (BTC) for the development of novel molecular-targeted therapies.
(9)	86 patients were enrolled in between March 1994 and May 1998. The study conducted in japan	The three novel biomarkers of the peripheral blood seemed to be of little value for screening of early malignant HPB neoplasms but may help to predict liver metastasis	The present study comprised a combination analysis of telomerase activity, Ki-ras codon 12 point mutation and P53 mutation of the peripheral blood in patients with HPB diseases, both benign and malignant, to determine the clinical implications of these three biomarkers
(12)	44 patients were enrolled in the study conducted in Germany.	In this study, we analyzed a large series of intraductal papillary neoplasms of the bile duct from the patients of European origin, with focus on molecular genetic changes in relation to morphology, distribution of different subtypes and their prognostic relevance	The development of biliary intraductal papillary neoplasms of the bile duct follows an adenomacarcinoma sequence that correlates with the stepwise activation of common oncogenic pathways.
	A total of 182 cases of biliary tact were enrolled in the study started from 1998 and 2012.	KRAS point mutations were detected in intrahepatic CC (7.6%), common bile duct cancer (13.3%), and gallbladder carcinoma (3.3%). BRAF gene amplifications were demonstrated in intrahepatic CC (4.3%), common bile duct cancer (3.3%), and gallbladder cancer (5%). No association was observed between mutation patterns and histopathological features. The analyses of risk factors for overall survival in patients with CC revealed no significant association in age, tumour site, genetic mutation, or amplifications. The tumour stage was the significant prognostic factor.	This study aimed to perform mutation analysis and copy number changes of KRAS and BRAF genes of CC in Taiwan.

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(13)	137 of Biliary tract carcinomas study converted in 1995 and 2004 in Taiwan.	EGFR and KRAS mutations are not uncommon in BTCs. BRAF mutation is rare in BTCs. EGFR mutation was an independent prognostic marker in BTCs in addition to tumour stage and differentiation. No simultaneous EGFR and KRAS mutations in extrahepatic cholangiocarcinoma and gallbladder carcinoma were found. AVC and KRAS mutations should be evaluated when tailoring molecular-targeted therapy to patients with BTCs.	We examined the correlation between these mutations and the overall survival, tumour location, stage, and differentiation in BTCs.
(14)	50 patients were enrolled in this study between May 2009 and August 2016 in Japan.	High MTV is associated with KRAS mutation and poor postoperative outcomes in patients with ICC, suggesting that the MTV of ICC measured by 18F-FDG-PET may provide useful information for tumour molecular profiles and prognosis.	This study aimed to identify clinical prognostic indicators by investigating the molecular profiles of ICC and evaluating the preoperative imaging data of 18F-fluorodeoxyglucose positron emission tomography (18F-FDG-PET).
(15)	34 patients in between 2001 and 2008. The study conducted in china.	Surprisingly, no BRAF mutation was detected in all 34CCA samples. Our findings indicate that somatic mutations in KRAS and PIK3CA but not BRAF oncogenes are closely associated with the development of CCA in Chinese population and provide new potential targets for future therapeutic treatments of the disease.	In this study, we evaluated the hotspot mutations of KRAS, BRAF, and PIK3CA genes in 34 Chinese CCA patients.
(16)	Molecular mechanism of cholangiocarcinoma	The recent analysis of genetic and epigenetic alterations occurring in cholangiocarcinoma (CA) has shed new light in the understanding of the molecular mechanisms leading to the malignant transformation of biliary cells.	The present study comprised a combination analysis of telomerase activity, Ki-ras codon 12 point mutation and P53 mutation of the peripheral blood in patients with HPB diseases, both benign and malignant, to determine the clinical implications of these three biomarkers
(17)	44 patients were enrolled in the study conducted in Germany.	In this study, we analyzed a large series of intraductal papillary neoplasms of the bile duct from the patients of European origin, with focus on molecular genetic changes in relation to morphology, distribution of different subtypes and their prognostic relevance	The development of biliary intraductal papillary neoplasms of the bile duct follows an adenomacarcinoma sequence that correlates with the stepwise activation of common oncogenic pathways.
(18)		KRAS point mutations were detected in intrahepatic CC (7.6%), common bile duct cancer (13.3%), and gallbladder carcinoma (3.3%). BRAF gene amplifications were demonstrated in intrahepatic CC (4.3%), common bile duct cancer (3.3%), and gallbladder cancer (5%). No association was observed between mutation patterns and histopathological features. The analyses of risk factors for overall survival in patients with CC revealed no significant association in age, tumour site, genetic mutation, or amplifications. The tumour stage was the significant prognostic factor.	In particular, the identification of how genetic and epigenetic modifications may play a major role in CCA development, progression, and metastasis may open a new era for the management of CCA, and may represent a potential strategy for the treatment of this devastating malignancy.

Abbreviations: phosphatidylinositol-4,5-bisphosphate 3-kinase, catalytic subunit α (PIK3CA), Biliary tract cancer (BTC), extrahepatic bile duct carcinoma (EBDC) , Gall bladder carcinoma (GBC), ampulla of Vater carcinoma (AVC), HPB, epidermal growth factor receptor (EGFR), metabolic tumour volume (MTV), MTV, intrahepatic cholangiocarcinoma (ICC), Positron emission tomography with 2-deoxy-2-[fluorine-18]fluoro- D-glucose integrated with computed tomography (18F-FDG-PET), v-raf murine sarcoma viral oncogene homolog B1 (BRAF), Cholangiocarcinoma (CCA), intraductal papillary biliary neoplasms (IPBN), Von Meyenburg complexes (VMC)

acceleration of development of intra-hepatic cholangiocarcinoma (IHCC) in mice with K-Ras mutation. While most of the mice with K-Ras mutation developed IHCC by the age of 15 months (75 weeks), p53 inactivation resulted in the acceleration of tumour genesis by initiating the development of IHCC in mice as early as 8 months (32 weeks). With p53 inactivation, the mean survival of mice decreased from 14 months (56 weeks) to 2 months (9 weeks), confirming the role of p53 inactivation in decreasing the latency of the disease in mice with K-Ras mutation. While mutation of K-Ras results in hyper-activation of extracellular signal-regulated kinases like Erk Ras/ Raf/ Mek pathway, p53 upon deletion further escalates this process by its loss of control on the hyperactive cell cycle and apoptosis of K-Ras mutated cells.[17] Moreover, it was noted that on the exposure of mutagens, the predominant cancer observed in the mice liver was of biliary phenotype with hepatic cholangiocarcinoma (HCC) being a rare finding. This suggests that either K-Ras and p53 shift cellular differentiation of hepatic cells to biliary phenotype or that biliary cells have an increased susceptibility to developing KRas mutation. Moreover, the histological features of cells implicated in IHCC showed a marked similarity to that in human IHCC, further strengthening the validity of the results stated above.[18]

Discussion

The frequency of K-Ras mutations varies from one ethnic group to other; Japanese, Europeans and Americans show a higher frequency of K-Ras mutations compared to the Taiwanese patients of cholangiocarcinoma.[13]

The OS of hepatobiliary carcinoma patients with K-Ras mutation was found to be significantly shorter than that of those with the wild-type K-Ras gene[6] as has also been reported by another study in Japan in 2018.[15] However, it is believed that this is probably due to the worse treatment outcomes in K-Ras mutation-positive patients.

Even though p53 overexpression is commonly detected in patients with BTCs, the association between p53 overexpression and the OS of patients after tumour resection has not been reported.[6] Studies confirm that p53 overexpression has no correlation with tumour prognosis or with pathogenesis.[6]

In Japan, it has been reported that the one-year survival rate after resection is zero in patients positive for K-Ras and p53 mutation, and it is 15% in those who were not, but the difference was non-significant (p=0.65).[9] This may be because most patients who had K-Ras and p53 mutation were diagnosed with stage IV of hepatopancreatobiliary disease and, therefore, had lower chances of survival.[9] Moreover, even though a correlation is observed between these biomarkers, no correlation has been established with the resectability rate or the one-year survival rate after surgical resection of the gall bladder or the biliary tree.[9]

BTCs follow the sequence of local invasion, vascular invasion and regional lymph node metastasis to distant metastasis to the liver, pancreas, hepatic portal vein, hepatic artery and lymphatic systems.[19] For such aggressive tumours, surgical resection is the final curative management. Neo-adjuvant therapy involving chemotherapy, radiation, chemo-radiation, or photodynamic therapy has been known to increase chances of curative resection.[20,21] However, in some studies, radiation and chemotherapy have not been considered to be the effective means of treatment for inoperable BTCs.[14] In fact, poor prognosis of the disease is associated with a high rate of relapse in the very few patients who manage to undergo a potentially curative surgical resection.[4]

Moreover, it has been reported that the frequency of p53 overexpression in BTCs increases with the grading of cancer.[12] The opposite, however, holds true for the frequency of K-Ras mutations in BTCs. Where, on the one hand, a lower frequency of K-Ras mutations is found to be associated with high-grade invasive carcinomas, and, on the other hand, a higher frequency of K-Ras mutations are recorded in low-grade non-invasive carcinomas. This shows that unlike p53 overexpression, the frequency of K-Ras mutation decreases with the increase in the grading of cancer.

Within intraductal papillary neoplasms, invasive tumours have a worse prognosis with a two-fold higher risk of death compared to the non-invasive tumours. Only the oncocytic type of invasive carcinomas show an exceptionally better prognosis than the other invasive types, like tubular and colloid-mucinous. This is primarily due to the fact that of all the invasive types of intraepithelial neoplasms of the biliary tract, only the oncocytic type showed no identification with K-Ras mutation.[12] The tubular and colloid-mucinous types, on the other hand, showed a high degree of association with K-Ras mutation, accounting for a poor prognosis.

Increased metabolic tumour volume[22] and increased total glycolysis have also been reported in tumour cells with increased GLUT-1 expression, causing increased glucose uptake by the cells and contributing to their increased tumour growth and formation. On the other hand, no significant difference is reported in the maximum standardised uptake value (SUV) of glucose between the cells with mutated K-Ras and those with non-mutated, wild-type K-Ras. The association between K-Ras mutation and GLUT-1 expression has also been identified in other types of carcinomas, including non-small cell lung carcinoma and oral squamous cell carcinomas.[23-25] The

higher the GLUT-1 expression in tumour cells, the higher the stage of the tumour, and the worse is the prognosis.[15] In oral squamous cell carcinomas, increased GLUT-1 expression is correlated with poor cancer prognosis.[24] Similarly, lung cancers with increased GLUT-1 expression also show a high correlation with decreased differentiation of tumour, increased lymph node metastasis and increased tumour size.[26]

Conclusion

K-Ras and p53 mutation both contribute to the pathogenesis of BTC. K-Ras mutation, however, has a much higher frequency compared to p53 inactivation in such cancers. Moreover, biliary tract tumours positive for K-Ras mutation have a worse prognosis compared to those with wild-type K-Ras. However, the molecular profile behind BTC pathogenesis still remains to be widely explored. Once the exact molecular pathways are identified, the goal of precision treatment of patients for better prognosis can be achieved.

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