Evaluation of Molecular Targets and Mismatch Repair Deficiency in Gallbladder Cancer

Niraj Kumari (nirajpath@gmail.com)
All India Institute of Medical Sciences, Raebareli https://orcid.org/0000-0002-9674-448X
Pooja Shukla
Sanjay Gandhi Post Graduate Institute of Medical Sciences
Narendra krishnani
Sanjay Gandhi Post Graduate Institute of Medical Sciences
Anu Behari
Sanjay Gandhi Post Graduate Institute of Medical Sciences
Vinay K Kapoor
Sanjay Gandhi Post Graduate Institute of Medical Sciences

Research Article

Keywords: gallbladder cancer, molecular targets, mismatch repair, KRAS

Posted Date: May 28th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-528091/v1

Abstract

Purpose

Gallbladder cancer (GBC) is most aggressive malignancy having very short survival having heterogeneous incidence, clinical and molecular profile. We evaluated molecular targets and mismatch-repair (MMR) protein expression in North-Indian patients.

Method

111 cases were subjected to high-resolution melt curve, followed by Sanger sequencing for KRAS, BRAF and PIK3CA. Immunohistochemistry was done for four MMR proteins.

Results

Six (5.4%) cases showed KRAS mutation while no mutation was found in BRAF and PIK3CA. Deficient MMR was seen in 27.6% of GBC. All KRAS mutant cases were >50 years having higher perineural invasion (67%), lymphnode metastasis (67%) and stage-III disease (67%). MMR deficient GBC were significantly associated with well differentiated histology. KRAS mutant GBC had shorter mean survival than wild patients. MMR deficient GBC showed longer mean survival than MMR proficient cases. 10% of MMR deficient compared to 4% MMR proficient GBC showed KRAS mutation.

Conclusion

KRAS mutation was lower in North Indian patients despite having high GBC incidence making these patients suitable for targeted therapy. It was associated with poor prognostic factors and lower mean survival. MMR deficiency was higher and harbored predominantly well-differentiated histology with higher mean survival. Molecular targets and MMR expression in GBC may guide towards more appropriate approach in these patients.

Introduction

Gallbladder cancer (GBC) is the fifth most common gastrointestinal cancer worldwide, which often goes undiagnosed until presents at advanced stage and is associated with extremely poor prognosis [1, 2]. It is the most aggressive malignancy of biliary tract with the shortest median survival from the diagnosis [3]. It has predilection for females with variable incidence and India particularly Northern India, is one of the region with highest incidence [4, 5]. Personalised therapy has become an interesting approach in oncology because of molecular heterogeneity and its role as predictive as well as prognostic in various cancers. Search for suitable targets in GBC is also being explored which may help to provide better management and improve selection of patients for different therapeutic strategies.

The RAS-RAF-MAPK kinase signalling pathway is the most frequently mutated pathway in cancer which is fortunately also the most common explored pathway for targeted therapy. The major downstream molecules of RAS-RAF-MAPK kinase that are being explored and are in use for personalized therapy include KRAS, BRAF and PIK3CA. Another important area specially in the field of gastrointestinal and pancreatobiliary malignancy is expression pattern of mismatch repair (MMR) protein or microsatellite instability (MSI) which influences the disease prognosis as well as therapeutic response of chemotherapeutic drugs [6–8]. In this study we evaluated the molecular targets in RAS-RAF-MAPK pathway namely KRAS, BRAF and PIK3CA and expression pattern of MMR protein in GBC by high resolution melt (HRM) curve confirmed by Sanger sequencing and find out any association between the two group of biomarkers.

Material Methods

One hundred eleven consecutive cases of resected GBC received in the Department of Pathology, were included and clinical and follow-up data were retrieved from the hospital records.

Hematoxylin and eosin stained slides were reviewed and following features were noted - tumor type, tumor grade, perineural invasion (PNI), lymphovascular invasion (LVI), necrosis, tumor infiltrating lymphocytes (TILs), mucinous or signet ring cell morphology, associated xanthogranulomatous inflammation, depth of infiltration, resection margin status, lymphnode and distant metastasis. DNA was extracted using Qiagen QiaAmp FFPE DNA kit. The mutation testing for KRAS, BRAF and PIK3CA (exons 9 and 20) were screened by HRM curve analysis in realtime PCR (Quant Studio 6Flex, Applied Biosystems) with sequence proven controls for point mutation and wild type samples. Positive control for PIK3CA was not available in our case cohort; however wild type samples were used as negative controls. The samples with abnormal curve patterns were confirmed on bi-directional Sanger sequencing. Primer sequences and annealing temperatures for HRM and Sanger sequencing are shown in Table 1.

Table 1
High resolution melt curve and sequencing primers

Gene	HRM-Primers	Annealing Temperature	Sequencing Primers	Annealing Temperature
KRAS-1	FP:5' GCCTGCTGAAAATGACTGAA 3'	60°C	FP:5'GGCCTGCTGAAAATGACTGA 3'	60°C
	RP:5'AGAATGGTCCTGCACCAGTAA 3'		RP:5' GTCCTGCACCAGTAATATGC 3'	
BRAF-15	FP:5' CTACTGTTTTCCTTTACTTACTACACCTCA 3'	60°C	FP:5' TGCTTGCTCTGATAGGAAAATG 3'	59°C
	RP:5' ATCCAGACAACTGTTCAAACTGATG 3'		RP:5' AGCATCTCAGGGCCAAAAAT 3'	
PIK3CA-9	FP:5' GTCCAAAGCAATTTCTACACGAGA 3'	60°C	FP:5' CATCTGTGAATCCAGAGGGGA 3'	55°C
	RP:5' TTCATTTTAGCACTTACCTGTGAC 3'		RP:5' AGCACTTACCTGTGACTCCA 3'	
PIK3CA-20	FP:5' GAGGCTTTGGAGTATTTCAT 3'	60°C	FP:5' CGACAGCATGCCAATCTCTTC 3'	57°C
	RP:5' AATCCATTTTTGTTGTCCAG 3'		RP:5' TCTTTTCAGTTCAATGCATGCTG 3'	

MMR protein expression was evaluated by immunohistochemistry for MLH1, MSH2, MSH6, and PMS2 proteins. Briefly the tissues were deparaffinized and antigen retrieval was done in Tris EDTA buffer at pH 8.0. The primary antibodies (ready to use) from DAKO (MLH-1 – Clone ES05, MSH-2 – Clone FE11, MSH-6 – Clone EP-49, and PMS-2 – Clone EP51) were incubated for 2 hours at room temperature followed by secondary antibody for 30 minutes. Complete absence of nuclear staining were interpreted as MMR deficient (loss of MMR protein expression) and any nuclear positive staining was taken as MMR proficient (intact expression). Expression of MMR protein in the normal intestinal epithelium, stromal cells and lymphocytes were taken as positive internal control. Chi-square test was used to find out correlation between categorical variables and survival analysis was done by Kaplan Meier log rank test using SPSS software version 20.

Result

There were 79 females and 32 males (M: F = 1:2.4) with age range 23–79 years (mean 53.7 years; median 54.5 years). Ninety-five cases (85.6%) were > 40 years of age while 16 patients were ≤ 40 years age. Gallstones were found in 66 (59.5%) patients.

Histopathology

Conventional adenocarcinoma was the predominant histology seen in 67.6% cases followed by papillary adenocarcinoma in 17%. Majority of the cases were grade I (55%) tumor however, T2 (67.6%) and T3 (23.4%) tumors surpassed T1 (9%) with overall advanced stage disease (stage III, IV) being present in 54% cases. The histopathological findings are mentioned in Table 2.

Table 2
Histopathological features in gallbladder cancer

Features	Number of cases (n = 111)	
Histological type	Conventional adenocarcinoma	75 (67.6%)
	Papillary adenocarcinoma	19 (17.1%)
	Signet ring cell adenocarcinoma	8 (7.2%)
	Adenosquamous carcinoma	6 (5.4%)
	Mucinous adenocarcinoma	3 (2.7%)
Tumor grade	Well	61 (55%)
	Moderate	10 (9%)
	Poor	40 (36%)
Perineural Invasion		49 (44.1%)
Lymphovascular		29 (26.1%)
Necrosis		69 (62.2%)
Xanthogranulomatous change		18 (16.2%)
Lymphnode Metastasis		46 (46%)
Pathological T-stage	T1	10 (9%)
	T2	75 (67.6%)
	T3	26 (23.4%)
TNM Stage	Stage I	9 (8.2%)
	Stage II	42 (37.8%)
	Stage III	52 (46.8%)
	Stage IV	8 (7.2%)

Mutation profile

HRM screening for KRAS showed mutant curve pattern in 6 cases and variant curve pattern in 5 cases, while 89 cases showed a wild curve pattern (Table 3). The cases with mutant curve pattern showed C > T nucleotide change at codon 13 in 5 cases (83%) and G > A at codon 12 in one case (17%). The cases with variant curve pattern confirmed to have wild sequence on Sanger sequencing (Fig. 1). HRM screening for BRAF showed wild curve pattern in 103 cases and variant curve pattern in 8 cases. Sanger sequencing confirmed all variant curve patterns to have wild type BRAF sequence (Fig. 2). HRM screening for PIK3CA showed wild curve pattern for both the exon 9 and 20 in all except two cases where PIK3CA exon 20 showed a variant pattern. Sanger sequencing of random 20 cases each for exon 9 and 20 confirmed wild PIK3CA sequence (Fig. 3a). The two cases with variant pattern in exon 20 showed synonymous variation at codon 1025 (Fig. 3b).

Table 3
Molecular profile of gallbladder cancer

Gene	HRM			Sanger Sequencing	
	Wild	Mutant	Variants	Wild	Mutant
KRAS	100	6	5	105	6
BRAF	103	0	8	111	0
PIK3CA exon 9	111	0	0	111	0
PIK3CA exon 20	109	0	2	111	0

MMR protein expression

MMR expression was evaluated by IHC in 105 cases. Rest of the 6 cases did not have sufficient tumor tissue for staining. Deficient MMR expression was found in 29 (27.6%) cases of which 14 (48%) cases had MLH1 and PMS2 loss followed by MSH2 and MSH6 loss in 9 (31%) cases. In another 6 (21%) cases individual loss of MHS6 expression was observed (Fig. 4a - d).

Correlation of KRAS mutation and MMR deficiency with clinicopathological features

KRAS mutation was largely seen in conventional adenocarcinoma (4/6 cases). All the 6 KRAS mutant cases were > 50 years of age (p value = 0.02), however no significant differences were found in distribution of KRAS mutation status among different tumor grades. PNI, LVI and lymphnode metastasis were seen in 67%, 33% and 67% of KRAS mutant cases respectively with no significant difference. Two-thirds (67%) of KRAS mutant cases were seen in stage III. MMR deficient cases were significantly associated with grade I tumors (p value = 0.02), however it did not show any significant association with other clinicopathological features. Correlation of histopathological features with KRAS mutation and MMR expression are shown **in** Tables 4 and 5 respectively.

Table 4
Clinicopathological characteristics and KRAS mutation in gallbladder cancer

ClinicopathologicalFeatures (n = 111)		KRAS		P-value
		Wild (n = 105)	Mutant (n = 6)	
Gender	Male (n = 32)	31 (29.5%)	1 (16.7%)	0.4
	Female (n = 79)	74 (70.5%)	5 (83.3%)	
Age	≤ 50 (n = 50)	50 (47.6%)	0	**0.02**
	> 50 (n = 61)	55 (52.4%)	6 (100%)	
Gallstone	Present (n = 64)	62 (59%)	4 (66.7%)	0.7
	Absent (n = 45)	43 (41%)	2 (33.3%)	
Tumor grade	Well (n = 61	58 (55.2%)	3 (50%)	0.7
	Moderate (n = 10)	9 (8.6%)	1 (16.7%)	
	Poor (n = 40)	38 (36.2%)	2 (33.3%)	
Depth of infiltration (T-stage)	T1 stage (n = 10)	9 (8.6%)	1 (16.7%)	0.7
	T2 stage (n = 76)	72 (68.6%)	4 (66.7%)	
	T3 stage (n = 25)	24 (22.8%)	1 (16.7%)	
Perineural Invasion (n = 49)		45 (42.8%)	4 (66.7%)	0.2
Lymphovascular Invasion (n = 29)		27 (25.7%)	2 (33.3%)	0.6
Xanthogranulomatous change (n = 18)		18 (17%)	0	0.2
Lymphnode Metastasis (n = 50)		46 (43.8%)	4 (66.7%)	0.2
Tumor stage	Stage I (n = 9)	8 (7.6%)	1 (16.7%)	0.5
	Stage II (n = 42)	41 (39%)	1 (16.7%)	
	Stage III (n = 52)	48 (45.8%)	4 (66.7%)	
	Stage IV (n = 8)	8 (7.6%)	0	

Table 5
MMR protein expression in gallbladder cancer

Clinicopathological features (n = 105)		MMR Expression Loss (n = 29)	MMR Intact Expression (n = 76)	P Value
Age (Year)	≤ 50	15 (51.7%)	32 (42%)	0.3
	≥ 50	14(48.3%)	44 (58%)	
Gender	Male	7 (24%)	22 (29%)	0.6
	Female	22 (76%)	54 (71%)	
Tumor grade	Well	19 (65.6)	38 (50%)	**0.02**
	Mod	5 (17.2%)	5 (6.6%)	
	Poor	5 (17.2%)	33 (43.4%)	
Pathological (T-) Stage	T1 Stage	4 (13.8%)	5 (6.6%)	0.4
	T2 Stage	18 (62%)	53 (69.7%)	
	T3 Stage	7 (24%)	18 (23.7%)	
TILs		10 (34.55)	25 (32.9%)	0.8
Perineural invasion		11 (38%)	36 (47.4%)	0.3
Lymphovascular invasion		7 (24%)	21 (27.6%)	0.7
Lymphnode metastasis		13 (44.8%)	35 (46%)	0.9
Necrosis		18 (62%)	48 (63%)	0.9
Presence of mucin		4 (13.8%)	25 (32.9%)	0.9
Stage	I Stage	3 (10.3%)	5 (6.6%)	0.1
	II Stage	9 (31%)	30 (39.5%)	
	III Stage	17 (58.6%)	33 (43.4%)	
	IV Stage	0	8 (10.5%)	
1. High resolution melt curve and Sanger sequencing confirmation for KRAS exon 1 showing wild, variant and mutant curve patterns.				
2. High resolution melt curve and Sanger sequencing confirmation for BRAF exon 15 showing wild and variant curve patterns.				
3. High resolution melt curve and Sanger sequencing confirmation for PIK3CA exon 9 (a), and PIK3CA exon 20 showing wild and variant curve patterns.				
4. Immunohistochemistry for mismatch repair proteins showing intact expression (a), and loss of expression (b) for MLH1; intact expression (c), and loss of expression (d) for MSH2.				
5. Survival plot showing longer mean survival in KRAS wild GBC.				
6. Survival plot showing longer mean survival in mismatch repair deficient GBC.				

Follow-up data was available in 100 cases with a range of 1-117 months. The overall mean and median survival was 18 and 25.9 months respectively. Eleven patients were lost to followup and 21 deaths were reported in 100 cases. KRAS mutant GBC had a mean survival of 68 months while KRAS wild patients had slightly longer mean survival of 89 months, however this was not statistically significant (p value = 0.5) (Fig. 5). MMR deficient cases showed a longer median survival of 26 months (mean 91 months) compared to MMR proficient cases with a median survival of 15.5 months (mean 76 months) (p value = 0.03) (Fig. 6).

Discussion

Gall bladder is the most aggressive malignancy of gastrointestinal tract with a wide geographical variation as well as heterogeneity at clinical and molecular level. Despite several developments in understanding the molecular pathogenesis of GBC, there has been no considerable progress in targeted therapy or personalized approach in GBC management. KRAS mutation which is one of the predictive marker for anti-EGFR (epidermal growth factor receptor) therapy varies widely in GBC from 0 to 41% in different studies [9–16]. We found 5.4% KRAS mutation in GBC. In another previous report from our group in which we studied 49 cases of GBC, KRAS mutation was seen in 2% cases [11]. Javle et al and Pai et al from United States also reported KRAS mutation frequency in similar range of 5.5% and 7% respectively in GBC. [12, 13]. There had been some reports from Japan with variable of KRAS mutation frequency by Asai et al, Yokoyama et al and Noguchi et al with 2.8%, 9.0% and 14.3% respectively [14–16]. BRAF, another predictive marker as well as candidate for targeted therapy shows most common mutation in codon 600 (90% of all BRAF mutations) with substitution of valine to glutamine (V600E) being the most common mutation (~ 90%) of all codon 600 mutations [17]. In GBC, BRAF mutation varies from 0–33% [18–20]. Yokoyama et al. from Japan, Goldenberg et al and Pai et al from United States, also did not show any BRAF mutation in GBC [13, 15, 19]. In the present study as well as a previous report from our group did not show any BRAF mutation in GBC [11]. PIK3CA mutation is another candidate for targeted therapy which has been reported in lower frequency than KRAS and BRAF in GBC in the range of 0 to 21.5% [14–16, 21]. Some reports from United States have shown PIK3CA mutation between 3.5 to 14% in GBC [9, 22] while Yokoyama et al. [15] did not find any PIK3CA mutation in their study. We also did not find any PIK3CA mutation in exons 9 and 20 in this study. Thus, the above findings suggest that molecular profile of GBC similar to its incidence, varies widely in different regions of the world. In India, a country with one of the highest GBC incidence, common candidates for targeted therapy such as KRAS, BRAF and PIK3CA show a very low mutation rate of KRAS mutation rendering these patients to be suitable for anti-EGFR therapy.

KRAS mutation has been regarded as a poor prognostic marker in different cancers. In GBC there are very few reports on association of KRAS mutation and clinicopathological features. It has been found to be more frequent in higher grade and advanced stage tumors. Asai et al showed that all KRAS mutant GBCs were poorly differentiated in his study [14]. In a recent report, Bagante et al reported that KRAS mutant GBC were more likely to show perineural invasion and positive resection margins as well as lower 5 year overall survival of 13% [23]. In one of our earlier report of 49 GBC cases, KRAS mutation was seen in 2% cases having poorly differentiated morphology with pathological stage of T2 [11]. In the present study we found a significant association of KRAS mutation with age where all 6 cases of KRAS mutant GBC were > 50 years age (p value = 0.02). Fifty percent of the KRAS mutant GBCs were moderate to poorly differentiated with nearly 67% of them harboring perineural invasion, lymph node metastasis and being stage III tumors. The KRAS mutant GBC also showed lower mean survival of 68 months than KRAS wild GBC with a mean survival of 89 months. These findings suggest that present of KRAS mutation confers a worse prognosis as well as these patients loose the opportunity of being candidates for targeted therapy.

MMR deficiency is another important prognostic and predictive biomarker for certain chemotherapy and immune check-point blockers [24, 25]. Microsatellite instability (MSI) as demonstrated by molecular methods like PCR and fragment length analysis or deficient MMR expression demonstrated by IHC has a comparable sensitivity (77–100%) and specificity (98–100%). The frequency of microsatellite instable or MMR deficient GBC ranges between 0 to 42% with IHC and / or molecular methods [26–31] where Saetta et al 18] did not find any MMR deficient tumor but Yanagisawa et al [29] reported 41% MMR deficient GBC. Moy et al reported nearly 8% MMR loss in GBC but did not find any significant association between MMR deficient GBC and clinicopathological features such as age, grade, stage, mucinous histology and TILs, however they found these patients to be older with a median age of 71 years having moderate to poorly differentiated histology but with an overall lower tumor stage 28]. Conversely Nagahashi et al reported a frequency of MSI high to be 42% (overall MSI 38%) with 75% of instable tumors being well differentiated, 19% moderately differentiated and 6% poorly differentiated tumors [30]. Geoppert et al reported 1.4% instable GBC with lymphnode metastasis, perineural invasion and stage III tumor but a longer overall survival [31]. We found MMR loss in 27.6% of GBC with 65% of tumors having well differentiated histology but 58% having advanced stage disease (stage III). Ten percent of MMR deficient GBC showed KRAS mutation in comparison to 4% MMR proficient GBC (p value = 0.2). The wide variability in MMR deficient cases in GBC and its inconsistent relation with histological features may be due to

differences in ethnic, geographical and dietary variations. No significant association has been found between KRAS mutation and MMR deficient GBCs. The TILs are regarded as one of the predictors for immune check-point therapy and since MMR deficient cancers have a tendency to harbour increased TILs, they are also potential candidates for immunotherapy, however we did not find any significant difference in TILs between MMR deficient and proficient tumors.

Conclusion

In this study we found a lower frequency of KRAS mutation in GBC in our population, however a higher prevalence of MMR deficiency was observed. KRAS mutant GBC showed an association with poor histological prognostic factors and lower overall survival. MMR deficient GBCs had a tendency for harboring predominantly well differentiated histology and a higher overall mean survival. The mutation profile for common molecular targets and MMR expression in GBC may help to devise more appropriate approach for managing this aggressive cancer.

Declarations

Acknowledgement – The authors acknowledge the financial support provided by Department of Biotechnology, Ministry of Science and Technology, New Delhi, India.

- To be used for non-life science journals – No
- Funding – The work was supported by Department of Biotechnology, Ministry of Science and Technology, New Delhi, India.
- Conflicts of interest/Competing interests - None
- Availability of data and material – All data are included in the manuscript.
- Code availability – No software code used
- Authors' contributions
    - Niraj Kumari – Study design, data interpretation and analysis, manuscript preparation
    - Pooja Shukla – Sample collection, performing tests, data analysis
    - Narendra Krishnani – Study design and planning, manuscript revision
    - Anu Behari – Clinical sample collection, data analysis
    - VK Kapoor – Clinical sample collection, data analysis
- Ethics approval - Cleared by Institute Ethics Committee

Consent to participate (include appropriate statements) - Yes

References

1. Zhu AX, Hong TS, Hezel AF, et al (2010). Current management of gallbladder carcinoma. Oncologist 15: 168–81.
2. Dutta U (2012). Gallbladder cancer: Can newer insights improve the outcome? J Gastroenterol Hepatol 27: 642–53.
3. Cariati A, Piromalli E, Cetta F (2014). Gallbladder cancers: associated conditions, histological types, prognosis, and Eur J Gastroenterol Hepatol 26: 562–69.
4. Chaurasia P, Thakur MK, Shukla HS (1999). What causes cancer gallbladder?: A review. HPB Surg. 11: 217–224.
5. Nandakumar A, Gupta PC, Gangadharan P, et al (2005). Geographic pathology revisited: Development of an atlas of cancer in India. Int J Cancer 116: 740–54.
6. Sinicrope F (2010). DNA mismatch repair and adjuvant chemotherapy in sporadic colon cancer. Nat Rev Clin Oncol 7: 174–77.
7. Zhang CM, Lv JF, Gong L, et al (2016). Role of Deficient Mismatch Repair in the Personalized Management of Colorectal Cancer. Int J Environ Re Public Health. 13: 892. doi:10.3390/ijerph13090892.

8. Eso Y, Shimizu T, Takeda H, et al (2020). Microsatellite instability and immune checkpoint inhibitors: toward precision medicine against gastrointestinal and hepatobiliary cancers, J Gastroenterol 55: 15–26.
9. Deshpande V, Nduaguba A, Zimmerman SM, et al (2011). Mutational profiling reveals PIK3CA mutations in gallbladder carcinoma, BMC Cancer. 11: 60. doi:10.1186/1471-2407-11-60.
10. Hanada K, Tsuchida A, Iwao T, et al (1999). Gene mutations of K-ras in gallbladder mucosae and gallbladder carcinoma with an anomalous junction of the pancreaticobiliary duct., Am J Gastroenterol 94: 1638–42.
11. Kumari N, Corless C, Warrick A, et al (2014). Mutation profiling in gallbladder cancer in Indian population. Indian J Pathol Microbiol 57: 9–12.
12. Javle M, Rashid A, Churi C, et al (2014). Molecular characterization of gallbladder cancer using somatic mutation profiling. Hum Pathol 45: 701–08.
13. Pai RK, Mojtahed K, R.K. Pai (2011). Mutations in the RAS/RAF/MAP kinase pathway commonly occur in gallbladder adenomas but are uncommon in gallbladder adenocarcinomas. Appl Immunohistochem Mol Morphol 19: 133-40.
14. Asai T, Loza E, Roig GVG, et al (2014). High frequency of TP53 but not K-ras gene mutations in Bolivian patients with gallbladder cancer. Asian Pac J Cancer Prev 15: 5449-54.
15. Yokoyama M, Ohnishi H, Ohtsuka K, et al (2016). KRAS Mutation as a Potential Prognostic Biomarker of Biliary Tract Cancers. Japanese Clin Med 7: 33–9.
16. Noguchi R, Yamaguchi K, Ikenoue T, et al (2017). Genetic alterations in Japanese extrahepatic biliary tract cancer, Oncol Lett 14: 877–84.
17. Oikonomou E, Koustas E, Goulielmaki M, et al (2014). BRAF Vs. RAS oncogenes: are mutations of the same pathway equal? Differential signalling and therapeutic implications. Oncotarget 5: 11752–77.
18. Saetta AA, Papanastasiou P, Michalopoulos NV, et al (2004). Mutational analysis of BRAF in gallbladder carcinomas in association with K-ras and p53 mutations and microsatellite instability. Virchows Arch. 445: 179–82.
19. Goldenberg D, Rosenbaum E, Argani P, et al (2004). The V599E BRAF mutation is uncommon in biliary tract cancers. Mod Pathol 17: 1386–91.
20. Simbolo M, Fassan M, Ruzzenente A, et al (2014). Multigene mutational profiling of cholangiocarcinomas identifies actionable molecular subgroups. Oncotarget 5: 2839–52.
21. Perkins G, Bouchet-Doumenq C, Svrcek M, et al (2017). Genomic profiling of ampullary adenocarcinoma (AA): Insights from a comparative analysis of pancreatic and intestinal adenocarcinoma and opportunities for targeted therapies use. J Clin Oncol 35: 300.
22. Borger DR, Tanabe KK, Fan KC, et al (2012). Frequent mutation of isocitrate dehydrogenase (IDH)1 and IDH2 in cholangiocarcinoma identified through broad-based tumor genotyping. Oncologist 17: 72–9.
23. Bagante F, Ruzzenente A, Conci S, et al (2019). Patterns of gene mutations in bile duct cancers: is it time to overcome the anatomical classification? HPB (Oxford) 21: 1648-55.
24. Mateo J, Carreira S, Sandhu S, et al (2015). DNA-repair defects and olaparib in metastatic prostate cancer. New Engl J Med. 373: 1697–708.
25. Le DT, Durham JN, Smith KN, et al (2017). Mismatch repair deficiency predicts response of solid tumors to PD-1 blockade. Science 357: 409-13.
26. Silva VWK, Askan G, Daniel TD, et al (2016). Biliary carcinomas: pathology and the role of DNA mismatch repair deficiency. Chinese Clin Oncol 5: 62. doi:10.21037/cco.2016.10.04.
27. Rashid A, Ueki T, Gao YT, et al (2002). K-ras mutation, p53 overexpression, and microsatellite instability in biliary tract cancers: a population-based study in China. Clin Cancer Res 8: 3156–63.
28. Moy AP, Shahid M, Ferrone CR, et al (2015). Microsatellite instability in gallbladder carcinoma. Virchows Arch 466: 393-402.
29. Yanagisawa N, Yanagisawa N, Mikami T, et al (2003). Microsatellite instability in chronic cholecystitis is indicative of an early stage in gallbladder carcinogenesis. Am J Clin Pathol 120: 413-7.

30. Nagahashi M, Ajioka Y, Lang I, et al (2008). Genetic changes of p53, K-ras, and microsatellite instability in gallbladder carcinoma in high-incidence areas of Japan and Hungary. World J Gastroenterol 14: 70-5.
31. Geoppert B, Roessler S, Renner M, et al (2019). Low frequency of mismatch repair deficiency in gallbladder cancer. Diagn Pathol 14: 36. doi: 10.1186/s13000-019-0813-5.

Figures

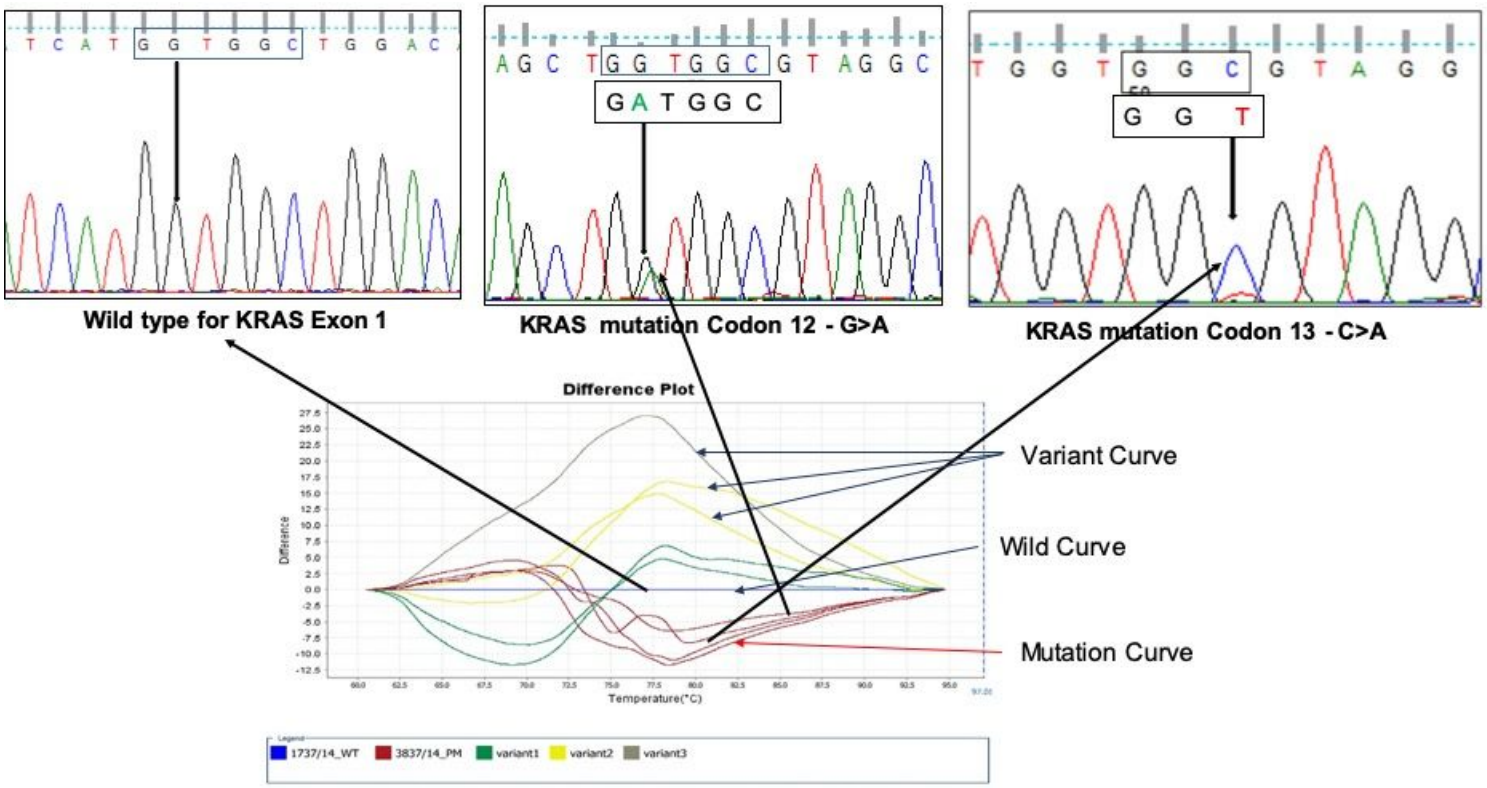

Figure 1

High resolution melt curve and Sanger sequencing confirmation for KRAS exon 1 showing wild, variant and mutant curve patterns.

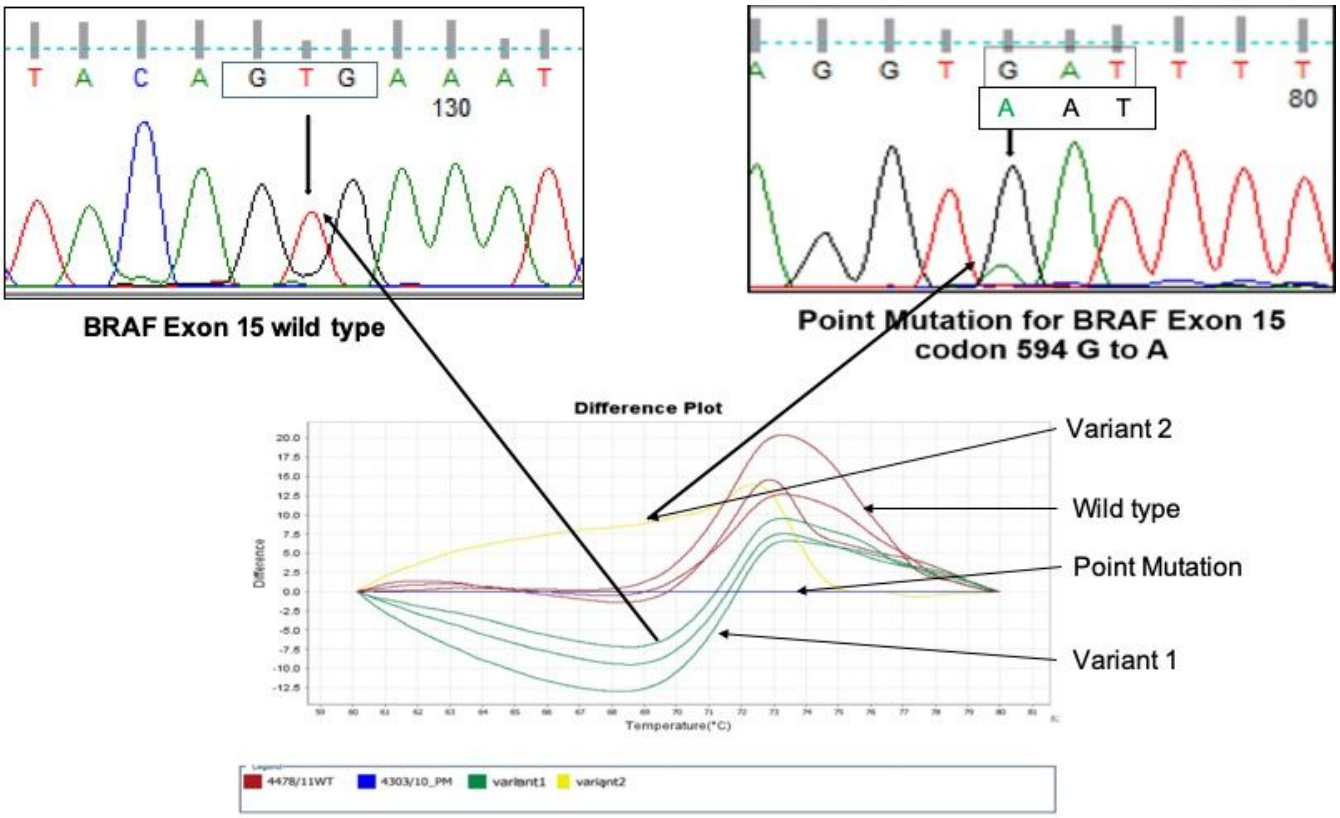

Figure 2

High resolution melt curve and Sanger sequencing confirmation for BRAF exon 15 showing wild and variant curve patterns.

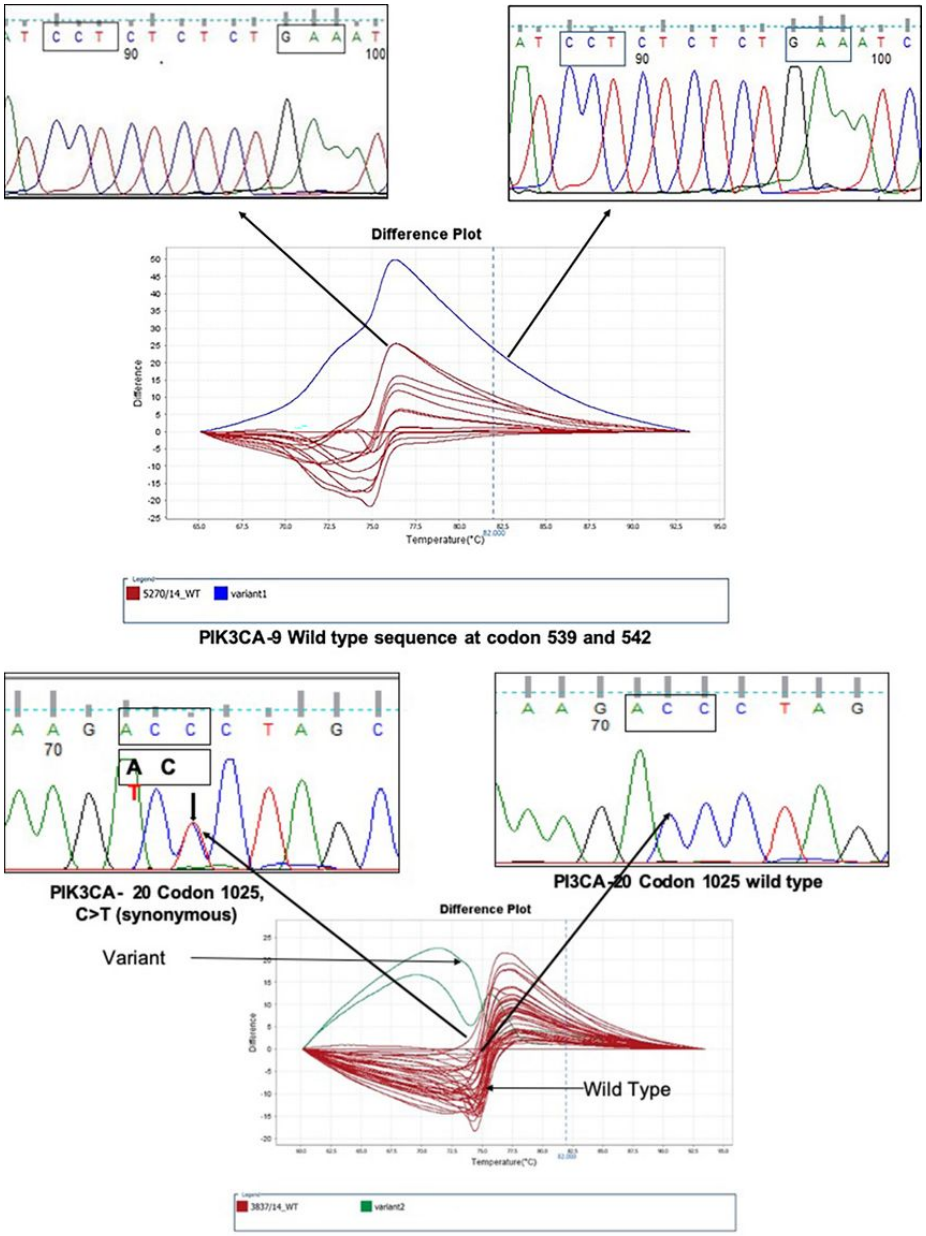

Figure 3

High resolution melt curve and Sanger sequencing confirmation for PIK3CA exon 9 (a), and PIK3CA exon 20 showing wild and variant curve patterns.

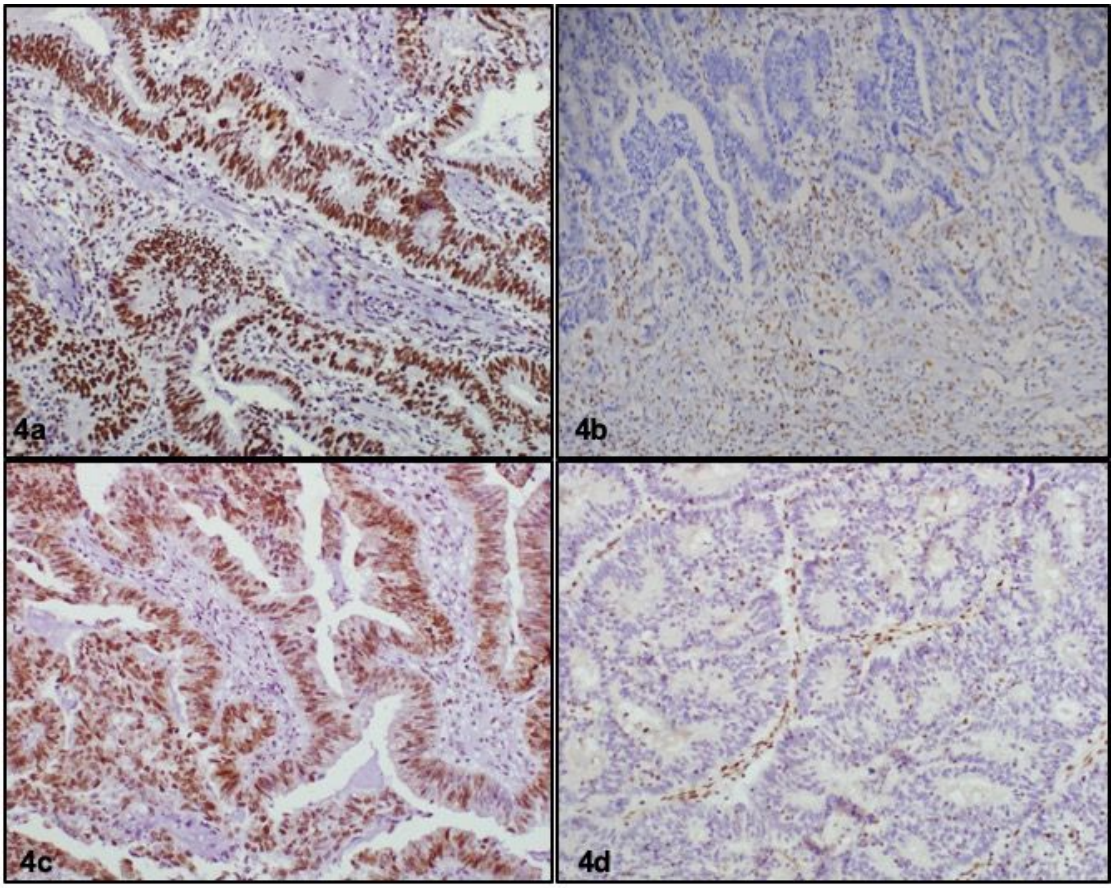

Figure 4

Immunohistochemistry for mismatch repair proteins showing intact expression (a), and loss of expression (b) for MLH1; intact expression (c), and loss of expression (d) for MSH2.

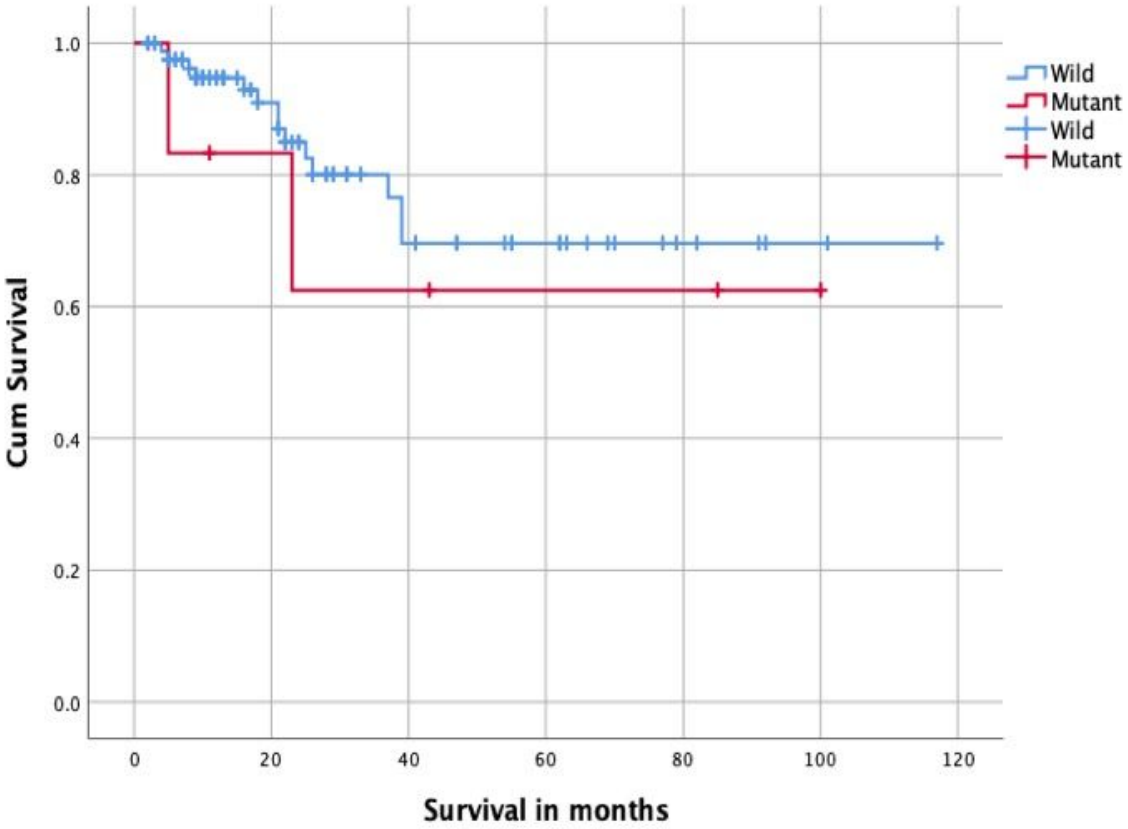

Figure 5

Survival plot showing longer mean survival in KRAS wild GBC.

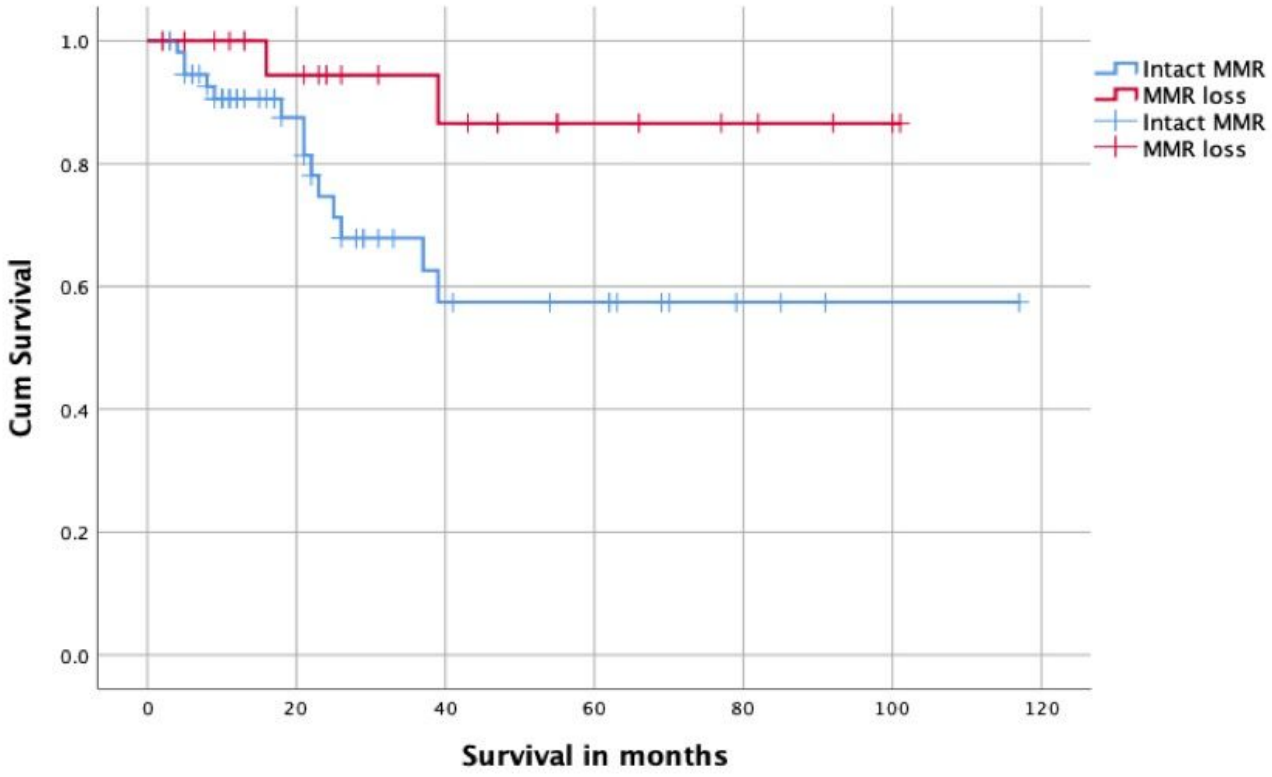

Figure 6

Survival plot showing longer mean survival in mismatch repair deficient GBC.