Multigene mutational profiling of biliary tract cancer is related with pattern of recurrence in surgical resected patients

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Abstract:

Background and Aims: Biliary tract cancer (BTC) is a heterogeneous group of malignancies with poor prognosis arising from the epithelial cells of biliary tree. Recently it has been reported that specific molecular mutations are associated with different types of biliary tree carcinomas, supporting their pathologic and molecular heterogeneity. However, the pathogenic pathways involved in carcinogenesis of BTC are still to be fully defined, and data regarding the relationship between molecular alterations and pattern or timing of recurrence is lacking. The aim of the present study was to investigate the relationship between the mutational gene profile and the pattern of recurrence in BTCs.

Patients and Methods: From September 1990 to December 2012, a total of 103 specimens of patients with BTC (56 PCC, 35 ICC, and 12 GBC), who underwent curative surgery in a single tertiary HPB surgery referral center, were assessed for mutational status in 56 cancer-related genes.

Results: Considering the different types of BTC, the 5-years RFS rate was 16.7%. (median RFS, 7 months) in GBC, 42.9%. (median RFS, 26.4 months) in ICC, 19.7% (median RFS, 16.5 months) in PCC, p=0.166 (figure 3).

The presence of mutations in ARID1A, BRAF, ERBB2, FGFR3, PIK3CA and TP53 genes was significantly associated with poor RFS compared with wild type tumors (median RFS of 11.5 months vs. 19.2 months, p=0.039; 3.0 months vs. 17.0 months, p= 0.002; 5.0 months vs 16.5 months, p=0.017; 5.1 months vs. 16.5 months, *p*=0.024; 11.1 months vs 18.5 months , p= 0.032 and 8.6 months vs 21.9 months , p = 0.003 respectively).

At the multivariate analysis including clinical, pathological and molecular characteristics, the factors independently related with survival were: Radicality of surgery (OR 2.050, C.I. 1.104-3.807, *p*=0.023), LN status (OR 1.835, C.I. 1.006-3.348, *p*=0.048), mutational status of *ARID1A* (OR 2.566, C.I. 1.174-5.608, *p*=0.018) and *TP53* (OR 2.805, C.I. 4.432-5.496, *p*=0.003).

Considering the pattern of recurrence, local recurrence occurred in 47 patients (73.4%) , while systemic recurrence occurred in 17 patients (26.4%) .

Regarding the prognostic genes identified at the univariate analysis: ARID1A mutation was associated with a local and systemic recurrence in the 43% and 29% of cases, respectively; BRAF mutation was associated with a local and systemic recurrence in the 33% and 33% of cases, respectively; ERBB2 and FGFR3 mutation were always associated with a local recurrence; PIK3CA mutation was related with a local and systemic recurrence in the 72% and 14% of cases, respectively; and TP53 mutation was associated with a local and systemic recurrence in the 29% and 41% of cases. Regarding other genes with relatively high rate of mutation: BAP1 mutation was associated with a local and systemic recurrence in the 57% and 29% of cases, respectively; KRAS mutation was related with a local and systemic recurrence in the 42% and 10% of cases, respectively; PBRM1 mutation was associated in the 64% of cases with a local recurrence.

Conclusion:

Our study reported specific prognostic genes for GBC, PCC and ICC that can identify patients with poor prognosis after curative surgery . Moreover, we analyzed the relationship between the mutational gene profile and the recurrence of BTCs. Disease-specific genes identified can be explored for new molecular therapies in clinical trial.

Introduction:

Biliary tract cancer (BTC) is a heterogeneous group of malignancies with poor prognosis arising from the epithelial cells of biliary tree [1,2]. They represent the second most common type of hepatobiliary cancer worldwide accounting for approximately 3% of all gastrointestinal malignancies [3] .

BTC are classified according to their site of origin: gallbladder cancer (GBC), intrahepatic cholangiocarcinoma (ICC) arising from intrahepatic bile ducts, perihilar cholangiocarcinoma (PCC) arising or involving the hepatic biliary confluence, and distal cholangiocarcinoma (DCC) arising from the bile duct distal to the cystic duct origin [4,5]. Regardless of its location BTCs are very aggressive diseases with high rate of local diffusion and metastatic spreading.

The global epidemiological trends in incidence of BTC vary according to geographic regions. Several conditions are considered risk factors for development of BTC such as chronic cholangitis [6,7], liver fluke infection [7,8] viral hepatitis [7,.8], aflatoxin exposure [9] or other chemical exposure [7,10]. Gallbladder cancer is more common in females, while intrahepatic and peri-hilar cholangiocarcinoma have a male predominance [11]. As a general rule, the incidence of BTC increases with age; the typical patient with these malignancies is between 60 and 70 years old. However, BTC arising in the setting of primary sclerosing cholangitis (PSC) and those with choledochal cysts occur in nearly two decades younger patients [12,13].

Surgical resection is the only treatment offering chance of long-term survival, but most cases are unresectable at the time of the diagnosis [14,15]. However, curative resection is possible in less than one-half of patients, and the majority does not achieve long-term disease control [16-21] . Resectability rates for BTC have increased over time, due to more aggressive operative strategies and extended criteria for resectability. The goal for surgical treatment of BTC is to achieve a radical resection with negative histologic margin (R0 resection). In literature, the rate of R0 resection varies according to the type of BTC from 70% to 90%. [22-29]

Nevertheless, even in case of complete radical resection, the majority of BTC recur either locally or with distant metastases [30-31]. Recurrence is a specific prognostic parameter that reflects the biological aggressiveness of these tumors.

The standard treatment for advanced unresectable cholangiocarcinoma are cisplatin or gemcitabine based chemotherapy regimen, but the response rate to these chemotherapy is low, consequently even in treated patients the prognosis is poor with only 5-10% five-year overall survival rate [32]. Nowadays, no effective molecular targeted agent has been approved for biliary tract cancers outside of clinical trial.

Recently it has been shown that specific molecular mutations are associated with different types of biliary tree carcinomas, supporting their pathologic and molecular heterogeneity.

A retrospective study [33] identified molecular subgroups of cholangiocarcinomas that can be explored for specific drug targeting in clinical trials. In that study the mutational status of 56 cancer-related genes in 153 biliary tract cancers was assayed. The genes most frequently involved were *KRAS* (28%), *TP53* (18%), *ARID1A* (12%), *IDH1/2* (9%), *PBRM1* (9%), *BAP1* (7%), and *PIK3CA* (7%). In particular *IDH1/2* (p=0.0005) and *BAP1* (p=0.0097) mutations were characteristic of ICC, while *KRAS* (p=0.0019) and *TP53* (p=0.0019) were more frequent in ECC and GBC. Furthermore *TP53* was identify as an independent prognostic factor in cholangiocarcinoma.

In a previous study [34] we reported specific prognostic genes in terms of overall survival that can identify patients with poor prognosis after curative surgery. In particular 91 patients with cholangiocarcinoma who underwent curative surgery were assessed for mutational status in 56 cancer-related genes. *ALK* and *IDH1* mutation had an exclusive prognostic impact for PCC, and ARID1A, PIK3C2G, STK11, and TGFBR2 for ICC. However, mutation of TP53 confirmed as a negative molecular prognostic factor. In fact the presence of mutations in ALK,

IDH1, and TP53 genes was significantly associated with poor prognosis in patients with PCC compared to wild type (median overall survival 5.0 vs. 34.9 months, p=0.001, 9.1 vs. 29.6 months, p = 0.043; and 15.4 vs. 32.5 months, p = 0.019, respectively). On the other hand mutations of *ARID1A*, *PIK3C2G*, *STK11*, *TGFBR2*, and *TP53* genes was significantly associated with poor prognosis in patients with ICC compared to wild type (median overall survival of 14.0 vs.52.0 months, p = 0.012; 11.8 vs. 40.1 months, p = 0.030; 11.8 vs. 40.1 months, p = 0.030; 9.3 vs. 40.1 months, p = 0.011; and 5.7 vs. 40.1 months, p = 0.011, respectively).

Wardell C et al [35] performed a large-scale genome sequencing analysis of 412 BTC samples from Japanese and Italian series (136 ICC, 101 DCC, 109 PCC, and 66 GBC) to investigate their somatic and germline driver events and characterize their genomic landscape. They identified 32 significantly and commonly mutated genes including *TP53*, *KRAS*, *SMAD4*, *NF1*, *ARID1A*, *PBRM1*, and *ATR*, suggesting that *KRAS*, *MUC17* and *ARID1A* negatively affected patient prognosis. Moreover, the authors identified somatic alterations and searched for driver genes in BTCs, finding pathogenic germline variants of cancer-predisposing genes predicting cell-of-origin for BTCs by combining somatic mutation patterns and epigenetic features.

There is evidence that ICC is more frequently due to alterations in genes involved in epigenetic regulation [33,36] whereas extrahepatic and gallbladder subtypes are driven by mutations in *TP53* and cell cycle genes. However, the pathogenic pathways involved in carcinogenesis of BTC are still to be fully defined, and data regarding the relationship between molecular alterations and pattern or timing of recurrence is lacking.

The aim of the present study was to investigate the relationship between the mutational gene profile and the pattern of recurrence in BTCs.

Patients and Methods:

Ethics statement

Data collection and analysis were performed according to the institutional guidelines and conformed to the ethical standards of the World Medical Association (Declaration of Helsinki). Specimens of resected patients were retrospectively retrieved from the formalin fixed paraffin embedded (FFPE) archives of the Department of Pathology-Diagnostics and the Arc-Net biobank of the University and Hospital Trust of Verona under a local ethics committee ARC-Net approval number prog. 1959.

Definition of Biliary Tract Cancers subtypes

Biliary Tract Cancer (BTC) were classified according to WHO 2010 and AJCC/UICC 7th edition criteria as gallbladder cancer (GBC), intrahepatic cholangiocarcinoma (ICC) and perihilar cholangiocarcinoma (PCC) [3,4].

Patients

From September 1990 to December 2012, a total of 194 patients with BTC submitted to surgical resection with radical intent in a single tertiary HPB surgery referral centre. In 103 BTC specimens the material was sufficient for the pathological and molecular analysis.

The clinical and pathological data were prospectively collected in all patients. For the 103 BTC, tissue microarrays (TMAs) were also prepared using two 1-mm cores for each case.

DNA extraction and PCR Amplifications

As previously described [33,34] DNA was prepared from tissues after enrichment for neoplastic cellularity using manual microdissection. A total of 5 to 15 consecutive 4-µm FFPE sections per case were used. DNA was extracted using the QIAamp DNA FFPE Tissue Kit (Qiagen). Purified DNA was quantified and its quality assessed using NanoDrop (Invitrogen Life Technologies) and Qubit (Invitrogen Life Technologies) platforms [38]. DNA quality was further evaluated by PCR analysis using the BIOMED 2 PCR multiplex protocol [39] with PCR products analyzed by DNA 1000 Assay (Invitrogen Life Technologies) on the Agilent 2100 Bioanalyzer on-chip electrophoresis (Agilent Technologies).

Two multigene panels were used: the 50-gene Ion AmpliSeq Cancer Hotspot Panel v2 (Life Technologies) and a 7-gene AmpliSeq Custom Panel. The first explores selected regions of the following 50 cancer-associated genes, in alphabetical order: *ABL1, AKT1, ALK, APC, ATM, BRAF, CDH1, CDKN2A, CSF1R, CTNNB1, EGFR, ERBB2, ERBB4, EZH2, FBXW7, FGFR1, FGFR2, FGFR3, FLT3, GNA11, GNAS, GNAQ, HNF1A, HRAS, IDH1, IDH2, JAK2, JAK3, KDR/VEGFR2, KIT, KRAS, MET, MLH1, MPL, NOTCH1, NPM1, NRAS, PDGFRA, PIK3CA, PTEN, PTPN11, RB1, RET, SMAD4, SMARCB1, SMO, SRC, STK11, TP53, VHL*. The details of the target regions may be found at http://www.lifetechnologies.com. The 7-gene custom panel was designed to target selected regions of a gene included in the 50-gene panel (*IDH2*) and six genes that were selected according to the results of a previously published ICC exome sequencing study (*ARID1A*, *BAP1*, *PBRM1*, *PIK3C2A*, *PIK3C2G*, *TGFBR2*)[40].

Sequencing was run on the Ion Torrent Personal Genome Machine (PGM, Life Technologies) loaded with 316 (50-gene panel) or 318 chips (7-gene panel).

Statistical analysis

Data were collected and analyzed with SPSS statistical software (SPSS version 21 Inc. Chicago Ill.) The differences between categorical variables were analyzed with a chi-square test. Comparisons between means were carried out with a t test. Recurrence Free Survival analysis was carried out using the Kaplan-Meier method. We considered the treatment day as time zero and patients recurrence free at the end of follow-up were considered censored. The mean follow-up period was 42.6 ± 36.2 months. Ten patients with 90-days postoperative mortality (1 GBC, 1 ICC and 8 PCC) were excluded from survival analysis.

A multivariate analysis including the clinical, pathological and molecular factors related to survival at the univariate analysis (with a p value < 0.05) were carried out with Cox's regression model with forward and backward analysis to identify factors that were independently related with survival. The p value < 0.05 was regarded as statistically significant.

Results:

The clinical and pathological features of the 103 patients included in the study are summarized in Table 1. The median age of the patients was 66 years (IQR 60 to 72) . The study population included 12 Gallbladder Cancer (GBC), 35 Intrahepatic Cholangiocarcinoma (ICC), and 56 Perihilar Cholangiocarcinoma (PCC). Sixty-seven patients were male (65.0%) and 36 were female (35.0%). Major hepatectomy was required in 72 patients (69.9%). R0 resection was achieved in 76 patients (73.8%). Lymph node dissection was performed in 96 patients (95.2%) and the rate of positive lymph-node was 46.7%. Microvascular invasion and perineural invasion was found in 74 patients (71.8%) and 64 (62.1%) of patients, respectively. The postoperative mortality was 9.7% (n 10).

A detailed description of gene mutations in GBC, ICC and PCC is reported in Table 2 and Figure 1. No mutations were identified in the following 19 genes: *ABL1, AKT1, ATM, CDH1, CSF1R, FGFR1, FGFR2, FLT3, HNF1A, JAK2, JAK3, MET, MPL, NOTCH1, NPM1,*

PDGFRA, RB1, RET, SMO and SRC. At least one gene mutation was identified in 77.7 % of the tumors (n=80). 51% of the tumors reported more than one gene mutation (n=41).

The most frequently mutated genes in GBC was *TP53*, with mutation in 41.7% (n 5) of the tumors. Other genes with a high rate of mutations (over 10%) in GBC were: *KRAS* in 25.0% (n 3), *ARID1A* in 16.7% (n 2) and *SMAD4* in 16.7% (n 2).

In ICC the most frequently mutated genes were N*RAS* and *IDH1* with mutation in 17.1% (n 6) of the tumors. Other genes with a rate of mutations over 10% in ICC were: BAP1 in 14.3% (n 5), *ARID1A* in 11.4% *(n 4) and PBRM1* in 11.4% (n 4).

The most frequently mutated gene in PCC was *KRAS*, with mutation in 41.1% (n 23) of the tumors. Other genes with a high rate of mutations (over 10%) in PCC were: *TP53* in 19.6% (n 11), *ARID1A* in 14.3% (n 8) and *PBRM1* in 10.7% (n 6).

Comparison in molecular profile between GBC, ICC and PCC

The results of the univariate analysis of comparison in molecular profile among the three groups are showed in table 2.

We observed a statistically significant higher frequency of mutation for *KRAS,* in PCC and GBC compared with ICC, in 41.1%, 25.0% and 8.6% of tumors, respectively, p=0.003. Moreover, TP53 resulted most commonly mutated in PCC and GBC compared with ICC, in 19.6%, 41.7% and 5.7% of tumors, respectively, p=0.015. Conversely, IDH1 and BAP1 were more commonly mutated in ICC compared with GBC and PCC, in particular IDH1 was mutated in 17.1% (n 6), 0% and 3.6% (n 2) of tumors, respectively, p = 0.035, and BAP1 was mutated in 14.3% (n 5), 0% and 3.6% (n 2) of tumors, respectively, p = 0.087.

APC, ERBB2, SMARCB1 and RB1 were mutated only in GBC. ERBB4, FGFR3 and NRAS were mutated only in ICC. ALK, CTNNB1, FBXW7, GNAS, KIT and PTPN11 were mutated only in PCC.

Relationship between gene mutations and clinic-pathological characteristics

The relationship between gene mutation and clinic-pathological features of patients is shown in Figure 2.

ARID1A had an higher frequency of mutation in patients < 70 years compared with ≥ 70 years (18.5% vs 5.3 % , p=0.050). *KRAS* mutation resulted in 35.9 % of resected tumors with perineural invasion compared with 15.4 % of resected tumors without perineural invasion (p= 0.020). *PIK3CA* mutation is always associated with microvascular invasion (p= 0.041).

TP53 was more frequently mutated in patients with positive LN compared with patients with negative LN (23.9 % vs 11.5 %, p=0.039), in high grade tumors (G3-G4) compare with low grade tumors (G1–G2) (31% vs 12.2 %, p= 0.027) and in tumor with perineural invasion compare with tumor without perineural invasion (23.4% vs 7.7 %, p= 0.034).

Factors related with Recurrence Free Survival (RFS) after surgery

The results of the univariate analysis and multivariate analysis of recurrence free survival (RFS) in the study population are shown in Table 3.

Considering the different types of BTC, the 5-years RFS rate was 16.7%. (median RFS, 7 months) in GBC, 42.9%. (median RFS, 26.4 months) in ICC, 19.7% (median RFS, 16.5 months) in PCC, p=0.166 (figure 3). The the 5-years RFS rate was 31.0% in patients with negative lymph nodes (median RFS, 26.4 months) compared with 24.9% (median RFS 11.0 months) in patients with positive lymph nodes, $p = 0.006$ (figure 4). The the 5-years RFS rate was 33.4% in R0 resection (median RFS, 30.5 months) compared with 5.9% (median RFS 17.1 months) in patients with positive margin (R1), $p = 0.002$ (figure 5). The the 5-years RFS rate was 38.5% in patients without microvascular invasion (median RFS 29.5 months) compare with 22.5% (median RFS 12.2 months) in patients with microvascular invasion, $p = 0.009$. Moreover, The

the 5-years RFS rate was 32.2% in patients with AJCC Stage I-II tumors (median RFS 26.4 months) compare with 23.4% (median RFS 12.0 months) in patients with AJCC Stage III-IV tumors, $p = 0.024$.

The presence of mutations in ARID1A (figure 6), BRAF, ERBB2, FGFR3, PIK3CA and TP53 (figure 7) genes was significantly associated with poor RFS compared with wild type tumors (median RFS of 11.5 months vs. 19.2 months, p=0.039; 3.0 months vs. 17.0 months, p= 0.002; 5.0 months vs 16.5 months, p=0.017; 5.1 months vs. 16.5 months, *p*=0.024; 11.1 months vs 18.5 months , p= 0.032 and 8.6 months vs 21.9 months , p = 0.003 respectively) (see table 3).

At the multivariate analysis including clinical, pathological and molecular characteristics, the factors independently related with survival were: Radicality of surgery (OR 2.050, C.I. 1.104-3.807, *p*=0.023), LN status (OR 1.835, C.I. 1.006-3.348, *p*=0.048), mutational status of *ARID1A* (OR 2.566, C.I. 1.174-5.608, *p*=0.018) and *TP53* (OR 2.805, C.I. 4.432-5.496, *p*=0.003).

Timing and pattern of recurrence

Recurrence occurred in 63 patients (67.7%), in particular, 31 patients (49.1%) recurred within 12 months from surgery (early recurrence), the trend of frequency of recurrence according with time after surgery is show in figure 8. In early recurrence group the frequency of mutation of BRAF, PIK3CA and TP53 were higher compared with other patients (13.3% vs 0%, p =0.027, 15.2% vs 1.8%, p=0.025 and 27.3% vs 10.7%, p=0.044, respectively).

Moreover BAP1 mutation was related with late recurrence (> 24 months after surgery), p=0.046.

Considering the pattern of recurrence, local recurrence occurred in 47 patients (73.4%) , while systemic recurrence occurred in 17 patients (26.4%) .

We observed a relationship between the pattern of recurrence and the mutational gene profile (Figure 9 and Figure 10). Regarding the prognostic genes identified at the univariate analysis:

ARID1A mutation was associated with a local and systemic recurrence in the 43% and 29% of cases, respectively; BRAF mutation was associated with a local and systemic recurrence in the 33% and 33% of cases, respectively; ERBB2 and FGFR3 mutation were always associated with a local recurrence; PIK3CA mutation was related with a local and systemic recurrence in the 72% and 14% of cases, respectively; and TP53 mutation was associated with a local and systemic recurrence in the 29% and 41% of cases. Regarding other genes with relatively high rate of mutation: BAP1 mutation was associated with a local and systemic recurrence in the 57% and 29% of cases, respectively; KRAS mutation was related with a local and systemic recurrence in the 42% and 10% of cases, respectively; PBRM1 mutation was associated in the 64% of cases with a local recurrence.

Discussion

Biliary tract cancers (BTCs) are clinically and pathologically heterogeneous malignancies with poor response to treatments .Surgical resection is the only potential cure for BTC . Although resectability has improved recently, the success rate remains poor and even in case of complete radical resection, the majority of BTC recurred. In our study the 67.7% of resected tumors recurred even if the R0 resection was achieved in 76 patients (73.8%). This is probably related to different mutational profile that influence the aggressive behaviour of these tumors.

Genomic profiling can offer a clearer understanding of their carcinogenesis, classification and treatment strategy.

In this study, we analyzed the molecular features of GBC, PCC and ICC of a series from a single tertiary HPB referral center and confirmed previously published data[33,34] . The main findings of our study showed: specific molecular characteristics for GBC, PCC and ICC and distinctive molecular prognostic factors for GBC, PCC and ICC.

From our data, the macroscopic type of GBC, PCC and ICC seem to have significant differences at the molecular level.

The most frequently mutated genes in GBC were *TP53*(41.7%), *KRAS* (25.0%), *ARID1A* (16.7%) and *SMAD4* (16.7). In ICC the most frequently mutated genes were N*RAS* (17.1%), *IDH1*(17.1%) , BAP1 (14.3%) , *ARID1A* (11.4%) *and PBRM1* (11.4%) The most frequently mutated gene in PCC were *KRAS*(41.1%) ,*TP53* (19.6%) , *ARID1A* (14.3%) and *PBRM1* (10.7%).

Moreover, our study confirmed that some gene mutations are specific for the different subtypes of BTC : *APC*, *ERBB2*, *SMARCB1* and *RB1* were mutated only in GBC, *ERBB4*, *FGFR3* and *NRAS* were mutated only in ICC, *ALK*, *CTNNB1*, *FBXW7*, *GNAS*, *KIT* and *PTPN11* were mutated only in PCC.

We observed a relationship between specific gene mutations and the clinic-pathological characteristics of the tumors. In particular *ARID1A* had an higher frequency of mutation in patients < 70 years old , *KRAS* in tumors with perineural invasion , *PIK3CA* in tumors with microvascular invasion . On the other hand *TP53* was more frequently mutated in patients with positive LN , high grade tumors (G3-G4) and in tumor with perineural invasion .

Analyzing the mutational profile and the clinic-pathological features of the patients in relation with the recurrence free survival rate, we found clinical and molecular independent prognostic factors. Our data confirmed that the surgical radicality , the LN status , the mutational status of *ARID1A* and *TP53* represent prognostic factor independently related with survival. Other gene significantly associated with poor RFS were *BRAF*, *ERBB2*, *FGFR3* and *PIK3CA*.

Moreover we observed a relationship between specific gene mutation and the timing of recurrence. In particular, *BRAF*, *PIK3CA* and *TP53* mutation was associated to early recurrence (within 12months), while mutation of *BAP1* was associated with late recurrence (after 12 months).

Furthermore we assessed a relationship between the pattern of recurrence and the mutations of the prognostic genes. Considering the mutations of the genes independently related whit a poor prognosis , we found that *ARID1A* mutation was more frequently associated with a local recurrence while *TP53* mutation was more frequently associated with a systemic recurrence;

A limitation of the current study is the small sample size, although data in the literature on the molecular profiling of BTCs are frequently multi-institutional and limited to a small number of patients. Moreover, statistical analysis on differences between subgroups and survival analysis could be suboptimal due to the low frequency rate of some gene mutations.

An external validation and further study are needed to confirm our results.

Conclusions

Mutational genes profiling identified different gene mutations between GBC, PCC and ICC. In particular, our study reported specific prognostic genes for GBC, PCC and ICC that can identify patients with poor prognosis after curative surgery . Moreover, we analyzed the relationship between the mutational gene profile and the recurrence of BTCs. Disease-specific genes identified can be explored for new molecular therapies in clinical trial.

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Tables and Figures

Table 1. Clinical and pathological features of 103 patients included in the study

		BTC (n=103)
Age, year, median (IQR)		66 (60-72)
Gender, M/F (%)		67 (65.0) / 36 (35.0)
Gallbladder Cancer, n (%)		12 (11.7)
Intrahepatic Cholangiocarcinoma, n (%)		35 (34.0)
Peri-hilar Cholangiocarcinoma, n (%)		56 (54.4)
Major Hepatectomy, n (%)		72 (69.9)
Bile Duct Resection, n (%)		56 (54.4)
Portal Vein Resecion, n (%)		15 (14.6)
Hepatic Artery Resection, n (%)		2 (1.9)
Postoperative Mortality, n (%)		10 (9.7)
R0 Resection, n (%)		76 (73.8)
Lymphnode Status, n (%)	**N/A**	7 (6.8)
	Negative	50 (48.5)
	Positive	46 (46.7)
Histologic Grading, n (%)	**G1-2**	74 (71.8)
	G3-4	29 (28.2)
Perineural Invasion, n (%)		64 (62.1)
Microvascular Invasion, n (%)		74 (71.8)
AJCC Stage, n (%)	**I**	10 (9.7)
	II	29 (28.2)
	III	43 (41.7)
	IV	19 (18.4)
	N/A	2

BTC, Biliary Tract Cancer; AJCC, American Joint Committee on Cancer Staging System;

Table 2. Frequency and comparison of gene mutations in the study population, including 12 Gallbladder Cancer (GBC), 35 Intrahepatic Cholangiocarcinoma (ICC), and 56 Perihilar Cholangiocarcinoma (PCC).

Gene mutations	TOT n 103	GBC n 12	ICC n 35	PCC n 56	*p* values
ALK	1 (0.9%)	0	0	1 (1.8%)	0.655
APC	1 (0.9%)	1 (8.3%)	0	0	0.112
ARID1A	14 (13.6%)	2 (16.7%)	4 (11.4%)	8 (14.3%)	0.879
BAP1	7 (6.8%)	0	5 (14.3%)	2 (3.6%)	0.087
BRAF	3 (2.9%)	0	2 (5.7%)	1 (1.8%)	0.453
CDKN2A	2 (1.9%)	1 (8.3%)	0	1 (1.8%)	0.194
CTNNB1	1 (0.9%)	0	0	1 (1.8%)	0.655
EGFR	2 (1.9%)	1 (8.3%)	0	1 (1.8%)	0.194
ERBB2	1 (0.9%)	1 (8.3%)	0	0	0.022
ERBB4	1 (0.9%)	0	1 (2.9%)	0	0.375
FBXW7	2 (2.9%)	0	0	2 (3.6%)	0.425
FGFR3	1 (0.9%)	0	1 (2.9%)	0	0.375
GNAS	1 (0.9%)	0	0	1 (1.8%)	0.655
HRAS	1 (0.9%)	0	0	1 (1.8%	0.655
IDH1	8 (7.7%)	0	6 (17.1%)	2 (3.6%)	0.035
IDH2	2 (1.9%)	0	1 (2.9%)	1 (1.8%)	0.819
KDR	3 (2.9%)	1 (8.3%)	0	2 (3.6%)	0.304
KIT	1 (0.9%)	0	0	1 (1.8%)	0.655
KRAS	29 (28.1%)	3 (25.0%)	3 (8.6%)	23 (41.1%)	0.003
MLH1	1 (0.9%)	0	0	1 (1.8%)	0.655
NRAS	6 (5.8%)	0	6 (17.1%)	0	0.002
PBRM1	11 (10.7%)	1 (8.3%)	4 (11.4%)	6 (10.7%)	0.956
PIK3CA	7 (6.8%)	1 (8.3%)	1 (2.9%)	5 (8.9%)	0.321
PIK3C2A	4 (3.9%)	0	1 (2.8%)	3 (5.4%)	0.635
PIK3C2G	6 (5.8%)	0	1 (2.8%)	5 (8.9%)	0.319
PTEN	4 (3.9%)	1 (8.3%)	1 (2.8%)	2 (3.6%)	0.687
PTPN11	1 (0.9%)	0	0	1 (1.8%)	0.655
SMAD4	5 (4.8%)	2 (16.7%)	0	2 (3.6%)	0.066
SMARCB1	1 (0.9%)	1 (8.3%)	0	0	0.022
STK11	2 (1.9%)	0	1 (2.8%)	1 (1.8%)	0.819
RB1	1 (0.9%)	1 (8.3%)	0	0	0.022
TGFBR2	5 (4.8%)	1 (8.3%)	1 (2.8%)	3 (5.4%)	0.723
TP53	18 (17.5%)	5 (41.7%)	2 (5.7%)	11 (19.6%)	0.015

Table 3. Univariate and multivariate analysis of recurrence free survival in the study population.

		Univariate Analysis			Multivariate analysis		
		Median survival (months)	5-years RFS rate (%)	p values	Hazard Ratio	95% C.I.	p values
Age, year	**< 70**	16.0	22.1	0.316			
	≥ 70	24.5	37.0				
Gender	**M**	15.0	28.4	0.818			
	F	19.2	26.3				
Gallbladder Cancer		7.0	16.7	0.166			
Intrahepatic Cholangiocarcinoma		26.4	42.9				
Peri-hilar Cholangiocarcinoma		16.5	19.7				
Radicality	**R0**	21.9	33.4	0.002	2.050	1.104-3.807	0.023
	R1	9.0	5.9				
Lymph-node Status	**Negative**	26.4	31.0	0.006	1.835	1.006-3.348	0.048
	Positive	11.0	24.9				
Grading	**G1-G2**	18.5	28.5	0.586			
	G3-G4	12.0	23.8				
Microvascular Invasion	**0**	29.5	38.5	0.009			
	1	12.2	22.5				
Perineural Invasion	**0**	26.7	28.2	0.340			
	1	14.7	27.0				
AJCC-UICC Stage	**I-II**	26.4	32.2	0.024			
	III-IV	12.0	23.4				
ARID1A	**wt**	19.2	31.5	0.039	2.566	1.174-5.608	0.018
	mut	11.5	0.0				
BRAF	**wt**	17.0	27.8	0.002			
	mut	3.0	0.0				
ERBB2	**wt**	16.5	27.5	0.017			
	mut	5.0	0.0				
FGFR3	**wt**	16.5	27.5	0.024			
	mut	5.1	0.0				
PIK3CA	**wt**	18.5	29.3	0.032			
	mut	11.0	0.0				
TP53	**wt**	21.9	31.2	0.003	2.805	1.432-5.496	0.003
	mut	8.6	0.0				

Figura 1. Detailed description on gene mutations in the study population. Each column represent a patient, genes tested are listed in rows. Red rectangles indicate mutations in a given gene and patient

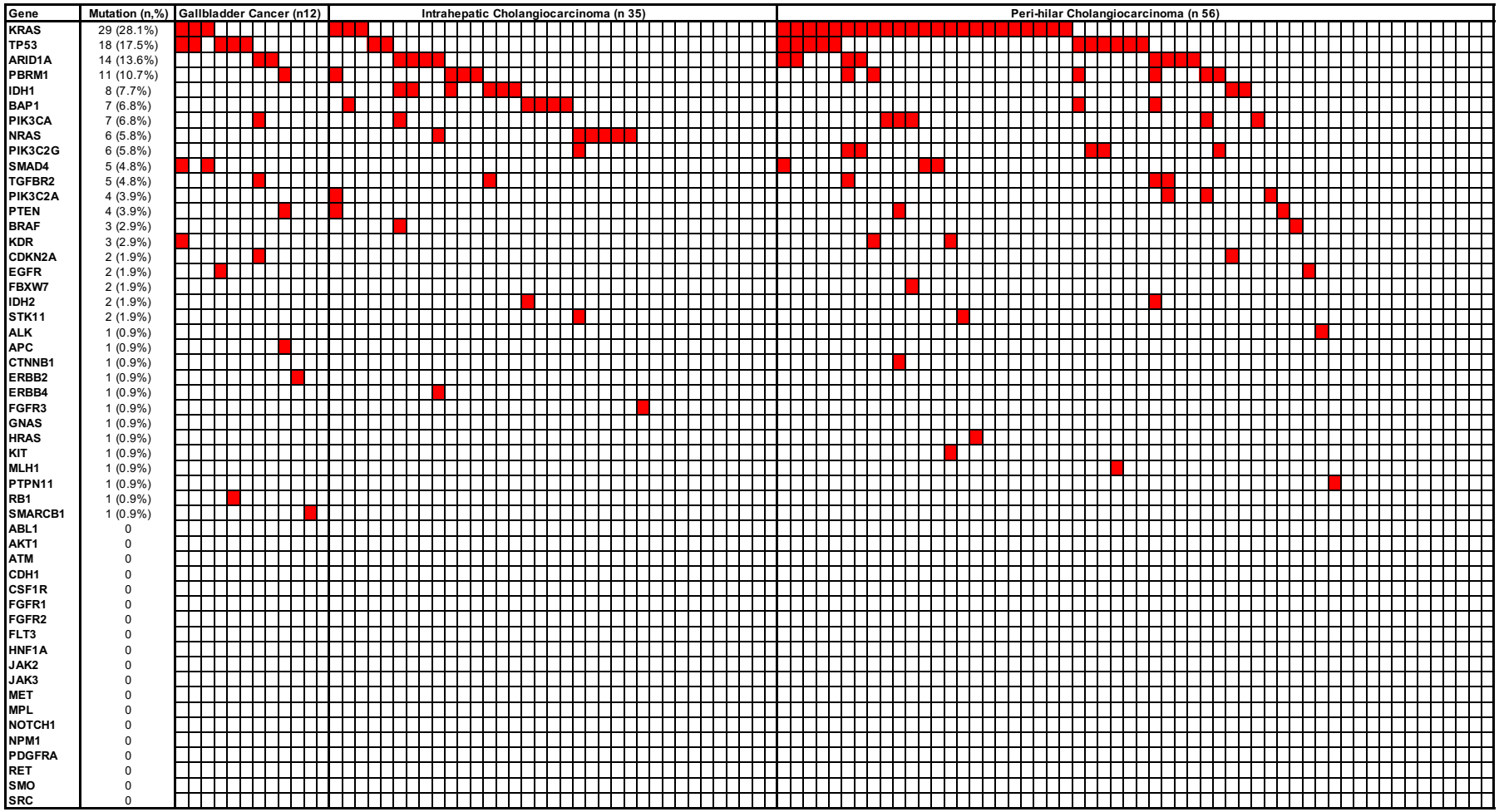

Figura 2. Relationship between gene mutation and clinical characteristics. Frequency of gene mutation in the study population are reported between round brake. Each column represent the frequency of specific gene mutation in the different subgroups.

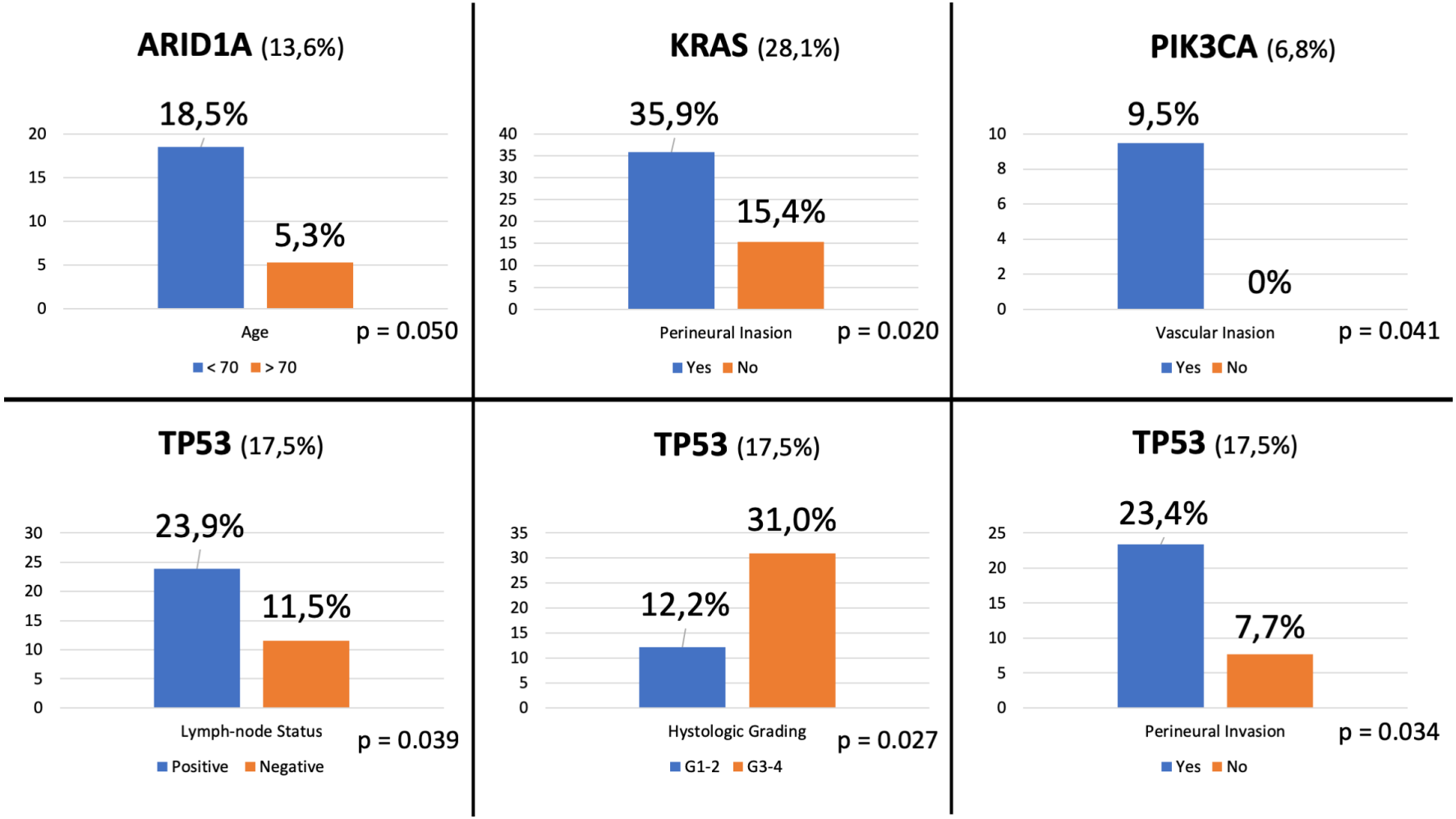

Figure 3. Recurrence Free Survival curves according to type of Biliary Tract Cancer (BTC): Intrahepatic Cholangiocarcinoma, green line; Peri-hilar Cholangiocarcinoma, red line; Gallbladder Cancer, blue line; p = 0.166.

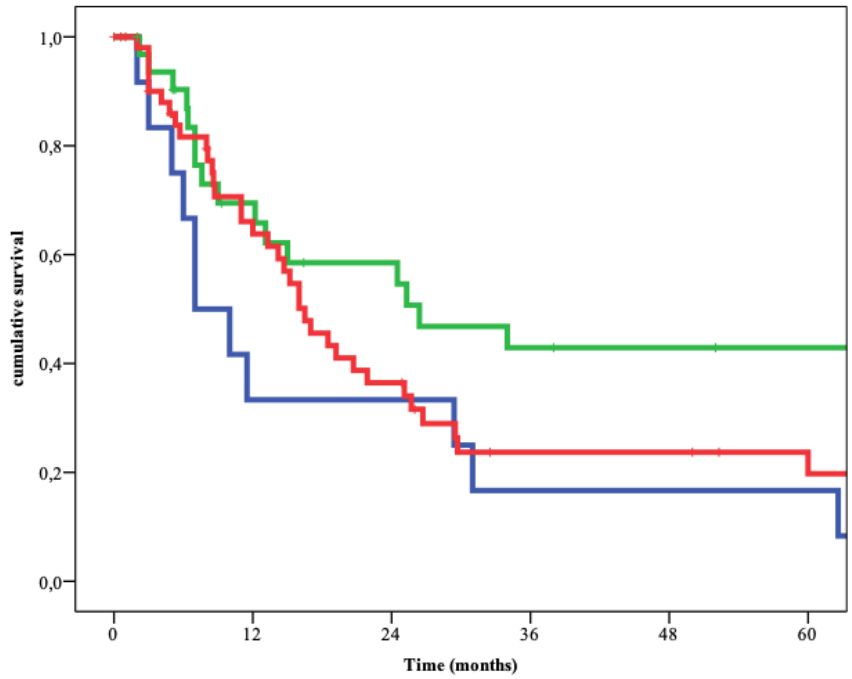

Figure 4. Recurrence Free Survival curves according to Lymph-node (LN) status: Negative LN, blue line; Positive LN, green line; p = 0.006.

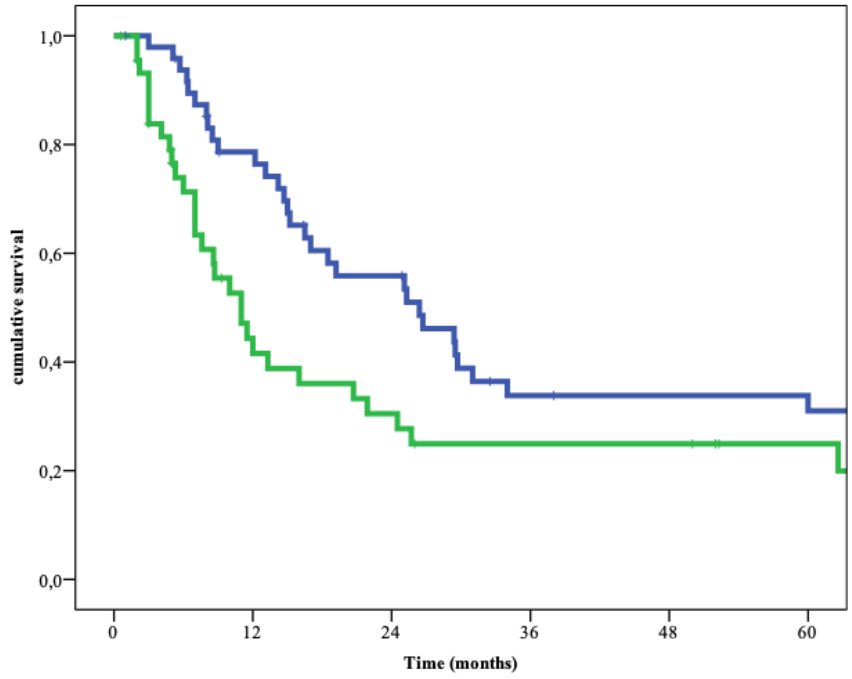

Figure 5. Recurrence Free Survival curves according to Radicality of surgery: R0 resection, blue line; R1 resection, green line; p = 0.002.

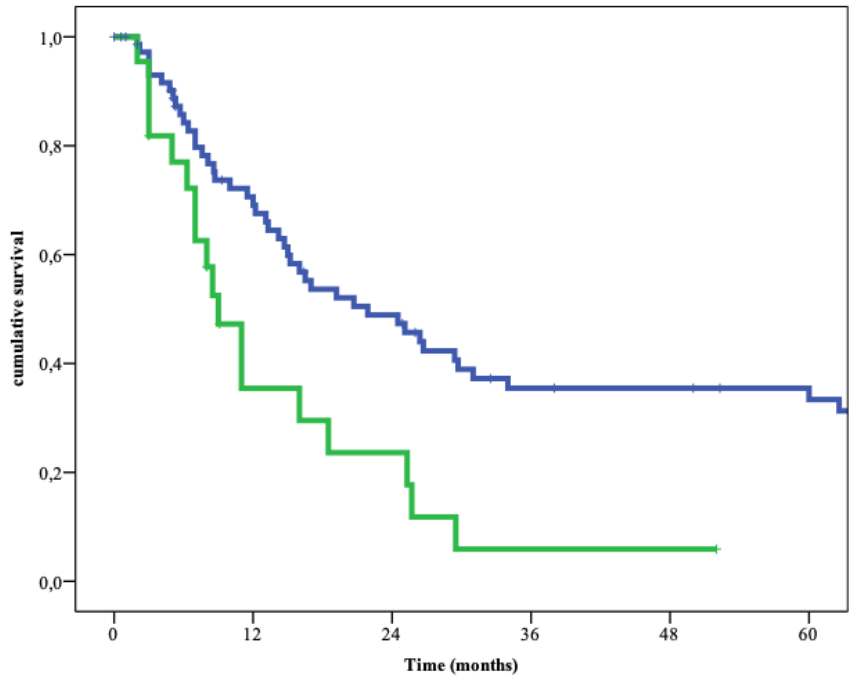

Figure 6. Recurrence Free Survival curves according to mutational status of ARID1A: Wild type, blue line; Mutated, green line; p = 0.039.

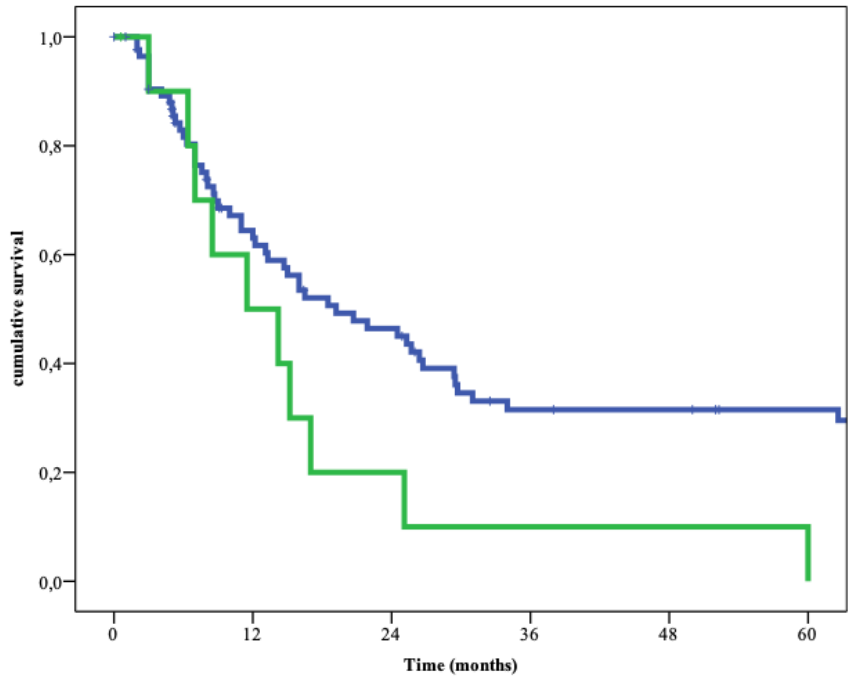

Figure 7. Recurrence Free Survival curves according to mutational status of TP53: Wild type, blue line; Mutated, green line; p = 0.003.

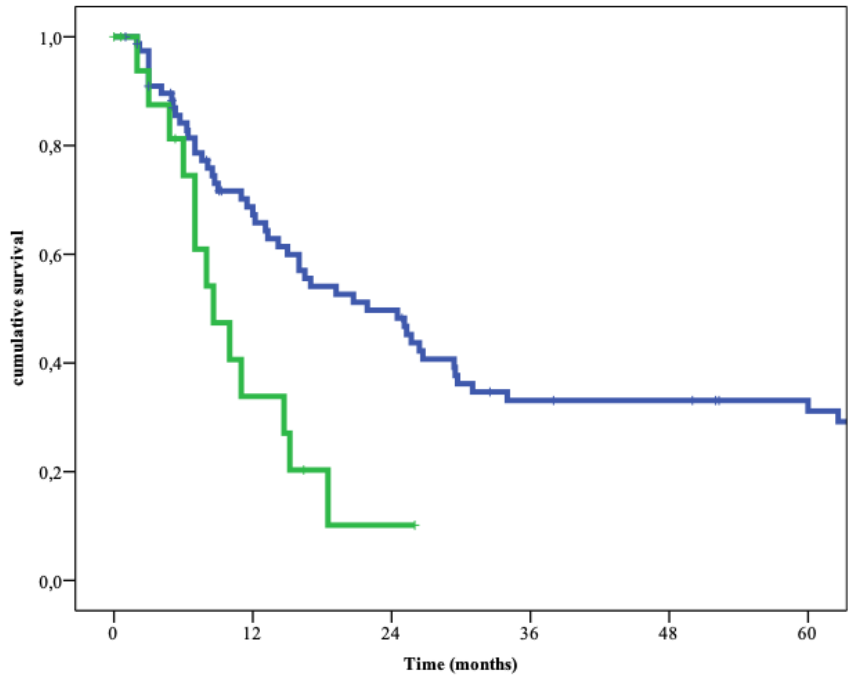

Figure 8. Frequency of recurrence during different time period after surgery and specific gene mutations.

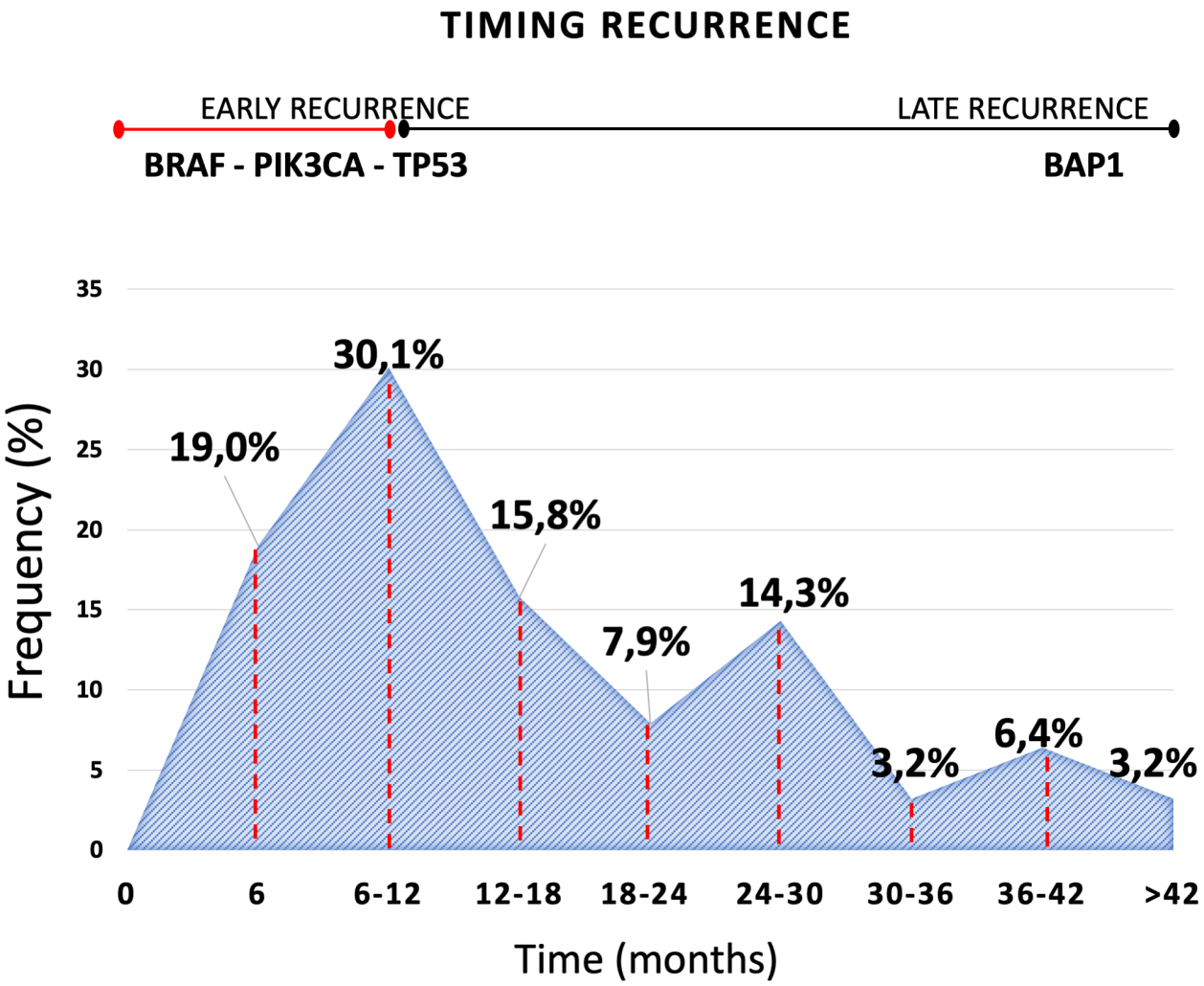

Figure 9. Type of recurrence according to mutation of prognostic genes identified at Recurrence Free Survival univariate analysis

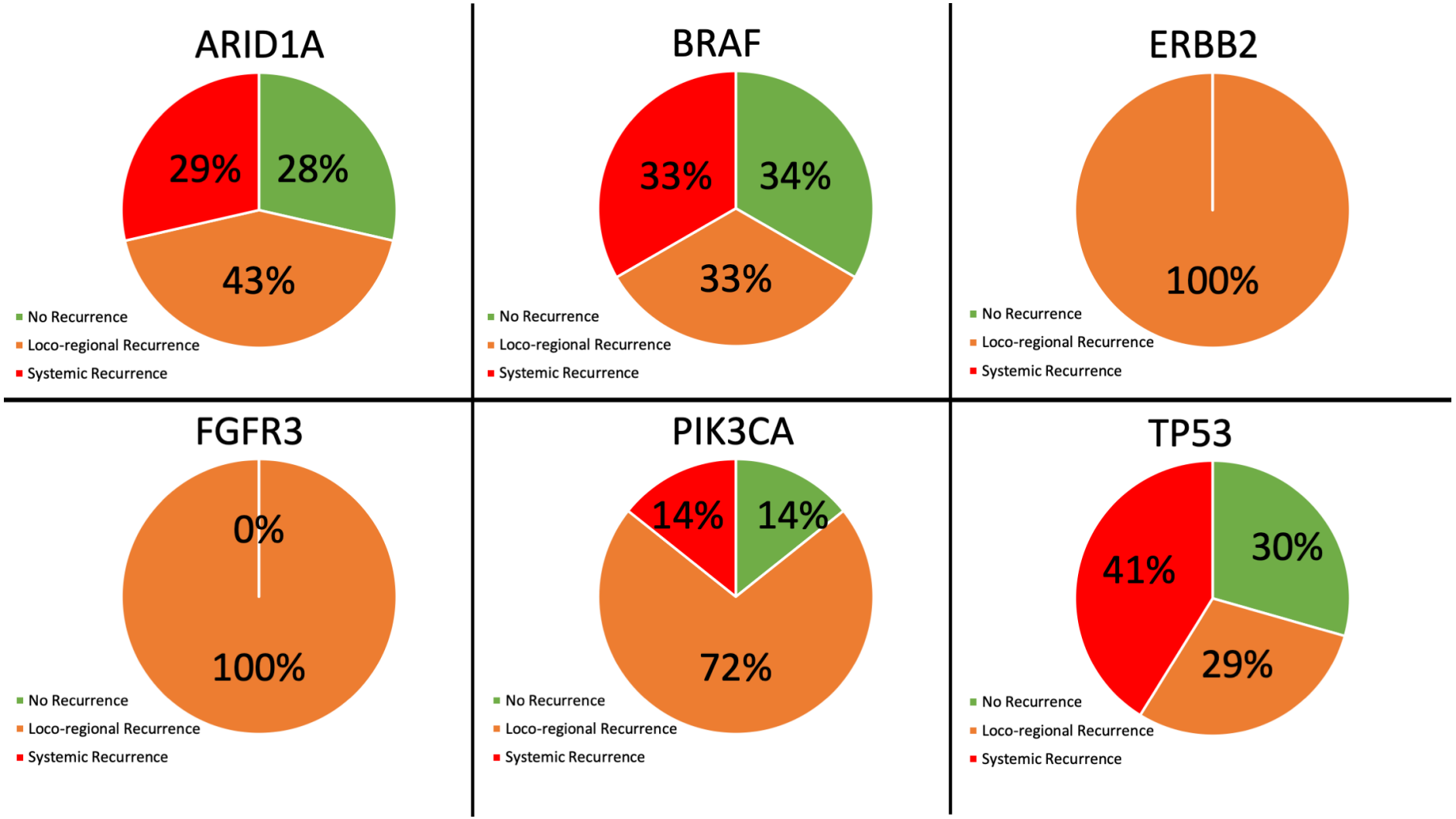

Figure 10. Type of recurrence according to mutation of other most common mutated genes.