Abstract. Peripheral blood of cancer patients “physiologically” presents cells and cellular components deriving from primary or metastatic sites, including circulating tumor cells (CTCs), circulating free DNA (cfDNA) and exosomes containing proteins, lipids and nucleic acids. The term circulating tumor DNA (ctDNA) indicates the part of cfDNA which derives from primary tumors and/or metastatic sites, carrying tumor-specific genetic or epigenetic alterations. Analysis of ctDNA has enormous potential applications in all stages of cancer management, including earlier diagnosis of cancer, identification of driver alterations, monitoring of treatment response and detection of resistance mechanisms. Thus, ctDNA has the potential to profoundly change current clinical practice, by moving from tissue to peripheral blood as a source of information. Herein, we review current literature regarding the potential role for ctDNA in biliary tract cancer (BTC) patients, with a particular focus on state-of-the-art techniques and future perspectives of this highly aggressive disease.

Biliary tract cancers (BTCs) include a heterogeneous group of malignancies usually classified in the following subgroups, according to anatomical location: intrahepatic cholangiocarcinoma (iCCA), extrahepatic cholangiocarcinoma (eCCA), gallbladder cancer (GBC) and ampulla of Vater cancer (AVC) (1-3). The term cholangiocarcinoma includes iCCA and eCCA, which in turn comprises perihilar cholangiocarcinoma (pCCA) and distal cholangiocarcinoma (dCCA) (4, 5). Although the anatomical classification of BTC may be considered simplistic, it faithfully reflects the differentiation of BTC subgroups in terms of epidemiology, etiology, clinical presentation, molecular features and therapeutic approaches (6, 7). BTC currently represents about 3% of all gastrointestinal malignancies and the second most common primary liver cancer (PLC), following hepatocellular carcinoma (HCC) (8, 9). Even though BTC is considered an uncommon cancer in Western countries, its incidence is increasing, and perhaps is associated with the increasing incidence of iCCA and partly as a result of better disease recognition (10-12). An important geographical variation in BTC epidemiology has been historically observed, with higher incidence rates in geographical areas where liver fluke infestation (Opisthorchis viverrini and Clonorchis sinensis) is more common such as Korea, Japan, China and Thailand (13, 14). More specifically, Northeast Thailand presents the highest BTC rate worldwide, with an annual incidence of 95/100,000 inhabitants and representing more than 80% of all PLCs in this region (15, 16). Other countries such as India and Chile depict high incidence of GBC, given the high prevalence of chronic hepatolithiasis (17, 18). Apart from these risk factors, primary sclerosing cholangitis (PSC), cirrhosis, chronic hepatitis C and B infection, fatty liver disease and asbestos exposure have been associated with an increased risk of developing BTC (19-22).

Although surgery remains the mainstay of cure in early stages, the majority of BTC patients are diagnosed with advanced-stage disease, therefore precluding any surgical management (23, 24). Cisplatin plus gemcitabine combination chemotherapy is considered the standard first-line treatment in advanced, unresectable BTC, following the results of the ABC-02 landmark trial (25). Despite ABC-02 trial representing a historical step forward in medical...
treatment for advanced BTC, the survival gain provided by first-line chemotherapy is modest since nearly all patients develop progressive disease following front-line treatment, with a median overall survival (OS) of less than a year (26). More recently, although outstanding advances in genomic sequencing have given hope to new treatment strategies, BTC patients still have a poor prognosis with short life expectancy (27-29).

In the last decade, liquid biopsy has received growing attention because of its promising applications in patients with cancer (30, 31). In fact, liquid biopsy, based on circulating free DNA (cfDNA), circulating tumor cells (CTCs), circulating cell-free RNA (ccRNA) and circulating tumor DNA (ctDNA), represents a potential tool which could bring a new insight into cancer diagnosis and management (Figure 1) (32, 33). More specifically, this new technology has the potential to reveal cancer-specific genetic and epigenetic features directly in the bloodstream (34, 35); if the term cfDNA indicates DNA which is freely circulating but not necessarily of tumor origin, ctDNA represents a tumor-derived fragmented DNA which is released into the bloodstream (36, 37). More specifically, the majority of cfDNA comes from normal cells; conversely, a small part of ctDNA directly comes from primary tumors, metastatic sites or CTCs, and is called ctDNA (38-40). The possibility to detect biological, tumor-derived material circulating in body fluids may have remarkable applications in any phase of cancer management in terms of earlier diagnosis, detection of relapse, identification of therapeutic targets, monitoring of treatment response and tracking emergence of resistance (41-44).

Herein, we review current literature regarding the potential clinical role of ctDNA in BTC management, with a particular focus on current state of art and possible future directions.

Current Limits in Diagnosis of BTC: Blood-based Markers, Imaging and Histology

Although multiple diagnostic methods are currently available, the diagnosis of BTC remains challenging (45, 46). In clinical practice, CA19-9 and carcinoembryonic antigen (CEA) are the most frequently used blood-based tumor markers (47, 48). However, CA 19-9 (with a cut-off >129 U/ml) represents the only recommended biomarker for clinical use, according to the ESMO guidelines for BTC (49); besides, overall sensitivity of CA19-9 remains controversial since high levels of CA19-9 may be encountered in several other malignancies, in benign cholestasis and after hepatic injuries (50). Lastly, various cut-off values have been proposed, usually between 100 U/ml and 200 U/ml (51).

Ultrasoundography, computed tomography (CT) and magnetic resonance imaging (MRI) are important techniques for diagnosis and staging (52). At ultrasonography, iCCA appear as solid mass lesions while pCCA and dCCA are more difficult to identify using ultrasound (53); conversely, MRI is considered the modality of choice in BTC diagnosis, given the high contrast resolution and the ability to determine the vascular, biliary and parenchymal extension of the neoplasm (54).

Pathological confirmation of diagnosis is necessary before any non-surgical treatment and can be challenging in BTC, particularly in patients affected by PSC and biliary strictures (55). Decisions to undertake biopsies should follow a multidisciplinary discussion, especially in potentially resectable tumors (56). Endoscopic imaging and tissue sampling are useful but, unfortunately, biopsy samples are often inadequate for molecular profiling (57), and in addition, tissue sampling has reported high specificity but low sensitivity in diagnosis of malignant biliary strictures (58). Lastly, the highly desmoplastic nature of BTC limits the accuracy of cytological and pathological approaches (59).

In this scenario, it is urgent to develop new strategies in order to anticipate the diagnosis identifying BTC at an early, resectable stage, and obtain sufficient material with which to perform genomic analysis.

Genomic Profiling of BTC

Recent efforts in genomic sequencing and molecular subtyping have paved the way towards a new era in BTC management (60). In fact, the advances in the comprehension of BTC molecular landscape have recently provided new keys to identify prognostic and predictive biomarkers as well as mechanisms of resistance and pathogenesis (61). More specifically, almost 50% of BTCs are supposed to harbor at least one driver mutation, and to date, several targeted agents have shown promising results in recent clinical trials (62, 63).

Firstly, Javle et al. suggested a correlation between genomic features and clinical outcomes, on the basis of data extracted from the FoundationOne platform (64). According to that study, KRAS was the most common aberration in eCCA (42% of cases), ERBB2 in GBC (16%) and IDH1 and FGFR in iCCA; moreover, FGFR mutations seemed to be associated with a good prognosis, according to the study. More recently, a multicenter study on 489 BTCs from 10 countries suggested the presence of 4 molecular clusters of BTC, on the basis of integrative clustering analysis of mutations, combined whole-genome, copy-number, gene expression and DNA methylation data (65). In this study, Cluster 1 mainly included fluke-positive malignancies with ERBB2 amplification, TP53 and ARID1A alterations; conversely, Cluster 4 identified fluke-negative iCCA with FGFR aberrations and CpG shore hypermethylation. Moreover, better OS was observed in Cluster 4, thus supporting previous findings from Javle et al. regarding the role of FGFR aberrations.
As stated above, these aberrations and molecular features represent potential therapeutic targets in specific anatomic subtypes. The recent prospective MOSCATO-1 trial analyzed 1,035 tumor samples and matched, on the basis of genetic aberrations, 199 patients to specific targeted therapies (66). Among them, 18 patients were affected by previously treated, advanced BTC; interestingly, in BTC patients receiving targeted therapies ORR was 33% and PFS and OS were 5.2 months and 17 months, respectively.

A plethora of previous studies on BTC have grouped together patients with different anatomical and molecular subtypes, something which represents the “original sin” of several clinical trials which do not do justice to the marked inter- and intra-tumoral heterogeneity of BTC (67-69). The modest survival benefit observed with current treatment options emphasizes the need for new affective agents and tailor-made trials based on genetic profile and histological features characterizing BTC (70). The emergence of targeted treatments in BTC is challenging previous treatment paradigms, especially for iCCA for whom targeting FGFR fusions and IDH1/IDH2 mutations is becoming part of current clinical practice (71-73).

**Between Two Worlds: ctDNA Assay and Tissue-based Assay**

Tumor biopsies are the gold standard for cancer diagnosis and the primary tool for molecular testing, guiding treatment selection (74). Nevertheless, sampling tissue is an invasive and often anatomically difficult method; moreover, conventional tissue biopsies are not always feasible, they frequently need to be repeated and it is not easy to obtain sufficient material of proper quality for cancer genome profiling (75).

Conversely, the analysis of ctDNA has the potential to overcome the abovementioned limitations, by capturing the outstanding spatial and temporal tumor heterogeneity and expanding the opportunity for real-time monitoring (76).
Thus, liquid biopsy is emerging as a promising, attractive molecular diagnostic tool with minimal invasiveness (77). Compared to classic tissue biopsies, the analysis of ctDNA is quick, simple and presents minimal procedural risk (Table I), considering that blood, saliva or urine are easier to access than tissue biopsy (78). In fact, although liquid biopsy is commonly referred to peripheral blood analysis, this term includes the collection and analysis of cancer-derived material from other bodily fluids such as saliva, bile, urine, stool, cerebrospinal fluid, ascites and pleural fluid (79). Overall, liquid biopsy is the natural “partner” of tailor-made, personalized oncology approach, having the potential to capture tumor spatio-temporal heterogeneity and providing a more “holistic” view of tumor (80-82).

Limitations of cfDNA/ctDNA analysis include lack of spatial specificity for anatomically critical and clinically relevant lesions, low shedding of ctDNA by certain malignancies and the lack of prospective validation for clinical practice for a majority of cancers (83, 84). Moreover, currently available ctDNA assays are not able to detect a number of genes compared to tissue-based panels, a critical issue which modern technologies are trying to face (85-87).

Clinical Applications of ctDNA/ cfDNA Analysis

In 1948, Mandel and Métais were the first to identify fragmented DNA in the non-cellular component of the blood, which was called cfDNA (88). Twenty-nine years after the first identification of cfDNA, Leon et al. observed increased levels of cfDNA in cancer patients compared to healthy controls (89). Since then, an accumulating body of literature has investigated CTCs, ctDNA and cfDNA as novel biomarkers, with the aim to facilitate early detection of malignancies and improve the prognosis of cancer patients (90-92). On the basis of current knowledge, the mechanisms of apoptosis and necrosis have been identified as important contributors to cfDNA release into the bloodstream (93). In physiological conditions, cfDNA derived from cells is found in plasma at low concentrations which may be influenced by several stressing situations (e.g. physical exercise, surgery, inflammation, etc.) (94). As previously stated, the proportion of cfDNA which is specifically released from tumor cells is currently called ctDNA, who in turn may represent from 0.1% to 90% of overall cfDNA (95). The applications of cfDNA/ctDNA can be schematically summarized by five categories (Table II): diagnosis, detection of tumor burden, prognosis, selection of treatment and monitoring for relapse/treatment efficacy.

With regard to diagnosis, early detection methods are under active investigation (96). In particular, the diagnosis of cancer at an early stage remains a challenge in several malignancies, given the frequent “silent” clinical character of early-stage disease and, in many cases, even of advanced cancer (97). Therefore, identifying early-stage malignancies would mean better chance of cure making cfDNA analysis an extremely attractive tool (98). Unfortunately, this approach would need an extremely sensitive method in order to detect minimal amounts of cfDNA released into the bloodstream and to date, no technology currently exists to reach this goal (99).

In the current era of precision cancer therapies, the choice of treatment is often based on tumor molecular profile and the clinical benefit of tailor-made agents is limited by the emergence of acquired resistance (100). In this landscape, ctDNA has the potential to assess molecular profile with a quick and minimally invasive procedure such as a simple blood draw (101). Despite early studies detected low concordance between tumor and plasma samples, recent and
larger studies have suggested concordance rates from 80% to 90% between the two samples, particularly in key driver genes (102, 103). Nevertheless, a proportion of patients affected by metastatic disease (estimated at about 10-15%) may not present sufficient cfDNA/ctDNA levels to permit mutational profiling from plasma, a key element to consider when interpreting the results of cfDNA/ctDNA analysis (104).

During medical treatment, liquid biopsy may detect emergent genetic alterations driving acquired therapeutic resistance (105); thus, serial liquid biopsies may be useful tools to identify resistant mutations and to change treatment in real time, avoiding invasive tumor biopsies (106). Therefore, liquid biopsy has rapidly emerged as an extremely promising technology, due to the ability to capture tumor molecular heterogeneity and the clonal outgrowth of resistant subclones (107). Interestingly, the main and earliest example of the application of cfDNA/ctDNA testing for the management of therapeutic resistance is epidermal growth factor receptor (EGFR)-mutated non-small cell lung cancer (NSCLC) (108, 109). In this setting, cfDNA analysis may detect the emergence of EGFR T790M mutation during EGFR inhibitor therapy – with a high level of concordance observed between the results of tissue testing and liquid biopsy – and also the coexistence of other resistance mechanisms, such as MET amplification (110). Other possible applications of cfDNA/ctDNA include the identification of prognostic and predictive biomarkers, detection of postsurgical residual disease, tracking of therapeutic response and the detection of recurrence (111, 112). Despite the fact that liquid biopsy may pave the way for a revolution in medical oncology, a careful understanding of limitations and advantages of this approach is mandatory to properly interpret the analysis and to correctly guide clinical decision making.

The Use of ctDNA in BTC

Although liquid biopsy may present an attractive diagnostic tool in early-stage BTC, very few data are currently available and the advances in the field have been hampered by technical challenges primarily due to the frequently low levels of ctDNA in patients with localized disease (113). As stated above, the difficulty in obtaining sufficient cytologic material to confirm the diagnosis and to perform genomic analysis is particularly challenging in BTC, whose poor prognosis is in part due to late diagnosis (114). Thus, ctDNA could play a particularly important role in BTC patients, since biopsy samples are often inadequate for molecular profiling, especially in eCCA and GBC (115).

In a prospective analysis of 26 pancreatobiliary malignancies, Zill et al. reported high concordance between mutations detected in tumor biopsies and cfDNA (116). This study included 8 patients with BTC and 18 with pancreatic cancer; cfDNA identified the 90.3% of mutations detected in tissue biopsies.

A study by Kumari et al. recently assessed the role of cfDNA in the diagnosis of GBC (117). Serum was collected from 34 GBC patients and 39 sex- and age-matched controls, 22 of which with cholecystitis and 17 patients without comorbidities. In this study, which represented the first to evaluate serum cfDNA in GBC, the authors used real-time PCR assay to quantify amount of cfDNA, comparing the three cohorts of patients (117). Interestingly, cfDNA was found to be significantly lower in cholecystitis controls and healthy subjects compared to the GBC group. Moreover, cfDNA was significantly associated with jaundice, metastatic lymph nodes and stage, according to TNM system (117). Thus, cfDNA quantitative analysis could play an important role in distinguishing inflammatory disorders and GBC and may serve as novel, noninvasive marker for GBC diagnosis.

Table II. Highlights of current and future applications of ctDNA in cancer management.

| Diagnosis | The majority of studies have shown poor sensitivity, especially for early stage disease. For small tumors, not enough ctDNA to provide an accurate test result. Need for further validation, in order to allow early intervention and curative surgery. |
| Tumor burden | Larger amount of ctDNA=advanced tumor stage/greater metastatic burden. ctDNA has the potential to “capture” tumor burden in different moments of the disease, giving the possibility to repeat blood testing more often than imaging/traditional biopsies. |
| Prognosis | Absence of ctDNA after radical surgery has been associated with better prognosis and smaller risk of relapse in several malignancies. Future perspectives: the possibility to determine the necessity of adjuvant therapy and the true risk of relapse. |
| Treatment | Sequencing the ctDNA may guide choice of therapy, targeting specific mutations. |
| Monitoring for relapse/treatment efficacy | ctDNA concentrations follow the evolution of the disease, indicating relapse/treatment failure before clinical practice/imaging. ctDNA has the potential to monitor response to treatment and the onset of new mutations. |
In another study on 69 cholangiocarcinoma patients (94% with pCCA) and 95 healthy sex- and age-matched controls, cfDNA analysis identified a panel of four genes (HOXA1, PRKCB, CYP26C1, and PTGDR) which had differentially methylated regions (DMRs) in CCA patients (118). The panel showed a specificity of 93% and a sensitivity of 83% in the detection of cholangiocarcinoma; interestingly, the DMR cfDNA panel detected 32 (80%) of the 40 CCAs which were deemed eligible for surgical resection or transplantation and 15 (60%). Overall, the sensitivity of cfDNA/cfDNA mutations for early stage BTC is currently unknown.

As previously stated, sequencing of tissue samples may be limited by low tumoral content, thus liquid biopsy is being harnessed for genomic profiling of BTC (119). In a study by Andersen and Jakobsen, the authors proposed a multiplex digital PCR method of screening for 31 mutations in KRAS, NRAS, BRAF and PIK3CA genes in patient plasma (120). Interestingly, the assay was firstly confirmed in pooled normal serum and positive controls; therefore, the assay was conducted on serum of six wild-type patients for the assayed mutations and five BTC patients with proven tumor mutations. Mutations found in the tumor were in parallel found in the plasma of all the “mutated” patients and, at the same time, there was a perfect agreement in wild-type status between tumor and plasma (120).

In another study, Mody et al. performed a ctDNA analysis on 138 samples of BTC patients, finding at least one genomic alteration in 89% of cases (121). Interestingly, the majority of cases included in this study were iCCAs, something which represents the main limitation of this study since iCCAs are the BTC subgroup for which liver biopsies and tissue sampling are easier. Although the most frequently detected alterations were TP53, KRAS and FGFR2, the proper and parallel concordance between ctDNA and tissue-based alterations has yet to be assessed in larger cohorts of patients (121).

Another role for ctDNA/cfDNA is represented by monitoring response to chemotherapy and targeted therapy, thus tracking emergence of resistance (122, 123). In a German study, ctDNA and tumor tissue samples were collected from 24 BTC patients before and during chemotherapy; the two samples were subjected to deep sequencing of 15 frequently mutated genes in BTC, including TP53, ARID1A, KRAS, IDH1, BAP1, PBRM1, SMAD4, PIK3CA, FBXW7, CDKN2A, ERBB2, NRAS, IDH2, BRAF and BLC2 (124). Interestingly, ctDNA in blood compared to tissue had a concordance of 74% in all patients and 92% in the iCCA cohort; moreover, 63% of chemotherapy-naive patients had their mutational profile changed during treatment. Lastly, ctDNA variant allele frequency (VAF) showed a strict correlation with progression-free survival (PFS) and tumor load.

As previously stated, FGFR2 genomic alterations are the most frequently observed aberrations in iCCA, with a prevalence ranging from 13-45% and a mutual exclusivity with KRAS/BRAF mutation (125, 126). In recent years, the role of FGFR-targeted therapies has been tested in a number of clinical trials and various agents have been evaluated or are currently under investigation including multtarget tyrosine kinase inhibitors as well as specific anti-FGFR2 antibodies including BGJ39 (127). Goyal et al. recently analyzed cfDNA collected by serial sampling in 4 patients enrolled in a Phase II trial assessing the role of BGJ39 (128). Among the 4 patients, 3 experienced significant tumor regression followed by short interval disease progression. Serial analysis of cfDNA at enrollment and after progression showed the presence of the V564F acquired mutation at the time of progression and, in 2 patients, multiple point mutations in the FGFR portion of the fusion genes (128). Moreover, a high concordance was observed between tissue and plasma measurements, since tumor biopsy of the post-progression lesions and postmortem analysis agreed with cfDNA analysis, identifying marked intratumor heterogeneity and de novo point mutations conferring resistance to the FGFR inhibitor (128). Although based on a small subgroup of patients, the study highlighted the potential advantages of cfDNA in BTC targeted therapy, where real-time detection of resistance mutations and monitoring of clonal evolution may provide extremely useful information to guide the selection of treatment.

Lastly, the option to use the bile as source for DNA sequencing in BTC has been recently investigated and deserves to be mentioned, since bile is another component of liquid biopsy. A recent study by Shen et al. from 10 BTC patients (including 4 cases of GBC) suggested that bile cfDNA could consist of long fragments, with a high correspondence between molecular features detected in bile and tissue sampling (129). Studies on larger cohorts of patients are needed to confirm the above results and to further assess the role of bile as source of cfDNA.

Conclusion

The applications of ctDNA/cfDNA on tumor detection, characterization and genetic assessment have the potential to pave the way towards a new era in cancer management. Although few data are currently available regarding ctDNA analysis in BTC, this cost-effective, fast and non-invasive test may contribute to the implementation of precision medicine and improve clinical outcomes in a highly aggressive and increasingly frequent disease.

Conflicts of Interest

No potential conflict of interest was reported by the Authors.
Authors' Contributions

AR, ADR: Made substantial contributions to the conception of the study and drafted the article; ST, GB: critically revised the article and gave final approval of the version to be published. All Authors critically revised the article, approved the final version to be published, and agree to be accountable for all aspects of the work.

References

1 Khan SA, Tavolari S and Brandi G: Cholangiocarcinoma: Epidemiology and risk factors. Liver Int 39(Suppl 1): 19-31, 2019. PMID: 30851228. DOI: 10.1111/liv.14095

2 Adeva J, Sangro B, Salati M, Edeline J, La Casta A, Bittoni A, Berardi R, Bruix J and Valle JW: Medical treatment for cholangiocarcinoma. Liver Int 39(Suppl 1): 123-142, 2019. PMID: 30892822. DOI: 10.1111/liv.14100

3 Rizzo A, Frega G, Ricci AD, Palloni A, Abbati F, DE Lorenzo S, Desserti M, Tavolari S and Brandi G: Anti-EGFR monoclonal antibodies in advanced biliary tract cancer: A systematic review and meta-analysis. In Vivo 34(2): 479-488, 2020. PMID: 32117144. DOI: 10.21873/inivo.11798

4 Rizvi S and Gores GJ: Pathogenesis, diagnosis, and management of cholangiocarcinoma. Gastroenterology 145(6): 1215-1229, 2013. PMID: 24104396. DOI: 10.1053/j.gastro.2013.01.013

5 Razumilava N and Gores GJ: Cholangiocarcinoma. Lancet 383(9935): 2168-2179, 2014. PMID: 24581682. DOI: 10.1016/S0140-6736(13)61903-0

6 Rizvi S, Khan SA, Hallemeier CL, Kelley RK and Gores GJ: Cholangiocarcinoma – evolving concepts and therapeutic strategies. Nat Rev Clin Oncol 15(2): 95-111, 2018. PMID: 28994423. DOI: 10.1038/nrclinonc.2017.157

7 Patel T: Worldwide trends in mortality from biliary tract malignancies. BMC Cancer 2: 10, 2002. PMID: 11991810. DOI: 10.1186/1471-2407-2-10

8 Forlano L, Cereda S, Aprili G, Di Girolamo S, Santini D, Silvestris N, Lonardini S, Leon F, Milella M, Vivaldi C, Belli C, Bergamo F, Lutrino SE, Filippi R, Russano M, Vaccaro V, Brunetti AE, Rotella V, Falcone A, Barbera MA, Corbelli J, Falasol G, Aglietta M, Zagonel V, Reni M, Vasile E and Brandi G: Multivariate prognostic factors analysis for second-line chemotherapy in advanced biliary tract cancer. Br J Cancer 110(9): 2165-2169, 2014. PMID: 24714745. DOI: 10.1038/bjc.2014.190

9 De Lorenzo S, Tovoli F, Barbera MA, Garuti F, Palloni A, Frega G, Garajova I, Rizzo A, Trevisani F and Brandi G: Metronomic capecitabine vs. best supportive care in Child-Pugh B hepatocellular carcinoma: a proof of concept. Sci Rep 8: 9997, 2018. PMID: 29968763. DOI: 10.1038/s41598-018-28337-6

10 Jepsen P, Vilstrup H, Tarone RE, Friis S and Sorensen HT: Incidence rates of intra- and extrahepatic cholangiocarcinomas in Denmark from 1978 through 2002. J Natl Cancer Inst 99(11): 895-897, 2007. PMID: 17551150. DOI: 10.1093/jnci/djk201

11 Brandi G, Farioli A, Astolfi A, Biasco G and Tavolari S: Genetic heterogeneity in cholangiocarcinoma: a major challenge for targeted therapies. Oncotarget 6(17): 14744-14753, 2015. PMID: 26142706. DOI: 10.18632/oncotarget.4539

12 Shaib YH, Davila JA, McGlynn K and El-Serag HB: Rising incidence of intrahepatic cholangiocarcinoma in the United States: a true increase? J Hepatol 40(3): 472-477, 2004. PMID: 15123362. DOI: 10.1016/j.jhep.2003.11.030

13 Saha SK, Zhu AX, Fuchs CS and Brooks GA: Forty-year trends in cholangiocarcinoma incidence in the US: intrahepatic disease on the rise. Oncologist 21: 594-599, 2016. PMID: 27000463. DOI: 10.1634/theoncologist.2015-0446

14 Smittenaar CR, Petersen KA, Stewart K and Moitt N: Cancer incidence and mortality projections in the UK until 2035. Br J Cancer 115(9): 1147-1155, 2016. PMID: 27727232. DOI: 10.1038/bjc.2016.304

15 Palmer WC and Patel T: Are common factors involved in the pathogenesis of primary liver cancers? A meta-analysis of risk factors for intrahepatic cholangiocarcinoma. J Hepatol 57(1): 69-76, 2012. PMID: 22420979. DOI: 10.1016/j.jhep.2012.02.022

16 Khan SA, Taylor-Robinson SD, Toledano MB, Beck A, Elliott P and Thomas HC: Changing international trends in mortality rates for liver, biliary and pancreatic tumours. J Hepatol 37: 806-813, 2002. PMID: 12445422. DOI: 10.1016/s0168-8278(02)00297-0

17 Lin CC, Lin PY and Chen YL: Comparison of concomitant and subsequent cholangiocarcinomas associated with hepatolithiasis: clinical implications. World J Gastroenterol 19(3): 375-380, 2013. PMID: 23372360. DOI: 10.3748/wjg.v19.i3.375

18 Tyson GL and El-Serag HB: Risk factors for cholangiocarcinoma. Hepatology 54(1): 173-184, 2011. PMID: 21488076. DOI: 10.1002/hep.24351

19 Zhang H, Zhu B, Zhang H, Liang J and Zeng W: HBV infection status and the risk of cholangiocarcinoma in Asia: a meta-analysis, Biomed Res Int 2016: 3417976, 2016. PMID: 29979994. DOI: 10.1155/2016/3417976

20 Brandi G and Tavolari S: Asbestos and intrahepatic cholangiocarcinoma. Cells 9(2), 2020. PMID: 32059499. DOI: 10.3390/cells9020421

21 Sastry AV, Abbassada B, Wayne MG, Steele JG and Cooperman AM: What is the incidence of biliary carcinoma in choleodochal cysts, when do they develop, and how should it affect management? World J Surg 39(2): 487-492, 2015. PMID: 25322697. DOI: 10.1007/s00268-014-2831-5

22 Jang MH, Lee YJ and Kim H: Intrahepatic cholangiocarcinoma arising in Caroli's disease. Clin Mol Hepatol 20(4): 402-405, 2014. PMID: 25548748. DOI: 10.3350/cmh.2014.20.4.402

23 Farioli A, Straif K, Brandi G, Curti S, Kjaerheim K, Martinesen JL, Sparen P, Tryggvadottir L, Weiderpass E, Bisagco G, Violante FS, Mattioli S and Pukkala E: Occupational exposure to asbestos and risk of cholangiocarcinoma: a population-based case-control study in four Nordic countries. Occup Environ Med 75(3): 191-198, 2018. PMID: 29133597. DOI: 10.1136/oemed-2017-104603

24 Forner A, Vidili G, Rengo M, Bujanda L, Ponz-Sarvise M and Lamarca A: Clinical presentation, diagnosis and staging of cholangiocarcinoma. Liver Int 39(Suppl 1): 98-107, 2019. PMID: 30831002. DOI: 10.1111/liv.14086

25 Valle J, Wasan H, Palmer DH, Cunningham D, Anthoney A, Maraveyas A, Madhusudan S, Iveson T, Hughes S, Pereira SP, Roughton M and Bridgewater J; ABC-02 Trial Investigators: Cisplatin plus gemcitabine vs gemcitabine for biliary tract cancer. N Engl J Med 362: 1273-1281, 2010. PMID: 20375404. DOI: 10.1056/NEJMoA0908721

26 Brandi G, Rizzo A, Dall'Olio FG, Felicani C, Ercolani G, Cescon M, Frega G, Tavolari S, Palloni A, De Lorenzo S, Abbati F,
Mollica V, Ricci AD and Serra C: Percutaneous radiofrequency ablation in intrahepatic cholangiocarcinoma: a retrospective single-center experience. Intl J Hyperthermia 37: 479-485, 2020. PMID: 32396398. DOI: 10.1080/02656736.2020.1763484

Robertson S, Hyder O, Dodson R, Nayar SK, Polking J, Beierl K, Eshelman JR, Lin MT, Pawlik TM and Anders RA: The frequency of KRAS and BRAF mutations in intrahepatic cholangiocarcinomas and their correlation with clinical outcome. Hum Pathol 44(12): 2768-2773, 2013. PMID: 24139215. DOI: 10.1016/j.humpath.2013.07.026

Voss IS, Holtegaard LM, Kerr SE, Frischer EG, Roberts LR, Gores GJ, Zhang J, Highsmith WE, Halling KC and Kipp BR: Molecular profiling of cholangiocarcinoma shows potential for targeted therapy treatment decisions. Hum Pathol 44(7): 1216-1222, 2013. PMID: 23391413. DOI: 10.1016/j.humpath.2012.11.006

Eckel F and Schmid RM: Chemotherapy in advanced biliary tract carcinoma: a pooled analysis of clinical trials. Br J Cancer 96: 896-902, 2007. PMID: 17325704. DOI: 10.1038/sj.bjc.6603648

Rizzo A, Mollica V, Ricci AD, Maggio I, Massucci M, Rojas Limpe FL, Fabio FD and Ardizzoni A: Third- and later-line treatment in advanced or metastatic cancer: a systematic review and meta-analysis. Future Oncol 16(2): 4409-4418, 2020. PMID: 31793342. DOI: 10.2217/onc-2019-0429

Mollica V, Di Nunno V, Santoni M, Cimadomo A, Scarpelli M, Lopez-Beltran A, Cheng L, Mariani C, Battelli N, Montironi R and Massari F: An evaluation of current prostate cancer diagnostic approaches with emphasis on liquid biopsies and prostate cancer. Expert Rev Mol Diagn 20(2): 207-217, 2020. PMID: 31640441. DOI: 10.1080/14737519.2018.1684265

Santoni M, Massari F, Del Re M, Ciccarese C, Piva F, Principato G, Montironi R, Santini D, Danesi R, Tortora G and Cascini S: Investigational therapies targeting signal transducer and activator of transcription 3 for the treatment of cancer. Future Oncol 15(2): 1753-1763, 2019. PMID: 31417921. DOI: 10.2199/wjjc.v7.i14.1753

Modena A, Ciccarese C, Iacovelli R, Brunelli M, Montironi R, Fiorentino M, Tortora G and Massari F: Immune checkpoint inhibitors and prostate cancer: a new frontier? Oncol Rev 10(1): 293, 2016. PMID: 27471580. DOI: 10.4081/oncol.2016.293

Komiya K, Nakashima C, Nakamura T, Hirakawa H, Abe T, Ogusu S, Takahashi K, Takeda Y, Egashira Y, Kimura S and Sueoka-Aragane N: Current status and problems of T790M detection, a molecular biomarker of acquired resistance to EGFR tyrosine kinase inhibitors, with liquid biopsy. Anticancer Res 38(6): 3559-3566, 2018. PMID: 29848710. DOI: 10.21873/anticancerres.12628

Brandi G, Venturi M, Pantaleo MA, Ercolini G and GICO: Cholangiocarcinoma: Current opinion on clinical practice diagnostic and therapeutic algorithms: A review of the literature and a long-standing experience of a referral center. Dig Liver Dis 50(3): 231-241, 2016. PMID: 26769568. DOI: 10.1016/j.dld.2015.11.017

Joo I, Lee JM and Yoon JH: Imaging diagnosis of intrahepatic and perihilar cholangiocarcinoma: recent advances and challenges. Radiology 288(1): 7-13, 2018. PMID: 29869699. DOI: 10.1148/radiol.2018171187

Xu MM and Sethi A: Diagnosing biliary malignancy. Gastrointest Endosc Clin N Am 25(4): 677-690, 2015. PMID: 26431597. DOI: 10.1016/j.gice.2015.06.011

Patel AH, Harnois DM, Klee GG, LaRusso NF and Gores GJ: The utility of CA 19-9 in the diagnoses of cholangiocarcinoma in patients without primary sclerosing cholangitis. Am J Gastroenterol 95(1): 204-207, 2000. PMID: 10638584. DOI: 10.1111/j.1572-0241.2000.01685.x

Valle JW, Borbath I, Khan SA, Huguet F, Gruenberger T and Arnold D: ESMO Guidelines Committee: Biliary cancer: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. Ann Oncol 27: v28-v37, 2016. PMID: 27664259. DOI: 10.1093/annonc/mdw324

CANCER GENOMICS & PROTEOMICS 17: 441-452 (2020)
Collisson EA: Cell-free DNA next-generation sequencing in pancreatobiliary carcinomas. Cancer Discov 5: 1040-1048, 2015. PMID: 26109333. DOI: 10.1158/2159-8290.CD-15-0274

117 Kumari S, Tewari S, Husain N, Agarwal A, Pandey A, Singhal A and Lohani M: Quantification of circulating free DNA as a diagnostic marker in gall bladder cancer. Pathol Oncol Res 23: 91-97, 2017. PMID: 27475647. DOI: 10.1007/s12253-016-0087-0

118 Andresen K, Boberg KM, Vedeld HM, Home H, Jebsen P, Hektoen M, Wadsworth CA, Clausen OP, Lundin KE, Paulsen V, Foss A, Mathisen Ø, Aabakken L, Schrumpf E, Lothe RA and Lind GE: Four DNA methylation biomarkers in biliary brush samples accurately identify the presence of cholangiocarcinoma. Hepatology 61(05): 1651-1659, 2015. PMID: 25644509. DOI: 10.1002/hep.27707

119 Eaton JE, Gossard AA and Talwalkar JA: Recall processes for biliary cytology in primary sclerosing cholangitis. Curr Opin Gastroenterol 30(03): 287-294, 2014. PMID: 24686433. DOI: 10.1097/MOG.0000000000000055

120 Andersen RF and Jakobsen A: Screening for circulating RAS/RAF mutations by multiplex digital PCR. Clin Chim Acta 458: 138-143, 2016. PMID: 27181912. DOI: 10.1016/j.cca.2016.05.007

121 Mody K, Kasi PM, Yang Y, Surapaneni PK, Bekaii-Saab T, Ahn DH, Mahipal A, Sonbol MB, Starr JS, Roberts A, Nagy R, Lanman R and Borad MJ: Circulating tumor DNA profiling of advanced biliary tract cancers. JCO Precis Oncol 3(3): 1-9, 2019. DOI: 10.1200/PO.18.00324

122 Lucci A, Hall CS, Lodhi AK, Bhattacharyya A, Anderson AE, Xiao L, Bedrosian I, Kuerer HM and Krishnamurthi S: Circulating tumor cells in non-metastatic breast cancer: a prospective study. Lancet Oncol 13: 688-695, 2012. PMID: 22677156. DOI: 10.1016/S1470-2045(12)70209-7

123 Ansari J, Yun JW, Kompelli AR, Moufarrej YE, Alexander JS, Herrera GA and Shackelford RE: The liquid biopsy in lung cancer. Genes Cancer 7(11-12): 355-367, 2016. PMID: 28191282. DOI: 10.18632/genesandcancer.127

124 Ettrich TJ, Schwerdel D, Dolnik A, Beuter F, Blätte TJ, Schmidt SA, Stanescu-Sieg mund N, Steinacker J, Marienfeld R, Kleger A, Ballinger L, Seufferlein T and Berger AW: Genotyping of circulating tumor DNA in cholangiocarcinoma reveals diagnostic and prognostic information. Sci Rep 9(1): 13261, 2019. PMID: 31519967. DOI: 10.1038/s41598-019-49860-0

125 Churi CR, Shroff R, Wang Y, Rashid A, Kang HC, Weatherly J, Zao M, Zinner R, Hong D, Meric-Bernstam F, Janku F, Crane CH, Mishra L, Vauthey JN, Wolff RA, Mills G and Javle M: Mutation profiling in cholangiocarcinoma: prognostic and therapeutic implications. PLoS One 9(12): e115538, 2014. PMID: 25536104. DOI: 10.1371/journal.pone.0115538

126 Lamarca A, Barriuso J, McNamara MG and Valle JW: Molecular targeted therapies: ready for "prime time" in biliary tract cancer. J Hepatol pii: S0168-8278(20)30165-3, 2020. PMID: 32171892. DOI: 10.1016/j.jhep.2020.03.007

127 Filippi R, Lombardi P, Quarà V, Fenocchio E, Aimag M, Milanesio M, Leon F and Aigletta M: Pharmacotherapeutic options for biliary tract cancer: current standard of care and new perspectives. Expert Opin Pharmacother 20(17): 2121-2137, 2019. PMID: 31550186. DOI: 10.1080/14656566.2019.1667335

128 Goyal L, Saha SK, Liu LY, Siravegna G, Leshchiner I, Ahronian LG, Lennerz JK, Yu P, Deshpande V, Kambadakone A, Mussolin B, Reyes S, Henderson L, Sun JE, Van Seventer EE, Gurski JM Jr, Baltschukat S, Schacher-Engstler B, Barys L, Stamm C, Furet P, Ryan DP, Stone JR, Iafirate AJ, Getz G, Porta DG, Tiedt R, Bardelli A, Juric D, Corcoran RB, Bardeesy N and Zhu AX: Polyclonal secondary FGFR2 mutations drive acquired resistance to FGFR inhibition in patients with FGFR2 fusion-positive cholangiocarcinoma. Cancer Discov 7(3): 252-263, 2017. PMID: 28034880. DOI: 10.1158/2159-8290.CD-16-1000

129 Shen N, Zhang D, Yin L, Qiu Y, Liu J, Yu W, Fu X, Zhu B, Xu X, Duan A, Chen Z, Wang X, Cao X, Zhao T, Zhou Z, Yu L, Qin H, Fang Z, Li JY, Liu Y, Xiong L, Yuan B, Li F and Zhang Y: Bile cell free DNA as a novel and powerful liquid biopsy for detecting somatic variants in biliary tract cancer. Oncol Rep 42(2): 549-560, 2019. PMID: 31173267. DOI: 10.3892/or.2019.7177

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