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The Final thesis

Non-Invasive Biomarkers for Early Diagnosis and Prognosis of Cholangiocarcinoma

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Content

Abstract:

Cholangiocarcinoma, also known as CCA, is a malignant tumour affecting the hepatobiliary system. Biliary cancer evolved over time to the second most commonly encountered type of liver cancer globally. Regardless diagnosis' improvements and management that surfaced in the previous years, one can say that the sensitivity as well as the specificity of the existing diagnostic tools are still lacking, and the prognosis of the patients has not substantially ameliorated. Moreover, in the last 20 years the global mortality rates have been increasing, placing CCA as a worldwide uprising health problem. The mainstay potentially curative therapy is surgical resection with or without adjuvant therapy, yet most patients with CCA do not present at an early, but at an advanced stage, where those regimens show less efficacy than they do in earlier phases of this malignant disease. Chemotherapy is a mainstay treatment for advanced-stage CCA and the role of targeted therapy for those patients is currently being studied extensively. A potential solution to this problem could be the utilization of biomarkers that are able to detect CCA at an earlier time than nowadays used tests are able to. Considering the potential of DNA methylation or the use of the N-Nose test to diagnose cholangiocarcinoma according to its chemotaxis, opens up many different approaches on how to diagnose CCA. Through the use of specific biomarkers, one could be able to identify certain gene mutations that might result in malignant growth, leading to a possible earlier diagnosis and a consequent efficient treatment before cancer develops further. Furthermore, those biomarkers proved to be efficient in other malignant diseases as well, but since these markers are a rather new discovery, their exact mechanism and potential in the clinical setting must be further understood. Therefore, biomarker discovery and a better understanding of its potential for chemotherapy are pivotal to improving patients' welfare and outcome.

Keywords: Cholangiocarcinoma, Diagnostic markers, DNA methylation, urine markers

Introduction:

Cholangiocarcinoma (CCA) is an infrequent, yet very dominant and often fatal type of cancer that involves the formation of malignant tissue within the gallbladder and can manifest as extrahepatic (eCCA), intrahepatic (iCCA), perihilar (pCCA), and distal cholangiocarcinoma (dCCA)(1,2). The most commonly encountered type of gallbladder cancer is perihilar cholangiocarcinoma (pCCA) (3) which can present in up to 60% of cases (4).

Even though CCA is considered a rare type of cancer, one can observe that it has alarming mortality with ~2% of all cancer-related deaths worldwide yearly, and its global incidence increased progressively over the past decades ((5,6) *op.loc.cit.*). An early diagnosis of this cancer is an immense challenge for medical doctors (7), due to its non-specific symptoms and its silent, highly aggressive nature: all of this contributes to a late diagnosis with surgery being possible in approximately 20% of patients and high mortality rates (8,9). Scientific research puts a great focus on finding a significant treatment and the ability to make an early, safe diagnosis for this type of cancer. The currently used non-invasive first-line regimen against CCA consists of radio- and chemotherapy (10). Within this review, the current approaches and aims should be highlighted and discussed, including the overall question if this cancerous growth can be diagnosed and treated as early as possible.

By undergoing the search for the literature review it became clear that many different approaches are currently performed in order to find a significant diagnostic marker to be able to diagnose CCA as early as possible. Certain markers will be highlighted in this work, such as DNA methylation, usage of biomarkers, chemoresistance markers, liquid biopsy, chromatin remodeling (IDH), and miRNR: beginning with the use of fluorescence in situ hybridization (FISH) which is a diagnostic modality that marks certain areas of the chromosome with a fluorescent dye. This turns out to be useful when one is searching for genetic abnormalities. This tool can detect dCCa and pCCa and is also considered as a significant modality for diagnosing gallbladder cancer (11).

When looking into the countries where the research facilities are located, one can realize that finding a regimen against cholangiocarcinoma is a highly desired goal as CCA has already evolved into a global burden (12). The general idea of this paper is to collect the current knowledge about the early diagnosis of cholangiocarcinoma, to improve the molecular

understanding of cancer development, as well as presenting trials about new approaches, diagnostic markers, and the possible realization of certain diagnostic tools in the medical regimen in order to be able to diagnose CCA at the earliest stage possible. Within this paper, we rather lay the focus on non-invasive procedures than on surgical procedures.

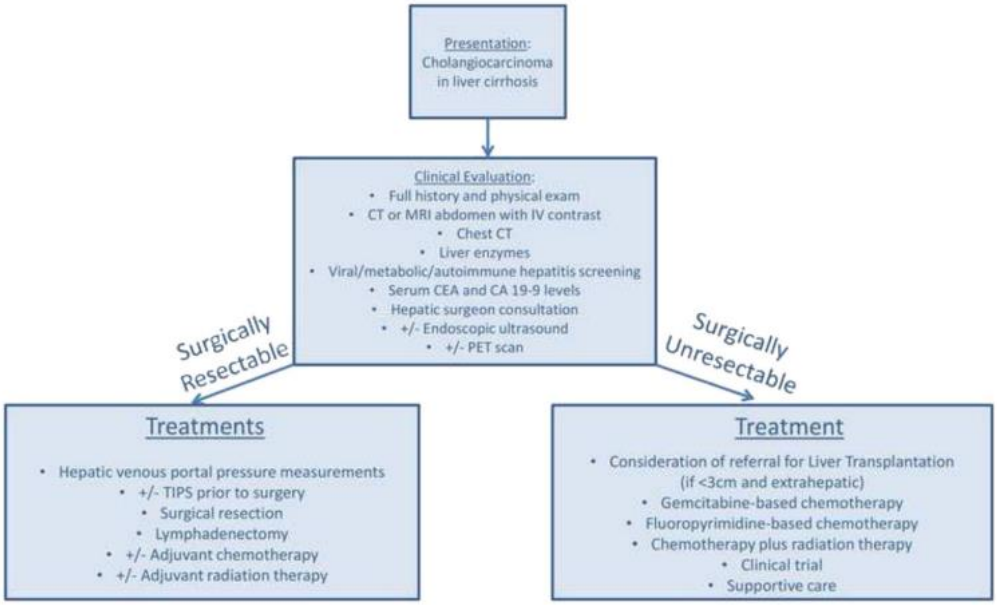


Fig. 2: Management of cholangiocarcinoma in cirrhosis. Treatment options differ in surgically resectable and surgically unresectable CCA (13).

Methodology:

In order to achieve a good overview of the current situation of diagnosing cholangiocarcinomas, the articles which were used as scientific sources for this review were collected within the last 5 years (from 2017 to 2022). The most utilized portals for scientific knowledge were PubMed, Google Scholar, Nature, and Springer. The focus of these articles lay on different approaches on how to diagnose CCA, as well as on some methods on how to treat it more efficiently, in a conservative or surgical way, depending on the CCA´s stage.

Literature review:

Diagnosing CCA in time is a great challenge nowadays due to its slow progression as well as the non-specific symptoms that are occurring during this disease process. Biomarkers for diagnosing cholangiocarcinoma are already used in the clinical setting, where cancer antigen 19-9/CA 19-9 and carcinoembryonic antigen/CEA are the most widely investigated and used biomarkers in diagnosing CCA (14). CA 19-9 and CEA are taken from the blood serum and show a diagnostic sensitivity between 47.2%- 98.2% and a specificity between 89.7%-100% which make them very specific biomarkers; however, their sensitivity is not as reliable as

the specificity ((14) *op.loc.cit.*). When observing newer approaches to CCA diagnosis, studies showed that DNA markers like cfDNA and methylation of certain DNA strands taken from tissue samples have a higher sensitivity (58-87%) and a higher specificity (98-100%) in comparison to CA 19-9 and CEA ((14–16) *op.loc.cit.*).

Promising drugs for CCA

Several drug trials were performed in the last years, which showed some promising results in treating cholangiocarcinomas. One of the most promising ones is Ivosidenib, also called AG-120, which targets and inhibits IDH-1. Isocitrate dehydrogenase 1 mutation plays a crucial role in developing cancerous diseases by overactivity of enzymes and overexpression of the oncometabolite 2-hydroxyglutarate/2-HG which in turn affects the cellular metabolism and is able to inhibit several alpha-ketoglutarate-dependent enzymes, consequently leading to malignant growths (17,18). The overall occurrence of IDH-1 mutations in CCA can go up to 15%, making this mutation a commonly encountered cause of cholangiocarcinoma (19). Ivosidenib is able to inhibit mIDH by deactivating histone demethylases, thus lowering the concentration of 2-HG within the tumor, which results in a shift of the IDH mutation, oncogenic regulation, and a restoration of the methylation and differentiation of cells (20,21). Studies showed that the use of Ivosidenib improved progression-free survival in comparison to the placebo (22). During the clinical trial of the mentioned drug, the progression-free survival was between 2-7 months, whereas for the placebo it was only 1-4 months ((22) *op.loc.cit.*). The most common adverse event of ivosidenib was ascites ((22) *op.loc.cit.*). Rarely, more serious reactions occurred; treatment-related deaths did not occur ((22) *op.loc.cit.*).

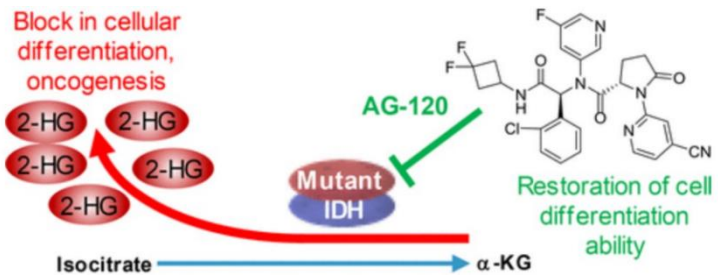


Image: mIDH leads to an accumulation of 2-HG, resulting in blocked cell differentiation and might lead to oncogenesis. AG-120 is able to prevent this state and restore cell differentiation (23)

Another study concerning the IDH-1 mutation (mIDH-1) was performed in March 2022 in Boston, Massachusetts. During this trial, it was proven with genetically modified mice that mIDH1 is involved in the development of CCA by suppressing the activity of CD8+ cells as well as by inhibiting TET2 DNA methylase, an enzyme that is closely connected to DNA

demethylation, which alters the transcription of protein sequences (24). TET2 is considered to be a cancer suppressor, therefore a mutation that results in its loss of function is often connected to malignant growth (25). By deactivation of IDH1 mutations in the mentioned mice, an increase in the amount of recruited CD8+ T cells and an increase in INF-γ was observable, which overall leads to an improved immunologic response against cancerous cells (18). The overall knowledge obtained by this recent study is that the correlation between INF-γ and TET2 methylase plays an immense role in the deactivation of IDH-1 mutations, it therefore could be a potential way of treating cholangiocarcinomas ((18) *op.loc.cit.*).

Finding a non-invasive biomarker for early diagnosis

DNA methylation

DNA methylation is considered to be an epigenetic controlling expression of genes and bears the future potential to detect and identify cancers at an earlier point of time (26). In oncogenesis, DNA methylation plays an essential role by primarily adding a methyl group to the CpG islands of a promotor region of a gene which can either result in silencing a gene or elevating the likelihood of mutations. (27,28). Nonetheless, DNA methylation can also have benefits, as seen in bacteria by the addition of a methyl group, as this renders the enzymes unable to destroy the bacteria's DNA ((27) *op.loc.cit.*). Hence, understanding the different DNA methylation profiles, DNA methylation can aid in the identification of malignant tumors efficiently and can also aid to find the appropriate treatment regimen ((27,29) *op.loc.cit.*). One of the advantages of DNA methylation is that it is able to detect any kind of dysregulation of DNA methylation, as it is seen in cancer, as well as it allows for a non-invasive approach unlike in biopsies ((26) *op.loc.cit.*). With the help of DNA methylation, one can detect changes in the pattern of DNA methylation, so if certain genes are hypo-or hypermethylated it can indicate a possible malignant cellular process that is occurring within the human body ((28) *op.loc.cit.*).

Tumor biopsies and MRIs are still considered the gold standard for cancer diagnosis and the primary method for molecular testing for genetic alterations (30–32). In CCA however, it can be challenging to obtain enough tissue for comprehensive molecular testing or even diagnosis (33–35). Hence, a diagnosis of CCA occurs most often in an already advanced disease state and the treatment options remain limited and do not secure necessarily a good outcome (36–38). Many of the currently used biomarkers obtained from fluids or biopsies are showing insufficient sensitivity and specificity ((36) *op.loc.cit.*). For this reason, new

biomarkers are required which are able to detect abnormalities faster than currently used markers, as well as aiding in an earlier diagnosis and treatment approaches of CCA. Several different studies across the globe took the goal to find a new biomarker to diagnose cholangiocarcinomas at an earlier time since CCA is regarded as the second most common liver cancer worldwide (39,40). Especially non-invasive biomarkers are intensively studied because they are more accessible compared to invasive markers, can detect cancer growth earlier and identify the origin more precisely, cost less money, have fewer sampling errors, do not have a high risk of infection compared to invasive biomarkers, and can be obtained more frequently to enable dynamic monitoring of disease status (41–45). The evaluation of solid tumor malignancies through the analysis of cell-free DNA (cfDNA), which can be efficiently isolated and analysed using advanced methods such as digital droplet PCR or next-generation sequencing is now envisioned. Detecting cfDNA might be helpful for (1) early detection of disease; (2) monitoring of patients at risk for cancer development; (3) identification of therapeutic targets and guiding therapeutic decisions (personalized medicine); (4) evaluation of treatment response, including prediction of prognosis (tumor relapse and disease progression); and (5) help to understand primary and secondary mechanisms of drug resistance (46–50). This was shown by a study that was performed in Thailand in 2019, where DNA methylation of the genes OPCML, HOXA9, and HOXD9 were quantified in cfDNA. The aim of this study was to differentiate CCA from other biliary diseases (51). The quantities of methylation were measured by methylation-sensitive high-resolution melting (MS-HRM), a PCR-based detection method to observe certain gene loci (52). The study showed that cholangiocarcinomas showed a higher methylation level in the mentioned genes compared to other biliary diseases ((51) *op.loc.cit.*). The positive predictive value/PPV was 80% and the negative predictive value/NPV was 90%, whereas the accuracy of this test was set at 85% ((51) *op.loc.cit.*). When combining the markers of OPCML and HOXD9, one can say that the specificity and the PPV were 100% which can be a useful tool to differentiate between gallbladder cancer and other pathologic biliary conditions.

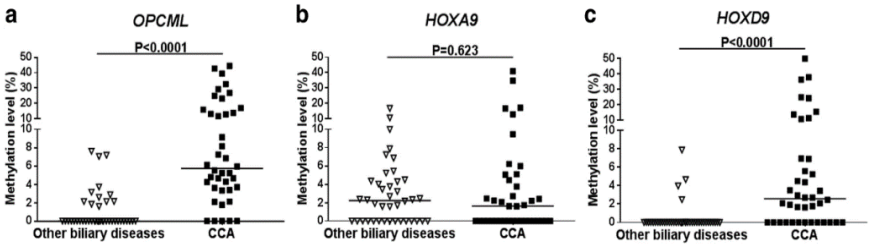


Fig.1: Shows the methylation levels of OPCML, HOXA9, and HOXD9 in CCA and other biliary diseases. The Mann-Whitney U test was carried out. (51)

Another example of the effectiveness of DNA methylation of certain genes was shown by an epigenetic study from Germany, where a quantitative methylation-specific PCR was utilized for the region of the TACSTD2 gene, which is termed the tumor-associated calcium signal transducer 2 gene. The overall task of this study was to find out if there is a correlation between the methylation levels in this gene and if there is hypermethylation in the case of renal cell cancer/RCC (53). As a result, the methylation-specific PCR presented an increased amount of methylation in RCC. These levels were strongly associated with a higher tumor stage, presence of metastases, and overall, an advanced disease course ((53) *op.loc.cit.*). Furthermore, the disease-free survival of patients with hypermethylation in RCC was decreased ((53) *op.loc.cit.*).

The utilization of DNA methylation for diagnosing several kinds of cancers showed in recent years promising results (54), since this testing is not as invasive and painful to the patient as for example biopsy extractions, because it can be obtained from body fluids like blood, bile, or urine by using biliary brushing or fine-needle aspiration (FNA) (55,56). By using DNA methylation, one is able to detect abnormalities within probes that would not be detected with commonly used tests mentioned above (56), since DNA methylation is able to find significantly different methylation gradients in CCA in comparison to healthy gallbladder tissue (57). An illustrative example for that is a study which was conducted in Thailand, where CCA is considered the most abundant primary liver malignancy in the northeastern regions (58). Within this study, the overall aim was to find possible relations between the methylation of certain gene sequences and the clinical expression of CCA ((58) *op.loc.cit.*). For the quantitative evaluation of the methylation levels, 54 CCA and 19 nearby tissues were utilized. In order to be able to detect a greater variety of differences in the methylation levels, several gene sequences were used, such as those involved in cell proliferation (CCND2, RASSF1), DNA repair (PPP4C), angiogenesis (HTATIP2), apoptosis (RUNX3, IRF4, UCHL 1, TP5313), and drug metabolism (ALDH1A3) ((58) *op.loc.cit.*). The methylation essay showed that elevated methylation levels were found in UCHL1, IRF4, CCND2, HTATIP2, and in TP5313, whereas low methylation levels of HTATIP2 and UCHL1 were observed in normal tissues ((58) *op.loc.cit*.). Furthermore, it could be said that the methylation level of the two previously mentioned parameters were in relation to the OS of patients and that the methylation of UCHL1 can be regarded as a self-standing parameter for cholangiocarcinoma which showed a hazard ratio of 1.81 within a high methylation group ((58) *op.loc.cit.*). In conclusion, the combined methylation status of HTATIP2 and UCHL1

proved to be a significant biomarker with a predictive value in CCA for those patients who presented with elevated HTATIP2 levels and decreased UCHL1 methylation levels since those patients had a longer OS than patients with reversed methylation levels ((58) *op.loc.cit.*).

The DNA methylation in mIDH1/2 is also regarded as insight-giving, as DNA methylation is able to highlight chromosome regions that are more affected than others (59). Mutations in IDH1/2 are abundant in many different types of cancer, especially in acute myeloid leukemia (60). mIDH1/2 can be found in cholangiocarcinomas as well, as a study from South Korea (2019) showed (61). For this study, genome sequencing in 124 patients with any kind of biliary tract cancer was performed. 25 patients of those participants suffered from CA, 55 patients from iCCA, and 44 from eCCA ((61)*op.loc.cit.*). The results showed genetic changes in 83,8% of all participants; the most commonly observed mutations were TP53 (44%), KRAS (29%), ARIDA (12%), and IDH1 (9%) ((61)*op.loc.cit.*). A significant finding was that IDH1/2 mutations were more frequently found in iCCA than in GBC (23,6%- 4%, respectively), whereas mutations in ERBB2/3 were solely found in CCA and eCCA ((61) *op.loc.cit.*). Furthermore, every patient with a mTP53 showed a shorter OS compared to other patients without this mutation (15.2 months -37.8 months); on the contrary, mIDH1 patients showed a prolonged PFS (10.6 months - 6.1 months) ((61) *op.loc.cit.*). An interesting finding was that in 63,5% of patient mutations in DNA damage repair/DDR was found and those were also linked to a prolongated PFS and OS in patients who underwent chemotherapies containing platinum ((61) *op.loc.cit.*). Hence, it was concluded from this study that mutagenic changes in DDR can be regarded as biomarkers with predictive potential for the patient's response to chemotherapy with platinum. ((61) *op.loc.cit.*).

Considering the frequent occurrence of mIDH1/2 in iCCA, a recent study performed in 2022 in England was searching for a connection between the prognosis of iCCA and TET2 (62). Therefore, TET2 was analysed in 52 cases of which 33 were small duct and 19 were large duct types. The enzyme TET2 was measured by PCR, sequencing-based methylation assay and immunohistochemistry and then compared to clinical aspects and changes in genes prone to malignant changes like KRAS or IDH1 ((62) *op.loc.cit.*). The application of the previously mentioned tests showed that in 42 cases (81% of all participants) TET2 was overexpressed in iCCA, whereas in normal bile ducts, TET2 was not overexpressed ((62) *op.loc.cit.*). The cases were divided into TET2-high and TET2-low; 25 TET2-high and 27

TET2-low cases were identified. Considering the immunohistochemistry of these two groups, one can say that for several parameters like histological type, size of the tumor, metastasis of lymph node, and mutation frequency of certain genes, their difference was not significant ((62) *op.loc.cit.*). Concerning the aim to find a correlation between the prognosis of iCCA and TET2 expression, it was observable that the overall survival/OS and recurrence-free survival/RFS were remarkably inferior in TET2-high iCCA patients compared to TET2-low iCCA patients ((62) *op.loc.cit.*). Hence, an elevated expression of TET2 can be seen as an independent factor in regard to the prognosis of iCCA. In addition to that, during the methylation assay, it became clear that in TET2-high iCCA the methylation degree at the CpG sites was inferior in comparison to TET2-low iCCA or healthy tissue. The study concluded that overexpression of TET2 is a frequent finding in both types and that this expression, which could be caused by hypomethylation of promoter regions, is regarded as a self-standing prognostic factor associated with poor outcome in iCCA ((62) *op.loc.cit.*).

According to another research on the topic of iCCA and mIDH which was conducted in 2021 in the USA, the aim was to find a possible correlation between tumor-induced changes in genes, their pathological variables, and the overall outcome of iCCA(63). For this purpose, next-generation sequencing/NGS was performed on primary tumours of 412 patients who had intrahepatic cholangiocarcinomas ((63) *op.loc.cit.*). NGS revealed that IDH (20%), ARIDA1 (20%), TP53 (15%), CDKN2A (15%), BRCA1 1(15%), FGFR2 (15%), PBRM1 (12%), and KRAS (10%) were identified as the most commonly encountered genetic alterations ((63) *op.loc.cit.*). In addition, mIDH1/2 was never occurring with mutations in FGFR2, and those mentioned were not associated with the outcome; on the contrary, the presence of TP52, KRAS, and CDKN2A was associated with inferior OS in all patients ((63) *op.loc.cit.*). The mentioned changes had a greater impact on advanced diseases but also affected all previous stages of the disease ((63) *op.loc.cit.*). It is important to mention that in 209 resected patients the deletion of mTP53 and mCDKN2A predicted a shorter OS as it has been the case with the mentioned high-risk variables ((63) *op.loc.cit.*). Moreover, mutations in TP53, KRAS, and deletion in CDKN2A predicted an inferior outcome in patients with iCCA which was unresectable ((63) *op.loc.cit.*). The results further showed that CDKN2A deletions with high-risk clinical aspects were associated with finite survival and no advantage of resection over the usage of chemotherapy ((63) *op.loc.cit.*). The research defined changes in TP53, KRAS, and CDKN2A as prognostic factors in the case of iCCA

concerning the control for clinical variants, stages of the disease, and treatment approach. It was further stated that the combination of those markers may aid to sort out patients with a poorer outcome than others, disregarding their treatment approach ((63) *op.loc.cit.*).

Expanding the possibilities of utilization of DNA methylation, one will come across the cysteine dioxygenase 1 gene/CDO1, which is observed in several studies concerning cholangiocarcinoma (56,64,65). The first study, which took place in 2018 in Japan, stated that the DNA methylation of CDO1 can be regarded as a marker in diagnosis and prognosis in a variety of different cancers; for this reason, the research aimed at the clinical importance of CDO1 methylation in the setting of biliary tract cancer/BTC ((64) *op.loc.cit.*). For this purpose, a quantitative methylation PCR was performed with 108 BTC tissues and 101 normal tissues in order to analyse the DNA methylation of CDO1 and to compare it with healthy tissue samples ((64) *op.loc.cit.*). The PCR unveiled that the samples of BTC consisted of 81 eCCA and 27 ampullary carcinoma/AC; the methylation levels of CDO1 were remarkably higher than in healthy tissues examined ((64) *op.loc.cit.*). Moreover, the OS of patients suffering from eCCA coexisting with elevated methylation levels were poorer than patients with hypomethylation, in AC a clear difference regarding methylation levels could not be found ((64) *op.loc.cit.*). The study further stated that during a multivariant analysis CDO1, as well as preoperative serum CA19-9 and perineural invasion were pointed out as unrestrained predictive elements in eCCA and that patients with eCCA and hypermethylation of CDO1 received a worse outcome than patients with hypomethylation ((64) *op.loc.cit.*). Concluding, the study brings forward that CDO1 methylation could be utilized as an outstanding biomarker in diagnosis and prognosis for primary eCCA and that patients with a worse prognosis can be sorted out more efficiently with the addition of preoperative CA19-9 levels ((64) *op.loc.cit.*).

A study similar to the previous one was performed in 2017 in Japan and dealt with the role of DNA methylation of the gene CDO1, which can be observed in various cancer tissues of a different kind (65). To this end, the study compared the methylation gradients of CDO1 in primary CCA with gallbladder disease of non-malignant origin by quantitative methylation-specific PCR in 203 patients, of which 99 patients had primary CCA, 78 patients had non-tumorous tissue, and 26 patients had non-malignant GB diseases (7 suffered from xantho-granulomatous cholecystitis) ((35) *op.loc.cit.*). The study brought forward that the mean value of CDO1 in primary CCA was between 23.5 and 26, which depicted them as

significantly elevated in comparison to non-malignant tissues where the CDO1 mean lay between 8 and 13, and in comparison to tissues with benign gallbladder diseases in which the CDO1 mean was between 0.98 and 1.6 ((35) *op.loc.cit.*). In addition, the elevated methylation grade was found in xantho-granulomatous cholecystitis as well ((35) *op.loc.cit.*). When a cut-off value of 17.7 was implemented, it could be observed that 47 cases of CCA with an increased methylation grade presented with a notable decline in the chance of recovery compared to 52 cases that had a hypomethylation ((35) *op.loc.cit.*). After the initiation of multivariate Cox proportional hazard analysis, the hypermethylation of the CDO1 gene was defined as a self-standing parameter with prognostic qualities, and especially in the second stage of CCA, the use of the degree of methylation of CDO1 showed prognostic significance ((35) *op.loc.cit.*). The study concluded that the DNA methylation of the CDO1 gene proved to cancer-associated in primary CCA as well as it owns the properties to function as a prognosing biomarker for high risk CCA patients in stage II ((35) *op.loc.cit.*).

In several studies concerning different types of cancer, it became clear that mutations in the KRAS gene predicted in many cases a worse prognosis and outcome, as it was shown in a recent study from 2022 in Italy, where the aim of the study was to analyse the KRAS mutation rate in hilar CCA to observe their impact on the prognosis of this disease (66). The study showed that KRAS mutations were present in 22.2% of all 54 cases and those were unrelated to any pathologic features of the tumor ((66) *op.loc.cit.*). In comparison, patients with a KRAS wild-type showed a significantly higher 5-year OS of 49.2% compared to patients with a mutant KRAS gene, who had a 5-year OS of 0% ((66) *op.loc.cit.*). In total, the study concluded that KRAS mutations can be regarded as an independent prognostic parameter in relation to a poor OS and that a pathologic screening for KRAS mutations should be implemented on a routine basis, as it can give insight into the prognosis of hilar CCA ((66) *op.loc.cit.*).

During the research, it was observable that DNA methylation bears the potential of being used as a routine regimen to identify cholangiocarcinomas earlier than with other procedures nowadays. With this epigenetic approach, it is also possible to differentiate CCA from other types of cancers or other diseases. The use of DNA methylation is clearly not limited to the identification of cholangiocarcinomas but can be applied to many other types of cancer as well. This was pointed out in a recent study from Japan in 2020, where the researchers used DNA methylation to quantify the levels of CpG islands in hepatocellular carcinoma/HCC

(67). CpG islands are believed to play an important part in the development of cancer ((67) *op.loc.cit).* To find an association between methylation levels and HCC, the study focused first on identifying altered genes in HCC. For this, the scientists compared the genes from 371 hepatic cancer tissues with 41 non-tumor tissues. Using the TCGA database for the DNA methylation, the study chose those genes where the promoter methylation levels were associated with gene expression. As a result, 115 genes were found in HCC tissues which were either over-or under-expressed. The overexpressed genes presented with cancerous traits like accelerated mitosis and proliferation. In addition, the underexpression of CpGis was found in certain gene sequences like KIF15, KIF4A, UBE2, and ERCC6L ((67) *op.loc.cit.*). These genes were linked to a worse prognosis than other sequences if upregulated.

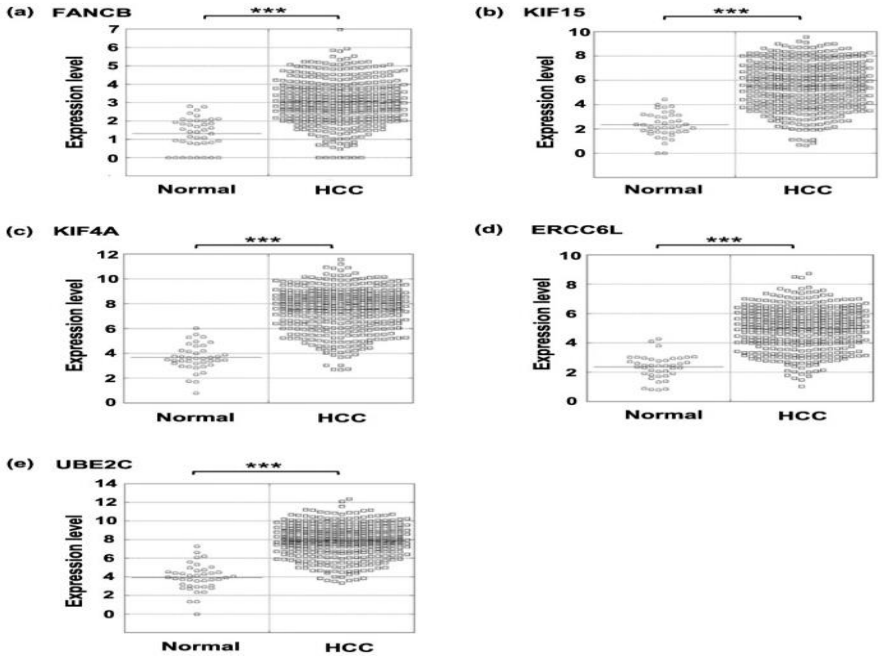
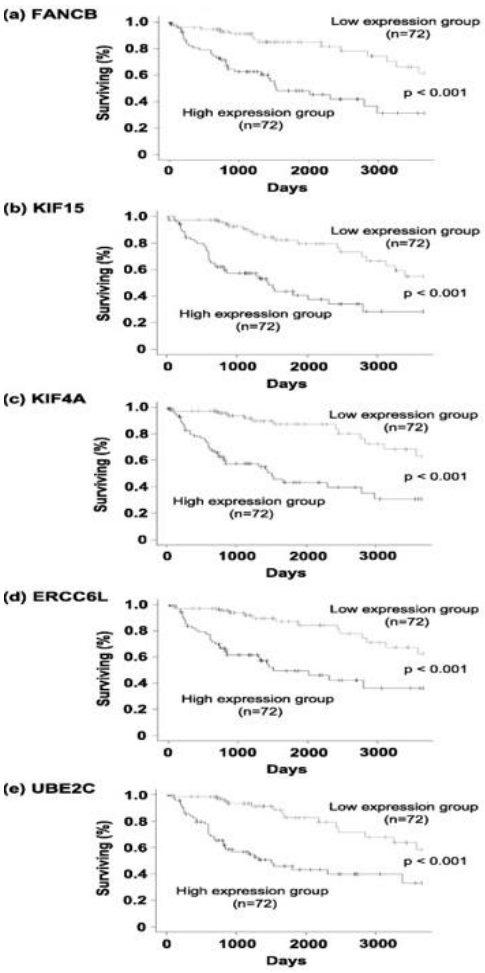


Fig.4 shows the expression levels of the chosen gene sequences in HCC and normal tissue. The expression levels were significantly higher in HCC compared to normal tissues ((67) *op.loc.cit.*).



Characteristics		HR	CI	p-value
FANCB expression (High / Low)		2.693	1.258 - 5.766	0.011*
Gender (Female / Male)		0.650	0.277 - 1.529	0.324
Age		1.004	0.977 - 1.031	0.787
History (/ None)	Hepatitis B	0.239	0.057 - 1.010	0.052
	Hepatitis C	1.254	0.410 - 3.837	0.692
	Alcohol consumption	0.527	0.180 - 1.544	0.243
	Complex	0.261	0.068 - 0.994	0.049*
	other	1.301	0.373 - 4.533	0.680
Histologic grade (G4+G3 / G1+G2)		1.460	0.702 - 3.037	0.312
Pathological stage (Stage III+IV / I+II)		1.614	0.764 - 3.413	0.210

Characteristics		HR	CI	p-value
KIF15 expression (High / Low)		2.286	1.112 - 4.700	0.025*
Gender (Female / Male)		1.416	0.635 - 3.158	0.396
Age		0.983	0.959 - 1.009	0.196
History (/ None)	Hepatitis B	0.365	0.095 - 1.400	0.142
	Hepatitis C	1.324	0.402 - 4.360	0.644
	Alcohol consumption	1.466	0.553 - 3.884	0.442
	Complex	0.518	0.182 - 1.474	0.218
	other	1.143	0.397 - 3.297	0.804
Histologic grade (G4+G3 / G1+G2)		1.074	0.558 - 2.067	0.832
Pathological stage (Stage III+IV / I+II)		3.128	1.607 - 6.089	< 0.001***

Characteristics		HR	CI	p-value
KIF4A expression (High / Low)		3.942	1.819 - 8.543	< 0.001***
Gender (Female / Male)		1.785	0.784 - 4.066	0.168
Age		0.996	0.972 - 1.020	0.754
History (/ None)	Hepatitis B	0.609	0.153 - 2.424	0.482
	Hepatitis C	1.905	0.583 - 6.224	0.286
	Alcohol consumption	0.938	0.351 - 2.509	0.899
	Complex	0.404	0.107 - 1.525	0.181
	other	0.958	0.288 - 3.191	0.945
Histologic grade (G4+G3 / G1+G2)		1.171	0.583 - 2.352	0.657
Pathological stage (Stage III+IV / I+II)		1.784	0.860 - 3.702	0.120

Characteristics		HR	CI	p-value
ERCC6L expression (High / Low)		2.781	1.251 - 6.182	0.012*
Gender (Female / Male)		1.504	0.625 - 3.620	0.362
Age		0.989	0.965 - 1.015	0.405
History (/ None)	Hepatitis B	1.000	0.252 - 3.965	0.100
	Hepatitis C	2.855	0.734 - 11.100	0.130
	Alcohol consumption	1.342	0.482 - 3.742	0.574
	Complex	0.345	0.065 - 1.815	0.209
	other	1.175	0.340 - 4.062	0.799
Histologic grade (G4+G3 / G1+G2)		1.457	0.680 - 3.122	0.333
Pathological stage (Stage III+IV / I+II)		4.398	1.970 - 9.816	< 0.001***

Characteristics		HR	CI	p-value
UBE2C expression (High / Low)		4.569	2.113 - 9.880	< 0.001***
Gender (Female / Male)		1.064	0.469 - 2.416	0.882
Age		0.990	0.969 - 1.012	0.381
History (/ None)	Hepatitis B	0.438	0.137 - 1.398	0.163
	Hepatitis C	1.333	0.441 - 4.031	0.616
	Alcohol consumption	0.825	0.302 - 2.252	0.707
	Complex	0.457	0.132 - 1.580	0.216
	other	2.755	0.584 - 12.990	0.200
Histologic grade (G4+G3 / G1+G2)		0.989	0.515 - 1.899	0.973
Pathological stage (Stage III+IV / I+II)		2.236	1.171 - 4.270	0.015*

Fig.5: The Kaplan-Meier analysis (left diagrams) and Cox regression analysis (right charts) of the 5-year OS of the chosen genes were carried out. A high expression of those genes was in all tests associated with a poorer outcome (67) *op.loc.cit.).*

Non-invasive biomarkers

It may seem surprising at first that urine biomarkers can help in diagnosing cholangiocarcinoma since the gallbladder and urinary tract are two different organs in the human body, but several studies have brought forward their potential effectiveness in the diagnosis of CCA or other cancers (68–71). One of the major advantages of urine as a source for biomarkers is the non-invasive procedure required to obtain a urine specimen that can be further inspected for the desired biomarkers and this process can be repeated as often as needed (72–74). Second, taking and analysing a urine sample is cost-effective in comparison to invasive procedures like a cystoscopy and contains many metabolites which can be investigated (75,76). A study performed in Japan in 2021 pointed out the effectiveness of a non-invasive urine test in order to diagnose CCA called N-NOSE. This new cancer screening method makes use of the nematode Caenorhabditis elegans, which is able to differentiate the urine of a healthy person from the urine of a person suffering from cancer (77). The 1 mm-

sized organism has an outstanding smelling sense and can smell volatile organic compounds (VOCs), which are believed to be cancer-specific odors (78,79). The end of this study showed that N-NOSE is a cheap, non-invasive test that has a high sensitivity regarding cancer detection (80) and even presents with a higher ROC curve than other currently used tumor markers (81). During this study, a 10-fold dilution and a 100-fold dilution of the collected urine samples were used for the Nematode-NOSE test to observe the chemotaxis of C. elegans ((77) *op.loc.cit*.). The combination of 10-fold with 100-fold dilution is also referred to as the N-NOSE combination method. A control sample was also prepared to compare the detection of the N-NOSE method in these different samples. In the patient selection for this trial, 32 patients were selected who fulfilled the criteria to be tested positive for different kinds of cancer (e.g., colon cancer, cholangiocarcinoma, pancreatic cancer). In the study, 143 cancer-negative volunteers participated in this study to give urine samples, in order to have a significant result ((77) *op.loc.cit.*). Chemotaxis assays were performed in order to observe to which urine sample the nematodes were more attracted. The results of this study presented an evident difference between the samples obtained from healthy individuals compared to the samples collected from cancer patients. With this method, it is more significant to classify patients as either positive or negative, since one uses not only one diluted sample but two differently diluted urine samples.

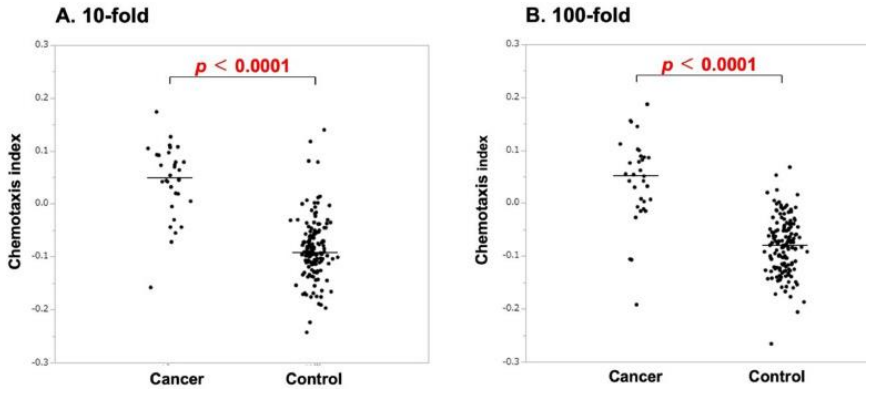


Fig.2: Comparison of the chemotaxis index in the cancer group and the control group using 10-fold and 100-fold. The cancer group shows an elevated chemotaxis index in both 10-fold and 100-fold compared to the control group. (77)

The measured sensitivity was set at 87.5%, which is more sensitive if one only uses a 10-fold diluted sample (78.1%) or only a 100-fold diluted urine sample (75%) ((77) *op.loc.cit*.). Nevertheless, the test also showed that by combining two differently diluted probes the specificity slightly decreased from 93.7- 95,1% to 90.2% ((77) *op.loc.cit.*).

		Positive	Negative	Total	Sensitivity	Specificity
10-fold	Cancer	25	7	32	78.1%	
	Control	9	134	143		93.7%
	Total	34	141	175		
100-fold	Cancer	24	8	32	75.0%	
	Control	7	136	143		95.1%
	Total	31	144	175		
Combination	Cancer	28	4	32	87.5%	
	Control	14	129	143		90.2%
	Total	42	133	175		

Table 2: The sensitivity and specificity by using 10-fold, 100-fold and combination were compared in this chart. Combination showed the highest sensitivity, whereas 100-fold showed the highest specificity (77).

A	Positive	Negative	Total	Sensitivity
Esophageal cancer	1	0	1	100.0%
Gastric cancer	4	0	4	100.0%
Colorectal cancer	8	1	9	88.9%
Gallbladder cancer	1	0	1	100.0%
Bile duct cancer	1	0	1	100.0%
Pancreatic cancer	1	1	2	50.0%
Breast cancer	10	0	10	100.0%
Malignant lymphoma	1	2	3	33.3%
Acute myeloid leukemia	1	0	1	100.0%
All types	28	4	32	87.5%
B	Positive	Negative	Total	Sensitivity
Stage 0	1	0	1	100.0%
Stage I	4	0	4	100.0%
Stage II	6	2	8	75.0%
Stage III	9	0	9	100.0%
Stage IV	2	0	2	100.0%
All stages	22	2	24	91.7%

Table 3: A listing of the sensitivity of different cancer types (seen in A) and different cancer stages (seen in B). The results show a total sensitivity of 87.5% in different cancer types and a total sensitivity of 91.7% in different cancer stages. (77)

Parallel to the N-NOSE method, other tumor markers like CEA or CAP 19-9 were measured in this study. CEA was detected in 27 patients suffering from gastrointestinal cancer, whereas CA19-9 was found in 17 samples ((77) *op.loc.cit.*). By comparing the sensitivity of N-NOSE to CEA, CA19-9, and CA15-3, it became clear that in nearly all kinds of cancer, the N-NOSE method turned out to be the most sensitive method ((77) *op.loc.cit.*), the only

exception being pancreatic cancer, which was more sensitive in CEA and CA19-9. Overall, this study presented the N-NOSE test as a possible future routine marker in order to diagnose many different types of cancer. Even though N-NOSE shows a lower sensitivity towards malignant lymphomas and pancreatic cancer in comparison to CEA and CA19-9, it overall has proven to have a significantly higher sensitivity rate than CEA and CA19-9 which are used as a standard regimen. Hence, this non-invasive urine test can contribute to the overall goal of being able to diagnose cancer, or in this case, cholangiocarcinoma earlier so a matching treatment can be still prescribed.

Discussion:

The role of non-invasive biomarkers for early diagnosis of CCA is currently under investigation. However, new approaches with DNA methylation, urine test, and miRNA have provided interesting results, with some non-invasive biomarkers having the potential to the overall goal of being able to diagnose cancer early. The early non-invasive diagnosis of CCA remains today's major challenge. This is crucial in order to enhance the number of patients eligible for surgical treatment, which is the only potentially curative option nowadays. Furthermore, non-invasive markers could contribute to a better understanding of appropriate chemotherapy for the late stages of CCA. For this goal, it is fundamental to develop novel diagnostic tools for monitoring patients at risk and early diagnosis of tumor development. Several approaches have been investigated in the search for non-invasive biomarkers for CCA, including DNA methylation, usage of biomarkers, chemoresistance markers, liquid biopsy, chromatin remodeling (IDH), and miRNA. However, the most promising diagnostic biomarkers are in need to be proven in large cohorts of patients, with appropriate control groups. It can be verified that most studies bring a new approach to how to diagnose CCA forward, but due to the limited number of participating individuals during these studies, the results cannot be seen as significant, trustworthy, and applicable to the general population as it is the case for nowadays' used tests. Furthermore, future research should investigate the potential biomarkers for all types of CCA, or for specific subgroups associated with known risk factors. Even though the search and potential use of non-invasive biomarkers seem promising, it is equally important to define their limits and possibilities and realistic application in the daily clinical field.

Conclusion:

To summarize one can say that the utilization of biomarkers in the diagnosis of cholangiocarcinoma bears the potential to improve the recognition of CCA compared with nowadays used diagnostic modalities. Though it was shown that these non-invasive biomarkers might promise a bright future in CCA diagnosis, the currently available data requires further research in larger cohort study groups in order to obtain more significant findings that would allow a non-invasive biomarker to be used in the clinical routine.

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