Expression of integrin alpha nu beta 6 differentiates perihilar cholangiocarcinoma (PHC) from benign disease mimicking PHC

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Expression of integrin αvβ6 differentiates perihilar cholangiocarcinoma (PHC) from benign disease mimicking PHC

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ABSTRACT

Background: Approximately 15% of patients undergoing resection for presumed perihilar cholangiocarcinoma (PHC) have benign disease at final pathological assessment. Molecular imaging targeting tumor-specific biomarkers could serve as a novel diagnostic tool to reduce these futile surgeries. Imaging agents have been developed, selectively binding integrin $\alpha_v\beta_6$, a cell receptor upregulated in pancreatobiliary malignancies, for both (preoperative) PET and (intraoperative) fluorescent imaging. Here, expression of integrin $\alpha_v\beta_6$ is evaluated in PHC, intrahepatic cholangiocarcinoma (ICC), hepatocellular carcinoma (HCC) and benign disease mimicking PHC using immunohistochemistry.

Materials & methods: Three tissue microarrays (TMA) including 103 PHC tumor cores and sixty tissue samples were selected from resection specimens of pathologically proven PHC (n = 20), ICC (n = 10), HCC (n = 10), metastatic PHC lymph nodes (n = 10) and benign disease (presumed PHC with benign disease at pathological assessment, n = 10). These samples were stained for integrin $\alpha_v\beta_6$ and quantified using the H-score.

Results: Immunohistochemical staining for integrin $\alpha_v\beta_6$ showed membranous expression in all twenty PHC whole mount slides (100%) and 93 out of 103 (92%) PHC tumor cores. Mean H-score of PHC samples was 195 ± 71, compared to a mean H-score of 126 ± 57 in benign samples ($p = 0.013$). In both benign and PHC samples, inflammatory infiltrates and pre-existent peribiliary glands showed integrin $\alpha_v\beta_6$ expression. The mean H-score across ten ICC was 33 ± 53, which was significantly lower compared to PHC ($p < 0.001$) but too weak to consistently discriminate ICC from HCC (H-score 0)($p = 0.062$).

Conclusion: Integrin $\alpha_v\beta_6$ is abundantly expressed in PHC and associated metastatic lymph nodes. Expression is significantly higher in PHC as compared to benign disease mimicking PHC, ICC and HCC, emphasizing its potential as a target for tumor-specific molecular imaging.

Introduction

Perihilar cholangiocarcinoma (PHC) is a tumor originating from the bile duct epithelium at the liver hilum, with an annual incidence of approximately 2 cases per 100.000 in the West [1]. Surgical resection, typically entailing hepatectomy combined with extrahepatic bile duct resection, is the only curative treatment for patients with PHC. This operation is known for its high severe morbidity (pooled 40%) and mortality (up to 18%) [2]. Radiological differentiation between (autoimmune) immunoglobulin G4(IgG4)-related cholangitis and PHC is challenging. Endoscopic retrograde cholangiopancreatography (ERCP) with brush cytology of the bile duct can help in confirming this diagnosis, but has a low sensitivity (25–52%) [3]. As a consequence, up to 15% of the patients undergoing resection for clinical suspicion of malignancy turn out to have benign diseases after pathologic assessment of the resected

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specimen [4]. A second diagnostic challenge encountered in the work-up of patients with primary liver tumors, is the differentiation of intrahepatic cholangiocarcinoma (ICC) from hepatocellular carcinoma (HCC). On imaging, HCC and ICC have overlapping characteristics, whilst treatment options and prognosis differ significantly. Liver transplantation is commonly performed in patients with HCC, while it is generally contraindicated in patients with ICC [5]. To improve diagnostic accuracy and avoid unnecessary surgery with associated risks, novel diagnostic tools with higher differentiating capacity are needed.

Molecular imaging targeting tumor-specific biomarkers is a relatively novel diagnostic tool. Integrin $\alpha_v\beta_6$ has been a target of research in pancreatic ductal adenocarcinoma (PDAC) [6–8]. Integrin $\alpha_v\beta_6$ is part of a large family of integrins, which are cell surface receptors that play a role in cell-cell and cell-extracellular matrix interactions [9]. Integrin $\alpha_v\beta_6$ expression is known to be specifically upregulated in various cancer types [9] and expression was shown to be highly upregulated in PDAC samples compared to surrounding normal pancreas tissue and chronic pancreatitis [10]. Several studies have suggested that integrin $\alpha_v\beta_6$ is also upregulated in ICC and PHC and is absent in HCC [11–13].

Imaging agents selectively binding integrin $\alpha_v\beta_6$ for preoperative PET/CT [14] and intraoperative near-infrared (NIR) fluorescent imaging have been developed [15]. If integrin $\alpha_v\beta_6$ is indeed overexpressed in PHC and IHC, this PET tracer might have the potential to preoperatively distinguish PHC from benign disease, PHC from ICC and HCC from ICC [14]. Additionally, intraoperative NIR fluorescent imaging could facilitate improved radical (R0) resection rates and help identify metastatic lymph nodes, important for intraoperative decision making. Therefore, we evaluated expression of integrin $\alpha_v\beta_6$ using immunohistochemistry to explore its potential as a target for tumor-specific molecular imaging. We hypothesize that integrin $\alpha_v\beta_6$ expression levels may be used to discriminate PHC (and associated lymph nodes) from benign disease mimicking PHC, PHC from ICC and ICC from HCC.

Materials and methods

Tissue samples

The need for ethical approval and individual consent was waived by the Institutional Medical Ethics Committee (W19_026). Well defined cases were arbitrarily selected based on operation reports, pathology records and availability of FFPE material from resection specimens of patients who had undergone resection at Amsterdam UMC, location AMC between 2000 and 2018. A total of 60 formalin-fixed paraffin-embedded tissue sections (FFPE) were selected by an HPB expert pathologist (JV) on the hematoxylin and eosin (H&E) stained slides. Fifty samples containing malignancy were selected, including pathologically proven PHC (ten PHC N0 and ten PHC N1 samples), ten malignant lymph nodes, ten samples of ICC and ten samples of HCC. Additionally, ten samples were selected of patients undergoing resection for presumed PHC, but with benign disease at pathological evaluation of the specimen. Benign samples included bile duct samples of six IgG4 mediated cholangitis, three fibrosing cholangitis and one chronic cholangitis.

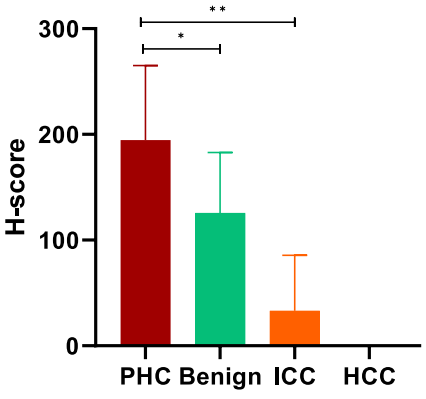

Fig. 1a. Expression of integrin $\alpha_v\beta_6$ in tissue samples. H-score in perihilar cholangiocarcinoma (PHC), benign disease mimicking PHC, intrahepatic cholangiocarcinoma (ICC) and hepatocellular carcinoma (HCC). *Difference between PHC (mean H-score 195 ± 71) and benign disease (mean H-score 126 ± 57), p = 0.013. **Difference between PHC (195 ± 71) and ICC (33 ± 53), p < 0.001.

Tissue microarray (TMA)

A TMA was constructed using additional cholangiocarcinoma samples. Tumor cores of 19 out of the previously mentioned 20 whole mount slides of pathologically proven PHC were also included in the TMA. An experienced HPB pathologist (JV) selected malignant areas of FFPE resection specimens of 103 patients who had undergone resection for pathological proven PHC between 2000 and 2017. From the selected specimens a single tumor core with thickness of 2 mm were collected using a TMA instrument (Beecher Instruments, Silver Springs MD, USA) and inserted in a recipient block. Each recipient block was sectioned at 4 μm, dried overnight at 37 °C.

Immunohistochemistry on tissue sections and TMA

Both whole- and TMA slides were stained for integrin $\alpha_v\beta_6$. Slides were deparaffinised with Xylene and rehydrated with ethanol. Endogenous peroxidase was blocked with peroxidase 0,3% hydrogen peroxide in methanol at room temperature for 20 min. Antigen retrieval was performed with 0.25% pepsine in 0.1 M HCl at 37 °C. Slides were subsequently incubated overnight at room temperature with the primary anti-$\alpha_v\beta_6$ antibody (Biogen Idec MA Inc., Cambridge, USA., Clone 6.2A1) 0.5 μg/ml with a dilution of 1:800. After washing with PBS-T, sections were incubated with secondary goat anti-mouse IgG1-HRP antibody (Southern Biotechnology) for 30 min at room temperature. Bound antibody

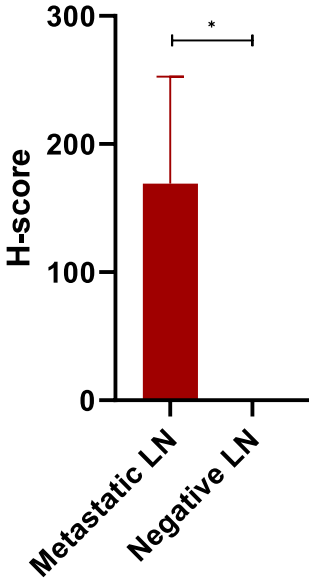

Fig. 1b. Expression of integrin $\alpha_v\beta_6$ in lymph nodes (LN) of patients with PHC. *Difference between metastatic LN (mean H-score 169 ± 84) versus negative LN (mean H-score 0), p < 0.001.

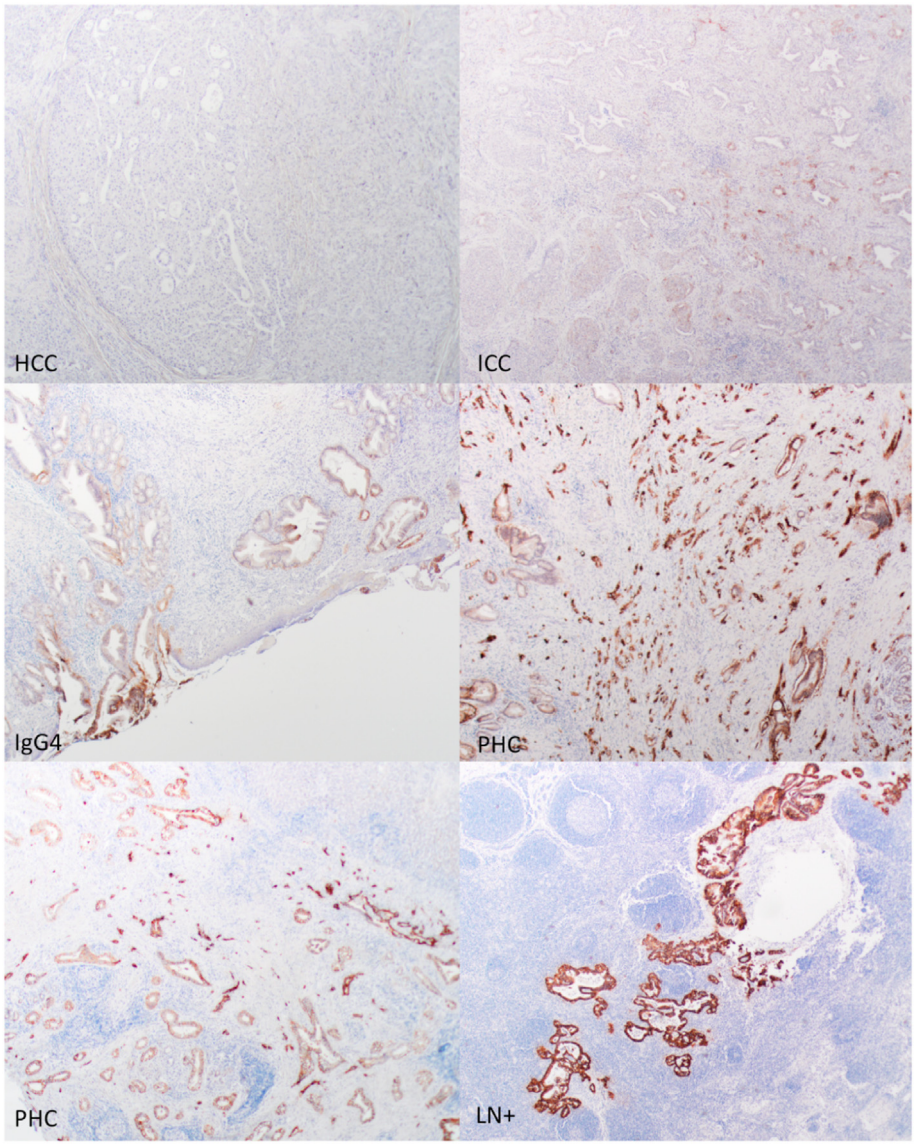

Fig. 2. Expression of integrin $\alpha_v\beta_6$ in different tissue samples. HCC; no expression in hepatocellular carcinoma, ICC; weak expression in intrahepatic cholangiocarcinoma, IgG4; mixed pre-existent expression in IgG4-cholangitis mimicking PHC, PHC (middle right); high expression in perihilar cholangiocarcinoma, PHC (bottom left); mixed expression in PHC with pre-existent expression, LN+; strong expression in malignant PHC lymph node. Original magnification 40×.

was detected using Bright DAB + detection kit (Immunologic) and slides were counterstained with 1:5 Hematoxylin (Klinipath).

Immunohistochemical staining quantification

H-score was determined for each slide and core by two experienced HPB pathologists (JV, AFS). The H-score is calculated by multiplying the percentage of positive tumor cells per slide (0–100%) by the staining intensity. Staining intensity was scored as: 0 (negative), 1 (weak), 2 (moderate) or 3 (strong). A score ranging from 0 to 300 was obtained [16]. Expression in preexistent tissue was observed in both benign as well as malignant samples. Therefore, only membranous expression in tumor cells was scored in malignant samples. Excessive staining of the basal membrane was considered as an aspecific artefact due to retraction. This was considered insignificant if the rest of the membrane showed no or weak expression. For the benign samples, staining of biliary and peribiliary glands in the inflamed region was scored.

Clinical data

Clinical data of PHC patients was derived from an existing prospectively maintained database. Patient and tumor characteristics were retrospectively collected including differentiation, lymph node status, angioinvasion, perineural growth and survival.

Statistical analysis

Descriptive statistics were used to describe the data. Mean H-scores were compared using an independent *t*-test and one-way ANOVA. H-scores obtained from whole tissue samples and TMA samples, were compared using a paired *t*-test. Statistical analysis was performed using SPSS version 25.0 (IBM, Armonk, New York, USA). P-values of <0.05 were considered statistically significant. Distribution of H-scores was displayed using Graphpad Prism version 8 (Graphpad inc, La Jolla, California, USA).

Results

Tissue samples

A total of 60 tissue samples were stained using immunohistochemistry. All twenty PHC samples showed membranous expression of integrin $\alpha_v\beta_6$, with a mean H-score of 195 ± 71. Although expression was observed in all samples, expression levels were heterogeneous within and between samples. In the benign samples, expression in inflamed peribiliary glands was observed, resulting in a mean H-score of 126 ± 57. In all ten HCC samples expression of integrin $\alpha_v\beta_6$ was absent. Out of ten samples of ICC, two were completely negative (20%). The mean H-score of ICC samples was 33 ± 53. Comparing mean H-scores, resulted in a significant higher H-score of PHC samples compared to that of benign disease ($p = 0.013$). H-score of PHC was also significantly higher compared to ICC ($p < 0.001$). A trend of a higher mean H-score of ICC samples compared to mean H-score of HCC samples was observed, although this difference was not statistically significant ($p = 0.063$)(Fig. 1a). All of ten (100%) PHC metastases in lymph nodes stained positive for integrin $\alpha_v\beta_6$ with a mean H-score of 169 ± 84, whilst all tumor-negative lymph nodes showed no expression of integrin $\alpha_v\beta_6$ ($p < 0.001$)(Fig. 1b). Although not quantified, expression of integrin $\alpha_v\beta_6$ was observed in pre-existent peribiliary glands and inflamed bile ducts of surrounding tissue in PHC samples. Peribiliary glands are only present in large bile ducts and pre-existent expression in small bile ducts surrounding HCC and ICC was scarce. Fig. 2 displays expression of integrin $\alpha_v\beta_6$ in different tissue samples.

Tissue microarray

In addition to full tissue samples, three TMAs were stained and scored, containing 103 PHC tumor cores (Fig. 3). Baseline characteristics of these patients are listed in Table 1. Ninety-three out of 103 (92%) tumor cores showed integrin $\alpha_v\beta_6$ expression, whilst eight tumor cores included in the TMA showed no expression of integrin $\alpha_v\beta_6$. Fig. 4 shows the distribution of H-scores of 103 tumor cores included in the TMAs, resulting in a mean H-score of 166 ± 93.6.

Tissue samples versus TMA

Supplementary Table 1 shows the relation between H-score as scored on the tissue samples of PHC, the corresponding malignant lymph nodes and H-scores derived from the corresponding tumor cores in the TMA. There was no statistical difference between H-score of the whole tissue samples and the corresponding tumor core in the TMA ($n = 19$) ($p = 0.395$).

Clinicopathological features and integrin $\alpha_v\beta_6$ expression in TMA

Out of the 103 samples included in the TMA, 76 were N0 tumors, 24 N1 tumors and 4 N2 tumors. Mean H-score did not significantly differ between groups, with a mean H-score of 158 ± 99 in N0-tumor samples, 176 ± 72 in N1-tumor samples and 245 ± 21 in N2-tumor samples ($p = 0.155$)(Fig. 5). Analysis based on differentiation grade resulted in an H-score of 154 ± 106 in poorly differentiated tumor samples ($n = 23$), 169 ± 93 for moderately differentiated tumor samples ($n = 50$) and 193 ± 60 for well differentiated tumor samples ($n = 20$), $p = 0.337$. The H-score of four intraductal tumors included in the TMA were 0, 0, 5 and 15, respectively. Additionally, there was one PHC N0 sample that showed low integrin $\alpha_v\beta_6$ expression (H-score of 5 on tissue sample, H-score of 0 on TMA). This tumor was classified as a small bile duct subtype.

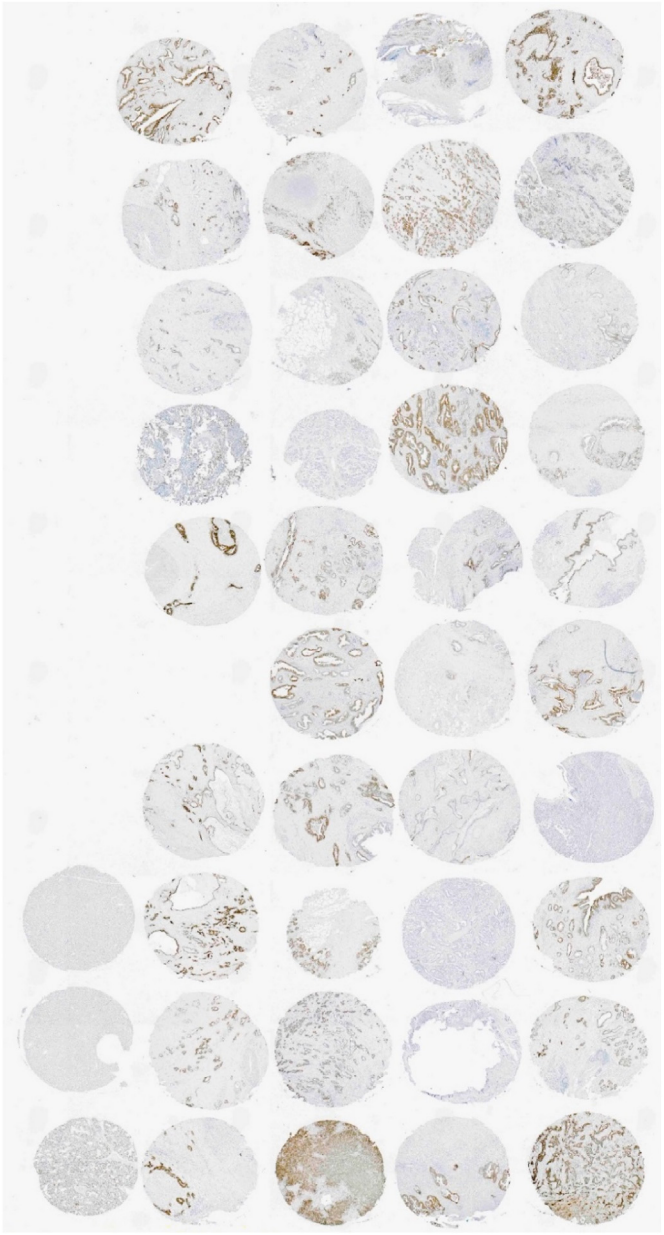

Fig. 3. Expression of integrin $\alpha_v\beta_6$ in PHC tumor cores included in TMA. Heterogeneity observed between expression levels in different tumor cores. Original magnification 2×.

Discussion

Expression of integrin $\alpha_v\beta_6$ in cholangiocarcinoma was studied using immunohistochemistry, with the aim to evaluate whether expression of integrin $\alpha_v\beta_6$ could discriminate benign disease in the hilar area from PHC, ICC from PHC and ICC from HCC. New diagnostic tools are needed to reduce futile surgery, as sensitivity of the current tests are known to be dismal. All twenty PHC samples showed membranous expression of integrin $\alpha_v\beta_6$, as well as 92% of PHC cores included in the TMA. Heterogeneous expression of integrin $\alpha_v\beta_6$ was observed in both malignant samples as well as in the bile ducts of benign cholangitis samples. The mean H-score of PHC samples was found to be significantly higher compared to the mean H-score of benign samples ($p = 0.012$) and ICC samples ($p < 0.001$). A trend in higher expression of integrin $\alpha_v\beta_6$ in ICC

Table 1
Baseline characteristics of patients and tumor cores included in tissue microarray (TMA). *Abbreviations: AJCC* American Joint Committee on Cancer, *PPPD* pylorus preserving pancreaticoduodenectomy.

	Perihilar cholangiocarcinoma in TMA (n = 103)
Age, *mean (SD)*	63
Females, *n (%)*	36 (35)
Operation, *n (%)*	
(ext) hemihepatectomy	91 (88)
Minor liver resection	2
Bile duct resection	10
+PPPD	6
Tumor diameter, *median (IQR)*	28 (20–40)
Differentiation, *n (%)*	N = 93
Poor	23 (25)
Moderate	50 (53)
Good	20
Vasoinvasiveness, *n (%)*	31/55 (56)
N-status (8th edition AJCC), *n (%)*	
N0	75 (73)
N1	24
N2	4

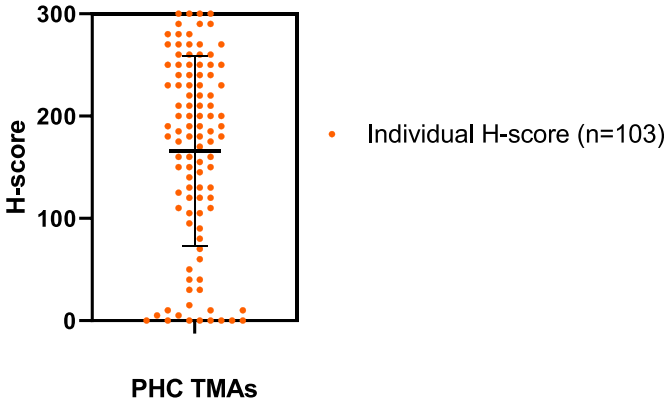

Fig. 4. Distribution of H-scores of individual tumor cores included in the three tissue microarrays (TMA). Expression of integrine avb6 in 93 out of 103 tumor cores (92%).

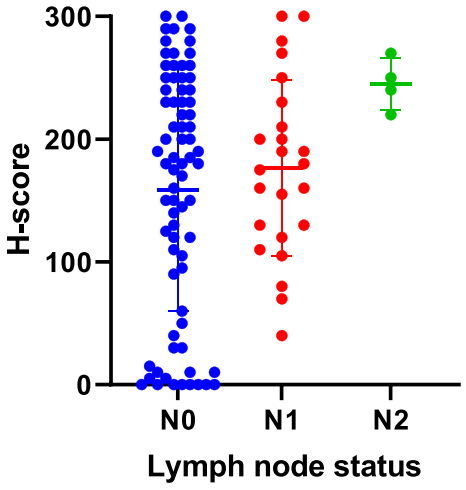

Fig. 5. Expression of integrin $\alpha_v\beta_6$ in relation to lymph node status. etc.

compared to HCC was observed in this study, however this difference was unable to consistently discriminate ICC from HCC.

Due to severe (chronic) biliary obstruction and inflamed bile ducts as typically observed in patients with PHC, expression in pre-existent bile ducts may be of major influence. This influence may be larger than expected in other hepatobiliary malignancies. For example, HCC samples showed no expression with limited staining in small pre-existent bile ducts. In PHC samples included in this study, only expression of integrin $\alpha_v\beta_6$ in tumor cells was scored, whilst (background) expression of inflammation and pre-existent ducts was ignored, leaving the pre-existent expression unaccounted for. In benign samples, specifically, expression of integrin $\alpha_v\beta_6$ in inflamed and bile ducts and pre-existent peribiliary glands was scored. For immunohistochemical staining, this may lead to an underestimation of the overall expression of the PHC samples and therefore potentially an underestimated difference between benign and PHC samples. Additionally, severe heterogeneity was observed in expression across and within PHC samples in this study. Heterogeneity within groups resulted in relatively high standard deviations. Nevertheless, expression levels were still significantly different between groups.

It is largely unknown whether expression assessed by immunohistochemistry in formalin fixed paraffin embedded (FFPE) tissue is one on one comparable with expression on PET/CT or NIR fluorescent imaging. However, immunohistochemistry expression analysis is the best available tool to test promising molecular markers in a preclinical setting. The effect of this heterogeneity and pre-existent expression for molecular imaging remains unclear and needs further investigation. In a recent study, several integrin $\alpha_v\beta_6$ PET tracers were evaluated in healthy volunteers and cancer patients, including two patients with pancreatic cancer. The lead tracer ([18F]FP-$R_0$1-MG-F2) was evaluated one pancreatic cancer patient, and compared to fluorodeoxyglucose (FDG). Whilst accumulation of FDG was concentrated around the biliary stent, uptake of the lead integrin $\alpha_v\beta_6$ tracer was more uniformly distributed over a larger volume [17]. If accumulation of the PET tracer selectively binding integrin $\alpha_v\beta_6$ is observed predominantly in the area with highest level of expression, this could suggest a more limited effect of 'background' expression compared to immunohistochemistry.

As far as we know, three studies have been published on integrin $\alpha_v\beta_6$ expression in cholangiocarcinoma. Comparing our results to those of other studies showed both similarities as inconsistencies that might need further research. Our study, to our knowledge, is the first to describe a difference between PHC and benign disease mimicking PHC and this finding is clinically most relevant. Although in a study Pratsenker et al. expression in primary

sclerosing cholangitis was investigated, this disease not only mimics PHC on imaging but is also known for the risk of malignant transformation [12]. In line with results from the study by Patsenker et al., we found no expression in HCC. However, their study showed that integrin $\alpha_v\beta_6$ stained positive in 88% of the 17 PHC samples and 87% of the 38 ICC samples, with comparable intensities [12]. In their study, expression of integrin $\alpha_v\beta_6$ could be used to discriminate HCC from cholangiocarcinoma (including ICC, PHC, gallbladder and unspecified CC). This is inconsistent with our results, where we found that PHC showed significantly higher expression compared to ICC and expression could not discriminate HCC from ICC. Differences in proportions of small and large duct types ICCs could play a role, since large bile duct type ICCs are more like PHC and may have higher integrin $\alpha_v\beta_6$ expression. Additionally, distinguishing ICC from PHC can be difficult in case an ICC is present nearby the hilar area [17]. As in our study, Soejima and colleagues also showed that expression of integrin $\alpha_v\beta_6$ was higher in 10 'non-peripheral' ICCs compared to 'peripheral' ICCs [18]. However, only ICCs were included in their study and the definition of '(non-)peripheral' in relation to our defined PHC versus ICC is difficult to trace. Major differences in classification and nomenclature were observed between all studies. Uniform nomenclature, based on international guidelines, is mandatory to directly compare results. Overall, expression in ICC in our study appears lower compared to these previously mentioned studies, resulting in the inability to differentiate ICC from HCC in our study. Different scoring systems with different cut-offs of 'high' and 'low' expression may also contribute to this lack of reproducibility.

In two studies, expression of integrin $\alpha_v\beta_6$ seemed to correlate with tumor characteristics. In the previously mentioned study by Soejima et al., higher expression was observed in well-differentiated tumors. In another study by Li et al., lymph node metastases correlated with expression of integrin $\alpha_v\beta_6$ in 95 cholangiocarcinoma samples. Unfortunately, the type of cholangiocarcinoma was not specified in this latter study [11]. Likewise, we observed a similar trend in higher expression of integrin $\alpha_v\beta_6$ in patients with lymph node metastases and well differentiated tumors. In the present study, only four N2-tumors were included, potentially explaining why this difference did not reach statistical significance.

A major limitation of this study is the inclusion of only one core per tumor in the TMA, potentially negatively effecting the quality of this analysis, mostly due to the heterogeneity in expression within tumors. However, H-scores on whole slides were comparable to H-score of corresponding tumor cores on the TMA, contributing to its validation. Strengths of this study include the evaluation of H-scores by two experienced pathologist (JV, AFS) and the well-defined subgroups with use of resection specimens, reducing the risk of bias. Furthermore, we studied expression of integrin $\alpha_v\beta_6$ in the largest number of patients with cholangiocarcinoma till date. As previously mentioned, the comparison of expression in PHC and benign disease radiologically mimicking PHC is new in our study and clinically most relevant. Also, the major heterogeneity and low expression in ICC were new in relation to existing literature. These findings are of major clinical relevance and deserve further research. The previously mentioned PET tracers and its use to confirm preoperative diagnosis in patients with hilar obstruction needs to be investigated. Also, the use of intraoperative near-infrared (NIR) fluorescent imaging to identify lymph nodes during staging laparoscopy or explorative laparotomy needs to be validated, as well as its potential during fluorescence cholangioscopy.

Conclusions

Expression of integrin $\alpha_v\beta_6$ seems significantly higher in PHC compared to benign disease. Expression of integrin $\alpha_v\beta_6$ was unable to consistently discriminate ICC from HCC. Based on immunohistochemical evaluation, integrin $\alpha_v\beta_6$ could be a promising target for molecular imaging to distinguish PHC from benign disease and ICC and to identify lymph node metastases of PHC.

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Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejso.2020.09.026.

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