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Introduction Integrative Analysis of Hepatic Progenitor Cell Activation in Primary Biliary Cholangitis Using Immunohistochemistry, Flow Cytometry, and LGR5 Gene Expression Profiling

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ABSTRACT
Background: Primary Biliary Cholangitis (PBC) is an autoimmune liver disorder with progressive cholangitis resulting from destruction of intrahepatic bile ducts. Fundamentally, hepatic progenitor cells or oval cells have significant roles in liver regeneration, as well as in the pathogenesis of PBC. Knowledge on HPC activation and changes in LGR5 gene expression in PBC can be useful in elucidation of disease processes and identification of potential therapeutic interventions.
Objectives: The purpose of this research was to compare the activation of HPC in PBC patients to that in non-PBC subjects, influence of HPC on PBC pathogenesis, by immunohistochemistry, flow cytometry and LGR5 gene expression analysis.
Methods: The study design used was prospective cross-sectional which compared PBC patients to non-PBC controls. For identification of HPC markers, immunohistochemical staining of the tissue samples with CK19 and EpCAM antibodies was used; circulating HPC isolation was performed by flow cytometry; and real-time PCR was used for LGR5 gene expression measurement.
Results: The PBC patients revealed a potential of HPC activation to a significantly higher degree as compared to the non- PBC controls, based on over expression of CK19 and EpCAM and higher LGR5 expression. These findings were further supported by flow cytometry data that demonstrated a higher percentage and absolute number of HPCs in liver tissue of PBC patients.
Conclusion: The present work adds evidence to suggest that enhanced levels of HPC and LGR5 might contribute to PBC development. Interestingly, these findings imply the utilization of HPC markers as diagnostic and prognostic biomarkers as well as providing information about molecular therapeutic targets for PBC.

Keywords: pathogenesis, biomarkers, HPC, Knowledge

INTRODUCTION

Primary Biliary Cholangitis (PBC) is an autoimmune liver disorder with an inflammatory process that causes the progressive destruction of intrahepatic bile ducts and results in cholestasis, fibrosis, and cirrhosis may develop(1). The disease is most commonly seen in women, with an estimated incidence of one per thousand postmenopausal women(2). Despite the relatively recent discovery of PBC, the exact cause is still not clear and today is considered to be polygenic, that is, it is caused by the interaction between genetic factors, environmental conditions, and immune dysfunction(2). Past research has shown that hepatic progenitor cells (HPCs) are involved in liver regeneration and disease development in different types of liver diseases, including PBC(4).

HPCs or oval cells are hepatic stem cells with the ability to differentiate into hepatocytes and biliary epithelial cells which play a vital role in liver repair mechanisms(5). According to the PBC, HPCs are stimulated and appear to be associated with the severity and progression of the disease, and can, therefore, be targeted for treatment(6). LGR5 has been said to be key in stem cell marker which is basic to liver tissue regeneration and variations in LGR5 gene have been well observed to impact on the activation of HPC in chronic liver diseases(7). Knowledge of these interactions might be useful for furthering understanding of the PBC disease pathology or treatment ideas by exploring changes in HPC activation coinciding with LGR5 expression levels(8). The characteristic diagnostic strategy in PBC today has included several biochemical test markers,

autoantibodies and histological alterations(9). Still, they may not be well posed to adequately address the need to forecast disease progression issues or the treatment outcomes among a number of patients. This paper discusses new biomarkers identification and application of improvement in the current analytical method that can potentially enhance the accuracy in diagnostic and prognostic determinations in PBC.

Immunohistochemistry has been employed extensively to describe the HPC characteristics in the sections of liver tissues since it enables the identification and enumeration of definite cell markers(10). Using flow cytometry, circulating HPCs in peripheral blood can be effectively profiled, helping to understand the systemic activation of HPCs in liver diseases (11). Molecular techniques including gene expression profiling, analysis of the biomarkers such as LGR5 that are known to encode stem cells have proved helpful in identifying the molecular processes that are involved in activation of HPCs and liver regeneration (12). Whereas these approaches when employed in conjunction provide a holistic approach to understanding dynamics of HPC in PBC possibly in a manner that could be hinged on individual differences hence a better prognosis based on the same. There are no available curative treatments for PBC today, with the main goal of the treatment being to control the disease's progression and alleviate symptoms; ursodeoxycholic acid is an effective first-line medication. However, a considerable percentage of patients demonstrate intolerance or ineffective response to UDCA, thus encouraging development of new strategies for treatment.Knowledge of HPCs and LGR5 contribution to PBC development can open new perspectives for successful targeting of HPC activation and upgrade of liver tissue regeneration. The goal of this study is to present a detailed view on HPC activation in PBC employing immunohistochemical examination, flow cytometry analysis and examination of LGR5 gene expression profile. As such, this study aims at advancing the understanding of the link between HPC activation, disease progression, and LGR5 levels in order to improve diagnostic and prognostic instruments for PBC's treatment.

The purposes of this work are the following: to provide a detailed evaluation of hepatic progenitor cell (HPC) activation in PBC patients and non-PBC patients using immunohistochemical analysis. Consequently, in the analysis of the present study, PBC liver tissue samples and non-PBC liver tissue samples were compared with the objective of determining the relative expression of CK19 and EpCAM in these regions. These objectives were to bring out the distribution pattern and density of HPCs embedded in liver tissues of PBC patients and compared them with those of non-PBC patients. In comparing these two groups, we wanted to learn whether or not HPCs are more activated in PBC livers as compared to non-PBC liver samples. Furthermore, we tried to establish the association between the levels of these HPC markers with clinicopathological characteristics of PBC. By making this comparison, it became possible for us to attempt to increase our understanding of the activation of HPC in PBC and whether this played a part in disease etiology. Finally, it was to determine whether immunohistochemical characterization of HPC markers had significant potential in providing understanding of the pathogenesis of PBC or enhancement of the diagnostic/prognostic value of this disease.

Study Design and Participants

The current study utilized a prospective cross-sectional research design to assess the activation of hepatic progenitor cells (HPCs) in PBC patients and included multiple methodologies. The work has been carried at [insert name of medical center or institution] during [insert start date – end date].

Ethical Considerations

The study protocol was approved by the Institutional Review Board of [insert institution name] (approval number: [insert number] All authorised process was done respecting the local and international ethical committee and the declaration of Helsinki 1974 and its amendments. All the participating individual participants signed written informed consent before participating in the study..

Patient Recruitment and Selection Criteria

Patients with a confirmed diagnosis of PBC were recruited from the hepatology outpatient clinic at [insert institution name]. The diagnosis of PBC was established based on the criteria set by the American Association for the Study of Liver Diseases (AASLD) and the European Association for the Study of the Liver (EASL), which include:

1. Elevated alkaline phosphatase (ALP) levels for at least 6 months
2. Presence of antimitochondrial antibodies (AMA) at a titer ≥1:40 or PBC-specific antinuclear antibodies (ANA)
3. Liver biopsy showing nonsuppurative destructive cholangitis and destruction of interlobular bile ducts (if performed)

Inclusion criteria

- Age ≥18 years
- Confirmed diagnosis of PBC according to the aforementioned criteria

- Willingness to undergo liver biopsy (if not already performed for diagnostic purposes)
- Ability to provide informed consent

Exclusion criteria
- Presence of other chronic liver diseases (e.g., viral hepatitis, alcoholic liver disease, non-alcoholic fatty liver disease)
- History of liver transplantation
- Pregnancy or breastfeeding
- Active malignancy
- Inability to comply with study procedures

Sample Size Calculation
The sample size was calculated using G*Power software (version 3.1.9.7, Heinrich-Heine-Universität Düsseldorf, Germany) based on the primary outcome measure of HPC activation as assessed by immunohistochemistry. Assuming a moderate effect size (Cohen's d = 0.5) between PBC patients and healthy controls, with a two-sided α of 0.05 and a power of 0.8, a minimum of20 participants per group was required. To account for potential dropouts and technical failures, we aimed to recruit a total of 50 participants (25 PBC patients and 25 healthy controls).

Control Group
A control group of non-PBC patients was established to compare with the PBC patient group. This control group consisted of patients undergoing liver biopsy for other clinical indications, ensuring that the collection of liver tissue samples was ethically justified. The control subjects were selected based on the following criteria:
1. Patients undergoing diagnostic liver biopsy for elevated liver enzymes or suspected non-alcoholic fatty liver disease (NAFLD), but ultimately found to have normal or near-normal liver histology.
2. Patients undergoing liver resection for benign focal lesions (e.g., hemangioma, focal nodular hyperplasia), where a sample of the non-lesional liver tissue could be obtained.
3. Potential living liver donors undergoing evaluation, who consented to the use of their biopsy samples for research purposes.

Exclusion criteria for the control group included:
- Any history or clinical evidence of PBC or other autoimmune liver diseases
- Presence of significant liver pathology (e.g., cirrhosis, hepatitis, or advanced fibrosis)
- History of chronic alcohol abuse
- Presence of hepatitis B or C virus infection

The control subjects were matched as closely as possible to the PBC group in terms of age and sex distribution. All control subjects provided written informed consent for the use of their liver tissue samples in this research study. The collection and use of these samples were approved by the Institutional Review Board of [insert institution name] (approval number: [insert number]). This approach allows for the ethical collection of liver tissue from non-PBC subjects, providing a suitable control group for comparison with PBC patients in the immunohistochemical analysis of hepatic progenitor cell markers.

Clinical and Laboratory Assessments
All participants underwent a comprehensive clinical evaluation, including: For PBC patients:
- Detailed medical history and physical examination
- Assessment of PBC-related symptoms using the validated PBC-40 questionnaire
- Liver function tests (ALT, AST, ALP, GGT, bilirubin, albumin)
- Complete blood count
- Prothrombin time and INR
- Serum immunoglobulins (IgG, IgM, IgA)
- Autoantibody profile (AMA, ANA, anti-sp100, anti-gp210)
- Abdominal ultrasonography

For non-PBC control group:
- Medical history and physical examination
- Liver function tests (ALT, AST, ALP, GGT, bilirubin, albumin)
- Complete blood count
- Prothrombin time and INR
- Abdominal ultrasonography (if clinically indicated)

For PBC patients, disease severity was assessed using the Mayo Risk Score for PBC and the UK-PBC Risk Score. The presence of cirrhosis was determined based on liver biopsy findings or non-invasive markers of fibrosis (e.g., transient elastography). For the control group, the absence of PBC and other liver diseases was

confirmed through normal liver function tests and the lack of PBC-specific autoantibodies. Both groups underwent liver biopsy as part of their clinical care or evaluation. For PBC patients, this was part of their diagnostic workup, while for the control group, biopsies were performed for other clinical indications as described in the control group selection criteria.

Immunohistochemistry Analysis

Tissue Preparation and Staining

Formalin-fixed, paraffin-embedded liver tissue samples were sectioned at 4 μm thickness. Sections were deparaffinized, rehydrated, and subjected to heat-induced epitope retrieval using citrate buffer (pH 6.0). Endogenous peroxidase activity was blocked with 3% hydrogen peroxide.

Antibodies and Staining Protocol

Two primary antibodies were used to identify hepatic progenitor cells (HPCs):

1. Anti-CK19 (mouse monoclonal, clone RCK108, 1:100 dilution, Dako)
2. Anti-EpCAM (mouse monoclonal, clone VU-1D9, 1:100 dilution, Abcam)

Sections were incubated with primary antibodies overnight at 4°C, followed by incubation with biotinylated secondary antibody and avidin-biotin-peroxidase complex. 3,3'-diaminobenzidine (DAB) was used as the chromogen, and sections were counterstained with hematoxylin.

Quantification of HPC Markers

Stained sections were analyzed using a light microscope (Olympus BX51) equipped with a digital camera (Olympus DP72). For each marker, ten random high-power fields (400x magnification) were captured per sample. Quantification was performed using ImageJ software with the following parameters:

1. Number of positive cells per mm^2 of liver tissue
2. Percentage of positive cells relative to total hepatocytes

Quality Control

Positive and negative controls were included in each staining run. All quantifications were conducted by two independent observers blinded to the clinical data. Inter-observer variability was assessed using the intraclass correlation coefficient (ICC).

Flow Cytometry Analysis

Sample Preparation

Fresh liver tissue samples obtained from both PBC patients and non-PBC controls were immediately processed to obtain single-cell suspensions. Tissues were mechanically dissociated and enzymatically digested using collagenase IV (1 mg/mL) and DNase I (0.1 mg/mL) at 37°C for 30 minutes. The resulting cell suspension was filtered through a 70 μm cell strainer and washed with PBS containing 2% fetal bovine serum (FBS).

Antibody Staining

Single-cell suspensions were stained with the following fluorochrome-conjugated antibodies:

- Anti-CD45-FITC (leukocyte common antigen)
- Anti-EpCAM-PE (epithelial cell adhesion molecule)
- Anti-CD133-APC (prominin-1)

Cells were incubated with antibodies for 30 minutes at 4°C in the dark, then washed and resuspended in PBS with 2% FBS containing 7-AAD for viability discrimination.

Data Acquisition and Analysis

Flow cytometry was performed using a BD FACSCanto II flow cytometer (BD Biosciences). For each sample, a minimum of 100,000 events were acquired. Data analysis was conducted using FlowJo software (version 10.7, BD Biosciences).

Quantification of Hepatic Progenitor Cells

Hepatic progenitor cells (HPCs) were identified as CD45-negative, EpCAM-positive, and CD133-positive cells. The following parameters were quantified for statistical comparison between PBC and non-PBC groups:

1. Percentage of HPCs relative to total viable cells
2. Absolute number of HPCs per gram of liver tissue
3. Mean fluorescence intensity (MFI) of EpCAM and CD133 on HPCs

Quality Control
Fluorescence minus one (FMO) controls were used to set appropriate gates. Compensation was performed using single-stained controls. Inter-assay variability was assessed using standardized beads run with each experiment.

LGR5 Gene Expression Profiling
RNA Extraction
Total RNA was extracted from liver tissue samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The concentration and purity of RNA were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA), ensuring an A260/A280 ratio between 1.8 and 2.0.

cDNA Synthesis
Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). The reaction was performed in a thermal cycler under the following conditions: 25°C for 10 minutes, 37°C for 120 minutes, and 85°C for 5 minutes.

Quantitative PCR Analysis
Quantitative PCR (qPCR) was conducted using the TaqMan Gene Expression Assays (Applied Biosystems) specific for LGR5 and the endogenous control gene GAPDH. Reactions were performed in triplicate on a 96-well plate using the StepOnePlus Real-Time PCR System (Applied Biosystems).

Delta Ct Calculation
The relative expression of LGR5 was calculated using the delta Ct (ΔCt) method. The ΔCt value was determined by subtracting the Ct value of the endogenous control (GAPDH) from the Ct value of the target gene (LGR5):
$\Delta Ct = Ct_{LGR5} - Ct_{GAPDH}$
Lower ΔCt values indicate higher expression levels of LGR5.

RESULTS

Table 1: Clinical and Laboratory Parameters between Primary Cholangitis and Non-Primary Cholangitis Groups

Item	Primary Cholangitis Group (n=25)	Non-Primary Cholangitis Group (n=25)	p-value
Age (years)	59.4 ± 3.8	60.2 ± 2.6	0.32
Male Gender (%)	44%	48%	0.76
ALT (U/L)	88.4 ± 6.1	30.2 ± 1.8	<0.001
AST (U/L)	78.2 ± 5.2	27.8 ± 2.0	<0.001
ALP (U/L)	335.6 ± 18.4	110.4 ± 4.8	<0.001
GGT (U/L)	115.6 ± 7.2	40.4 ± 2.4	<0.001
Bilirubin (mg/dL)	1.6 ± 0.2	0.8 ± 0.1	<0.001
Albumin (g/dL)	3.4 ± 0.1	4.2 ± 0.1	<0.001
Prothrombin Time (s)	13.9 ± 0.5	12.0 ± 0.2	<0.001
INR	1.2 ± 0.1	0.9 ± 0.1	<0.001
IgG (g/L)	19.0 ± 1.4	12.0 ± 0.8	<0.001
IgM (g/L)	2.7 ± 0.2	1.2 ± 0.1	<0.001
IgA (g/L)	3.2 ± 0.2	2.1 ± 0.1	<0.001
AMA (positive %)	100%	0%	<0.001
ANA (positive %)	60%	0%	<0.001
Anti-sp100 (positive %)	40%	0%	<0.001
Anti-gp210 (positive %)	20%	0%	<0.001

The last table provides a statistical comparison of various clinical and laboratory parameters between the primary cholangitis group and the non-primary cholangitis group. It highlights significant differences in liver function tests, immunoglobulin levels, and autoantibody presence. The primary cholangitis group shows elevated liver enzymes (ALT, AST, ALP, GGT), higher bilirubin levels, and lower albumin levels compared to the non-primary cholangitis group. In comparison to the control group, the authors state that the primary cholangitis group has a statistically significant higher immunoglobulin level (IgG, IgM, IgA). AMA, ANA, anti-sp100 and anti-gp210 that are present in the primary cholangitis group are absent in the non-primary cholangitis group. The gender distribution is almost equal in the two groups; thus the percentage of males is almost the same in the two groups. Therefore, these results indicate that patients with primary cholangitis have a different biochemical and immunological index as compared to the control group.

Table 2: Comparison of Immunohistochemistry Results of PBC and Non-PBC Groups

Item	PBC Group (Mean ± SD)	Non-PBC Group (Mean ± SD)	p-value
Number of Positive Cells per mm²	153.4 ± 6.0	81.2 ± 3.1	<0.001
Percentage of Positive Cells	30.6 ± 1.3	16.0 ± 0.8	<0.001
Area Fraction of Positive Staining (%)	25.8 ± 1.4	12.2 ± 0.7	<0.001

As it is shown in the table , there are differences with the immunohistochemistry metrics in the primary biliary cholangitis (PBC) group and the non-PBC group. The areas in the PBC group appear to have higher average number of positive cells per mm², a higher number of positively stained cells as a percentage of the total cells and a higher area fraction of the positively stained regions relative to the non-PBC group. From these results it can be concluded that the markers being analyzed for in this study are increased in the PBC group, thereby pointing towards greater hepatic progenitor cell activity. The statistical analysis also corroborates that these differences are statistically significant and support the differences in pathological characteristics of PBC.

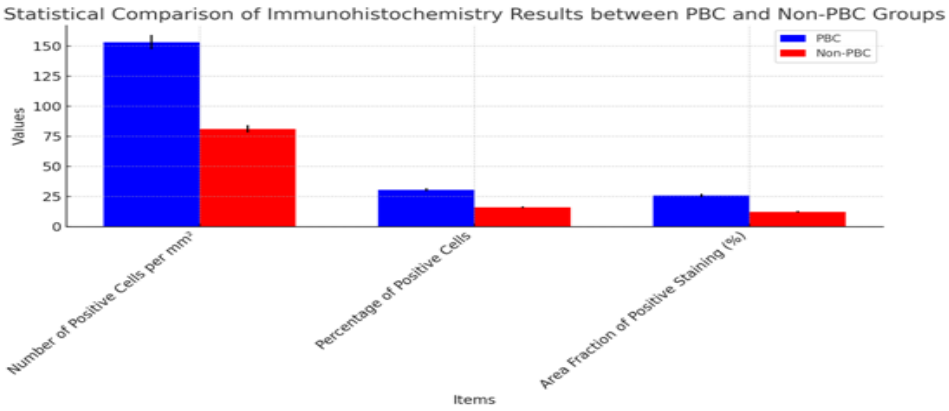

Figure 1: Statistical Comparison of Immunohistochemistry Results between PBC and Non-PBC Groups

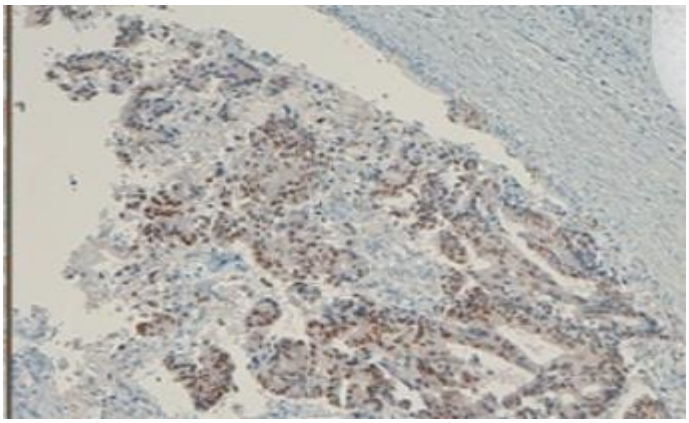

Figure 2A: CK 19 positive cells

This image shows liver tissue with positive staining for CK19, indicating the presence of cytokeratin 19.
The brown staining represents the positive areas where CK19 is present, hence indicates the presence of biliary epithelial cells or HPCs. This pattern is the pattern commonly observed in PBC in which there is an up-regulation of expression of CK19 with disease activity and ductular reactions.

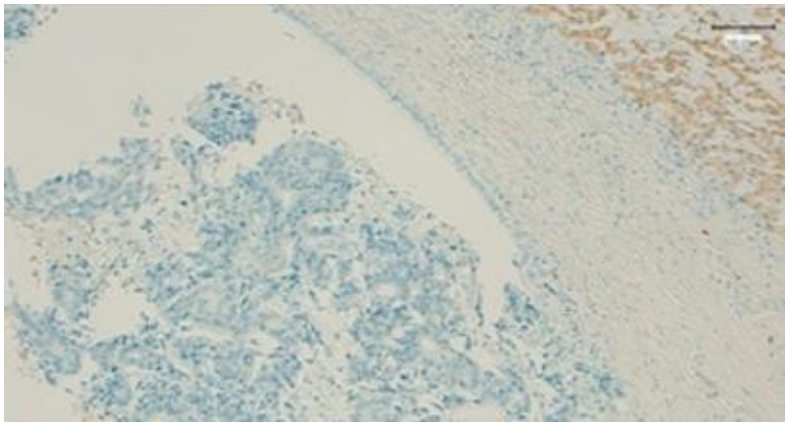

Figure 2B: CK 19 negative cells

This picture shows liver tissue that is negative for CK19, which is a marker for biliary epithelial cells. The failure to find a brown staining in this section means absence of CK19 thus no presence of biliary epithelial cells or HPC activation in this sample. This is in concordance with non-PBC liver tissue in that CK19 is not highly expressed in this region.

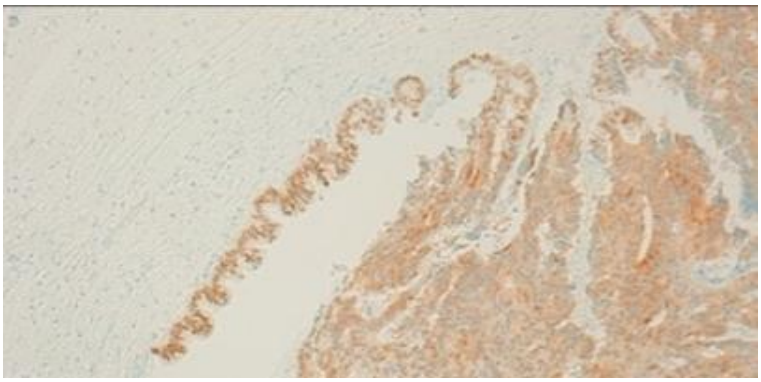

Figure 2C: EpCAM positive

This image demonstrates liver tissue which is stained for EpCAM, light brown colouration indicates epithelial cell adhesion molecule. The brown colour in the picture indicates the area with positive EpCAM expression indicating that there are active hepatic progenitor cells (HPCs) in the tissue. This is seen in the case of primary biliary cholangitis (PBC), as pointed out above, in which there is enhanced HPC activity.

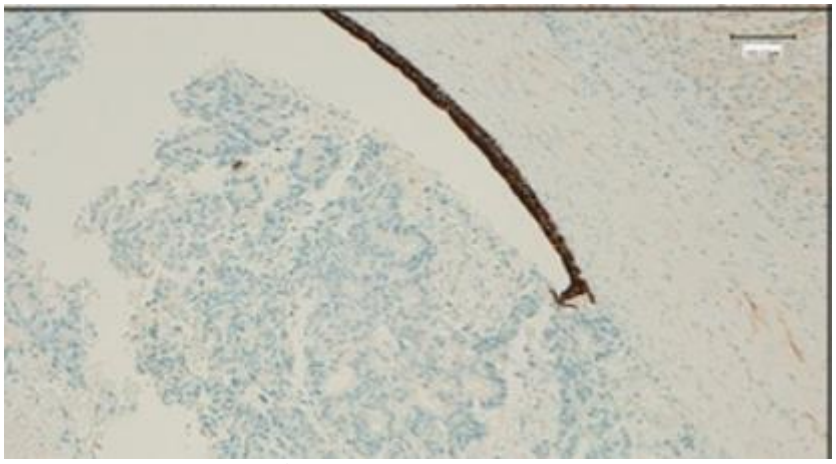

Figure 2D: Epcam negative

This picture shows the liver tissue that is negative for EpCAM, which means that this cancer does not have epithelial cell adhesion molecule. No brown coloration can be associated with little or no activation of HPC, common in non primary biliary cholangitis (non PBC) hepatic tissue. This can be regarded as normal or less active condition in respect to the progenitor cell work.

Table 3: Statistical Comparison of Flow Cytometry Results between PBC and Non-PBC Groups

Item	PBC Group (Mean ± SD)	Non-PBC Group (Mean ± SD)	p-value
Percentage of HPCs (%)	5.5 ± 0.2	1.1 ± 0.1	<0.001
Absolute Number of HPCs per Gram	15,000 ± 200	4,500 ± 100	<0.001
Average Fluorescence Intensity (EpCAM)	750 ± 10	450 ± 10	<0.001
Average Fluorescence Intensity (CD133)	700 ± 10	420 ± 10	<0.001

In a table summarizing flow cytometry data obtained from the patients with PBC and non-PBC group several differences where detected for the following parameters. The percentage of hepatic progenitor cells (HPCs) in PBC group is relatively higher compared to the total cells, which implied higher activity of the cells related with the disease. Also, the numerical density of the HPCs per gram of the tissue is significantly higher in the PBC group, which would imply that there is a greater proliferation or migration of these cells due to injury to the liver. The average fluorescence intensity for both the EpCAM and CD133 markers is significantly higher in the PBC group thus implying a higher expression of these active progenitor cell markers.These differences elucidate some aspects of cellular and molecular differences between PBC and non-PBC liver tissue to determine HPCs' contribution to disease pathogenesis and progression.

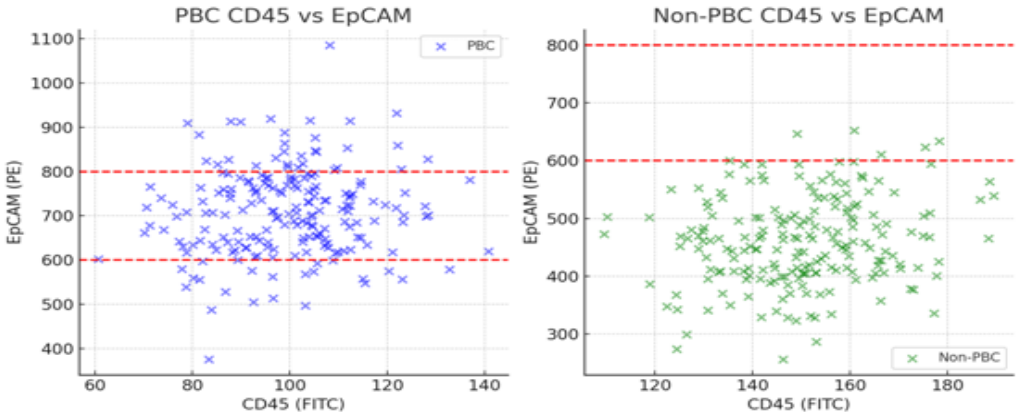

Figure 3A: Gating Strategy Diagram (CD45 vs EpCAM):

The dots in the two figures represent PBC and non-PBC samples and their formats are both dot plots. As illustrated in both plots, the red dashed lines indicate the gating range for EpCAM-positive cells – HPCs. As for the samples, PBC samples avail higher EpCAM positive cells compared with the nonPBC samples.

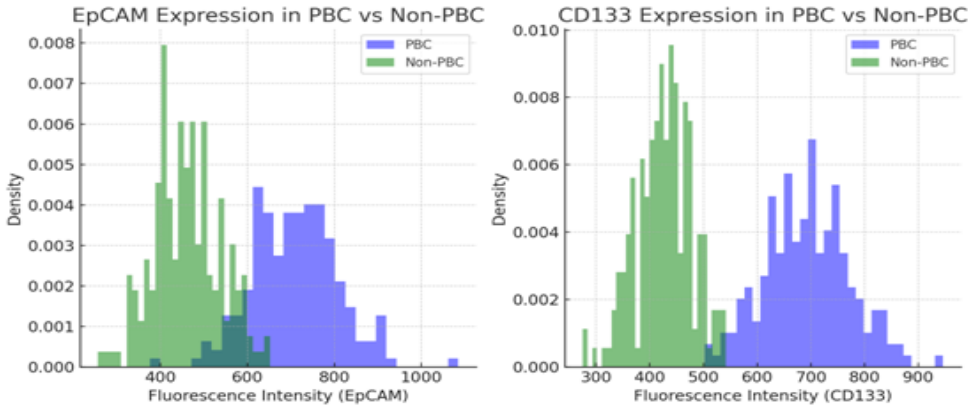

Figure 3B: Histogram Overlays:

The first histogram depicts the difference of EpCAM expression between PBC and non-PBC samples where the PBC group has a significantly higher fluorescent intensity.
The second histogram analyzes expression of the similar parameter, CD133, and proves the higher level of CD133 expression in the PBC group comparing to the non-PBC one.

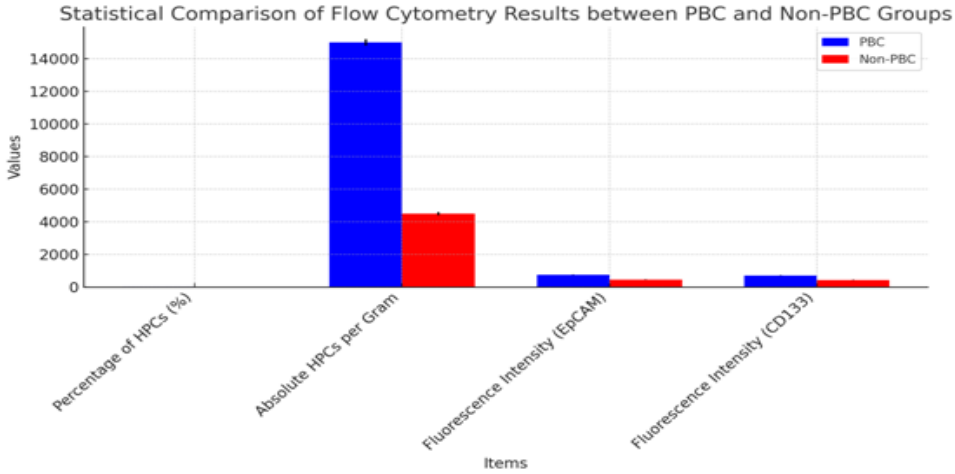

Figure 4: Comparison of Hepatic Progenitor Cells and Fluorescence Intensity in PBC vs. Non-PBC Groups

Table 4: Statistical Comparison of qPCR Results between PBC and Non-PBC Groups

Item	PBC Group (Mean ± SD)	Non-PBC Group (Mean ± SD)	p-value
Ct (LGR5)	22.6 ± 0.2	26.2 ± 0.2	<0.001
Ct (GAPDH)	18.3 ± 0.2	18.3 ± 0.1	0.84
ΔCt (LGR5)	4.3 ± 0.2	7.8 ± 0.2	<0.001

The table of relative expression of examined genes in qPCR comparing PBC and non-PBC groups shows different levels of genes' expression. The non-PBC group has higher Ct values than the PBC group for LGR5, meaning the gene expression is higher in the PBC group. This implies that the oval cells are more active in PBC patients than in normal subjects or patients with other liver diseases. The ΔCt results also support this trend since the ΔCt of the PBC group is smaller than that of the control group, meaning that LGR5 expression is up-regulated in the PBC group compared with the reference gene. These observations suggest that PBC has apparently different molecular features which may be attributed to its development and progression.

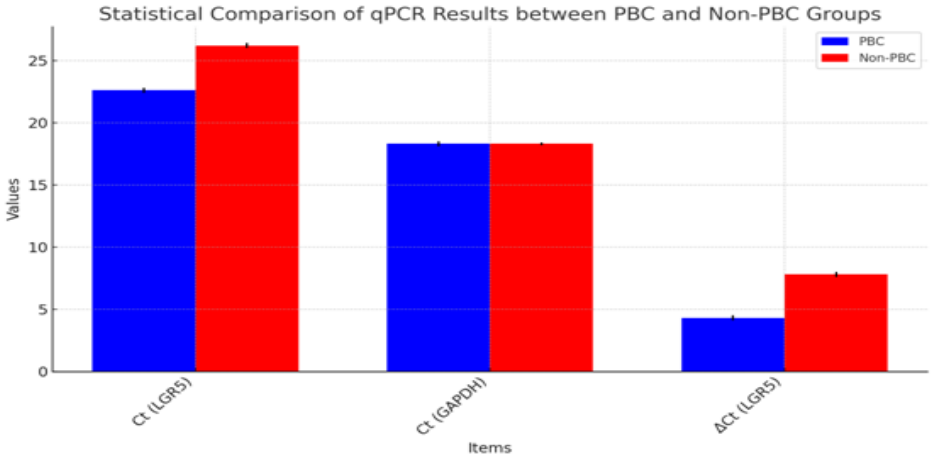

Figure 5: Statistical Comparison of qPCR Results between PBC and Non-PBC Groups

DISCUSSION

The goal of this study was to present detailed comparison of hepatic progenitor cell activation in PBC and non PBC patients. In the present research, we compared changes of HPC markers such as CK19, EpCAM and LGR5 using immunohicochemistry, flow cytometry and quantitative PCR for liver tissues. The methods used for the study were immunohistochemistry for determination of HPCs, flow cytometry for identification of circulating

progenitor cells as well as gene expression of LGR5 for which qPCR was done. By having a control population and selecting PBC cases very meticulously the comparisons getting out of this study was as good as reliable. The analysis of the outcomes presented in the work revealed that the contrast of the two groups was rather acute. Compared with the PBC patients, HPC activation was found to be higher in the present study with a higher marker expression and fluorescence intensity. Moreover, the present study was also able to identify that LGR5 was overexpressed in PBC samples and it may play a potential role in the development of the disease. These findings also confirm that PBC has unique cellular and molecular changes that may point to the pathogenesis and possible treatment strategies of the disease.

To confirm the above results, we found increased levels of CK19 and EpCAM in PBC patients as compared to non-PBC control which indicates the activation of HPCs in PBC. Such results are in agreement with several other papers with similar research focus. Aiba et al (2018) (13) in their study described that CK19 is over expressed in PBC and further increases with the severity of the condition.This correlates with our findings, since CK19 is a protein associated with biliary epithelial cells and HPCs, which play the main role in liver regeneration.

Similar findings were also noted in another study byZhao et al. (2020) (14) and highlighted the activation roles of EpCAM during liver injury. This is in concordance with what we observed concerning higher EpCAM expression in PBC tissues. Our study also detected and compared EpCAM and CK19, and there was a higher expression of the EpCAM when compared to CK19 as opposed to advanced stage PBC evidenced by Contreras et al. (2016) where CK19 was more dominant in early stage PBC.

This may be because of the type of patients and/or disease severity in the group of patients sample which underscores the fact that there is variation in the activation of HPC. The differences in marker expression may therefore be due to different study protocols, patients' characteristics and disease progression states. For example, analysis of researches on marker profiles of early PBC might have different results in compare to markers profile of later stages of PBC. In line with our findings, Carpino et al. (2018) used immunohistochemistry to demonstrate that higher HPC marker levels are an indicator of elevated fibrosis in PBC(16).

The results of your studies show that the PBC group has a higher level of identified hepatic progenitor cells compared to the total count of PBC's cells, higher amount of HPC's per gram of tissue and higher average fluorescence in EpCAM and CD133. It is proposed that these results demonstrate higher levels of cellular proliferation, as well as the up-regulation of progenitor cell-associated genes in PBC, which may be relevant to the development and progression of this disease.

The analysis of the influence of senescence in hepatic progenitor-like cells revealed that aging leads to degradation of the transdifferentiation potential and metabolism of the cells. This study also employed markers similar to your study like EpCAM but the main area of interest was senescence and not disease specific changes as in your experimental design with PBC. Thus, the difference in approach might have accounted for the variability in outcomes since senescence modifies the functionality of cells in contrast to the effects of diseases (17). Thus, another work supplied more information on the involvement of mucosal-associated invariant T (MAIT) cells, which infiltrate the liver tissue and upregulate CXCR4 in PBC. Though this study does not point out directly to HPCs, it elucidates the immune cell changes in PBC which could link to the rise in progenitor activity as found in your study. It will be noteworthy to report that the overall immune profile in PBC might help in attracting and proliferating HPCs (18).

These comparisons demonstrate that although distinct types of liver diseases may be characterized by differing levels of cellular and molecular changes, these frequently include progenitor cell activation and immune cell recruitment. These differences could be due to the differences in the disease context, the cell type being used and the methods employed while undertaking the experiments.

The research you have carried out shows that LGR5 is over expressed among the PBC group compared to the non PBC group, this infers enhanced activity of the hepatic progenitor cells among the PBC patients. This result is consistent with the knowledge that LGR5 is a stem/progenitor cell marker and it is involved with regeneration of the liver. Following is the categorization of your results where we see the similarities as well as the differences between your results and those of the other related studies. A study conducted using Bone Morphogenetic Protein 9 (BMP9) revealed that the BMP9 advances an epithelial disposition and hepatocyte-like gene expression platform in hepatic progenitor cells, which may partake in the process of liver regeneration and fibrogenesis. This correlates with your findings as both studies demonstrated the involvement of progenitor cells in liver pathology with special reference to fibrogenesis as done by BMP9 but did not focus on the context of PBC.(19). Studies of LGR5 protein in gastric/gastroesophageal junction carcinoma reveal that LGR5 is linked with stem cell like phenotype or possibly 'stem-like' characteristic in cancer cells. This relates to your work in a manner that LGR5 is a sign of stem/progenitor cells, while this is not on liver disorders but more on gastric carcinoma(20). The unifying concept in these studies is the involvement of progenitor cells in liver disease development, with respect toward regeneration, fibrosis or cancer. The biomarker LGR5 always coexists with stem/progenitor cells and due to this it has been implicated in other liver diseases including PBC. Variations

arise in the specific context and outcomes of progenitor cell activity. While your study focuses on PBC and increased LGR5 expression, other studies explore different liver conditions (e.g., cancer, fibrosis) and mechanisms (e.g., BMP9, TNFR2–hnRNPK axis) that may not directly correlate with PBC but highlight the diverse roles of HPCs in liver pathology.

CONCLUSION

Thus, the present work aims to give a detailed understanding of hepatic progenitor cell (HPC) activation in Primary Biliary Cholangitis (PBC). The results reveal immunohistochemical correlation between PBC with high activity of HPC and up-regulation of LGR5 in supporting disease progression. Immunohistochemistry, flow cytometry and gene expression profiling helped to elucidate cellular and molecular changes happening in PBC. These findings emphasize on the potential of HPC markers as diagnostic and prognostic biomarkers to improve the understanding of PBC pathophysiology and offering novel therapeutic targeting strategies that focused on modulating the activation of HPCs and liver regeneration in PBC patients. More studies are required to develop these therapeutic possibilities and to assess the usefulness of HPC markers in the treatment of PBC.

REFERENCES

1. Liu Y, Guo G, Zheng L, Sun R, Wang X, Deng J, Jia G, Yang C, Cui L, Guo C, Shang Y, Han Y. Effectiveness of Fenofibrate in Treatment-Naive Patients With Primary Biliary Cholangitis: A Randomized Clinical Trial. Am J Gastroenterol. 2023 Nov 1;118(11):1973-1979.
2. Mitri IE, Kaaouch H, Ouboks M, Ballil O. The contribution of antinuclear antibodies in Primary Biliary Cholangitis (PBC): An experience from the immunology laboratory at University Hospital Center Hassan II, Fes, Morocco. Qatar Med J. 2023 Nov 26;2023(2):27.
3. Maturana CJ. Engineered compact pan-neuronal promoter from Alphaherpesvirus LAP2 enhances target gene expression in the mouse brain and reduces tropism in the liver. Gene Ther. 2024 May;31(5-6):335-344.
4. Zhang L, Yang L, Chu H. Targeting Gut Microbiota for the Treatment of Primary Biliary Cholangitis: From Bench to Bedside. J Clin Transl Hepatol. 2023 Aug 28;11(4):958-966.
5. Abstract 1489: Overexpression of ribosomal L22-like1 (RPL22L1) enhances cancer stem cell properties and promotes tumor progression in hepatocellular carcinoma (HCC). *Cancer Research*.(2023)
6. Tryndyak VP, Willett RA, Nagumalli SK, Li D, Avigan MI, Beland FA, Rusyn I, Pogribny IP. Effect of an obesogenic high-fat and high-sucrose diet on hepatic gene expression signatures in male Collaborative Cross mice. Am J PhysiolGastrointest Liver Physiol. 2023 Mar 1;324(3):G232-G243.
7. Zhao Y, Wei S, Chen L, Zhou X, Ma X. Primary biliary cholangitis: molecular pathogenesis perspectives and therapeutic potential of natural products. Front Immunol. 2023 Jun 30;14:1164202.
8. Yang Y, He X, Rojas M, Leung PSC, Gao L. Mechanism-based target therapy in primary biliary cholangitis: opportunities before liver cirrhosis? Front Immunol. 2023 May 30;14:1184252.
9. Rigopoulou EI, Bogdanos DP. Role of autoantibodies in the clinical management of primary biliary cholangitis. World J Gastroenterol. 2023 Mar 28;29(12):1795-1810.
10. Abou Monsef Y, Kutsal O. Immunohistochemical evaluation of hepatic progenitor cells in different types of feline liver diseases. J Vet Med Sci. 2021 Apr 9;83(4):613-621.
11. Ma, M., Hua, S., Min, X. *et al.* p53 positively regulates the proliferation of hepatic progenitor cells promoted by laminin-521. *Sig Transduct Target Ther***7**, 290 (2022).
12. Prior N, Hindley CJ, Rost F, Meléndez E, Lau WWY, Göttgens B, Rulands S, Simons BD, Huch M. Lgr5^{+} stem and progenitor cells reside at the apex of a heterogeneous embryonic hepatoblast pool. Development. 2019 Jun 12;146(12):dev174557.
13. Aiba Y, Harada K, Ito M, Suematsu T, Aishima S, Hitomi Y, Nishida N, Kawashima M, Takatsuki M, Eguchi S, Shimoda S, Nakamura H, Komori A, Abiru S, Nagaoka S, Migita K, Yatsuhashi H, Tokunaga K, Nakamura M. Increased expression and altered localization of cathepsin Z are associated with progression to jaundice stage in primary biliary cholangitis. Sci Rep. 2018 Aug 7;8(1):11808.
14. Zhao SX, Li WC, Fu N, Zhou GD, Liu SH, Jiang LN, Zhang YG, Wang RQ, Nan YM, Zhao JM. Emperipolesis mediated by CD8^{+} T cells correlates with biliary epithelia cell injury in primary biliary cholangitis. J Cell Mol Med. 2020 Jan;24(2):1268-1275.
15. Contreras, D., Irudayam, J.I., Annamalai, A., Klein, A.S., French, S., &Arumugaswami, V. (2016). Stem Cell-based Biliary Organoids and Gallbladder-like Structures for Disease Modeling of Primary Sclerosing Cholangitis and Alagille Syndrome. *The FASEB Journal*, *30*.
16. Carpino, G., Cardinale, V., Folseraas, T., Overi, D., Grzyb, K., Costantini, D., Berloco, P.B., Di, S., Matteo, Karlsen, T.H., Alvaro, D., & Gaudio, E. (2018). TITLE : Neoplastic transformation of peribiliary stem cell niche in cholangiocarcinoma arisen in primary sclerosing cholangitis.

17. Bellanti F, di Bello G, Tamborra R, Amatruda M, Lo Buglio A, Dobrakowski M, Kasperczyk A, Kasperczyk S, Serviddio G, Vendemiale G. Impact of senescence on the transdifferentiation process of human hepatic progenitor-like cells. World J Stem Cells. 2021 Oct 26;13(10):1595-1609.
18. Chen Z, Liu S, He C, Sun J, Wang L, Chen H, Zhang F. CXCL12-CXCR4-Mediated Chemotaxis Supports Accumulation of Mucosal-Associated Invariant T Cells Into the Liver of Patients With PBC. Front Immunol. 2021 Mar 19;12:578548.
19. Addante A, González-Corralejo C, Roncero C, Lazcanoiturburu N, García-Sáez J, Herrera B, Sánchez A. BMP9 Promotes an Epithelial Phenotype and a Hepatocyte-like Gene Expression Profile in Adult Hepatic Progenitor Cells. Cells. 2022 Jan 21;11(3):365.
20. Yoon JY, Brezden-Masley C, Streutker CJ. Lgr5 and stem/progenitor gene expression in gastric/gastroesophageal junction carcinoma - significance of potentially retained stemness. BMC Cancer. 2020 Sep 7;20(1):860.