Phenotypical characterization of intrahepatic cholangiocarcinomas stroma : the more fibrous, the less pejorative.

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INTRODUCTION

Intrahepatic cholangiocarcinoma (iCC) is a rare liver tumor showing a growing incidence (1). Its prognosis is poor due to a late diagnosis and the lack of therapeutic options available. The 5 years survival rate does not exceed 10%. The surgery which is the only curative option, is feasible in 20% of the case (2). Morphologically, iCC is characterized by an abundant fibrous stroma, composed of extracellular matrix (ECM) proteins and different types of cells : myofibroblasts, endothelial and immune cells.

The cancer associated fibroblasts (CAFs) are mostly derived from activated hepatic stellate cells, and produce ECM components. These cells interact with tumoral cells through many paracrine ways. Stroma is becoming a major focus in cancer studies. Its prognostic value is still controversial and can be considered either as a defense from the host (3) or a tumoral supportive tissue (4). As stroma is considered as a niche contributing to tumoral progression, targeting it is an attractive therapeutic approach (5).

AIM

We aim to characterize stromal pattern of a set of iCC with complementary approaches in order to assess its prognostic value, and define new potential therapeutic targets.

MATERIAL & METHODS

Fifty cases of iCC, free from neo-adjuvant therapy, resected in Beaujon Hospital between april 2002 and september 2013, were studied. Immunohistochemistry (IHC) was performed on representative tissue sections from formalin fixed, and parrafin embedded (FFPE) samples, using anti-pan-cytokeratin, -αSMA, -CA9, -CD31 antibodies, and Picrosirius staining for stroma quantification. All slides were scanned and automatically quantified using dedicated algorithms (Aperio® software).

To better characterize the collagen component, 26 cases were studied using multiphotonic microscopy (HistoIndex® analytical software). Assessment of quantity and quality of collagen was obtained by exciting tissue at 405nm wavelength. This analysis was made on tissue microarray (TMA) from FFPE samples, built for the purpose of the study, by selecting 5 to 7 spots / tumor (Fig. 1). A total of 126 tumoral spots were analysed, as well as 7 non tumoral spots used for controls. Statistical analysis was done on ExcelStats®, Morpheus® and Prism®.

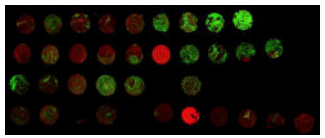


Figure 1 : Example of TMA analysed with multiphotonic microscopy.

Table 1 : Clinical and pathological data of the 50 cases of iCC

Clinical and pathological data	iCC n=50
Sex (m/f)	22/28
Median age (years)	61
Chronic liver disease n (%)	20 (4%)
Median size (cm) (range)	8.0 (1,9-15)
Satellites nodules n (%)	27 (54%)
Perineural involvment n (%)	20 (40%)
Vascular invasion n (%)	27 (54%)
Resection R0 n (%)	38 (76%)
Differentiation grade 1/2/3 n (%)	19 (38%) / 23 (46%) / 8 (16%)
TNM T1 / T2a / T2b / T3 / T4 n (%)	9 (18%) / 15 (30%) / 22 (44%) / 3 (6%) / 1 (2%)
Median time to recurrence (months) (range)	18.36 (1,2-162)
Median overall survival (months) (range)	32,26 (2-162)

RESULTS

Fibrosis amount

Surface of collagen in tumor samples was quantified by 2 morphometric approaches: picrosirius staining (%) and multiphotonic microscopy (MM) (Collagen Area Ratio). Results were similar with the 2 methods.

Median collagen value was 40,1% (9,7-76,6%) and 32,4% (6,9-69,1%) for PS staining and MM, respectively.

•Picrosirius (PS) staining assessment : *Pourcentage of surface of collagen*

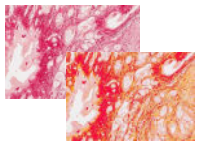


Figure 2 : Picrosirius staining showing collagen area stained in red (Aperio®), x20.

•Multiphotonic microscopy (MM) assessment: *Collagen Area Ratio (CAR)*

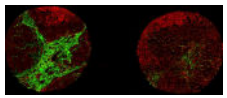


Figure 3 : 2 spots from a case of iCC analysed by MM, collagen fibers are shown in green, tissue auto-fluorescence in red (HistoIndex®).

•Clustering based on fibrosis amount

Hierarchical clustering of 26 iCC highlighted 3 tumor groups according to their collagen extent: rich (10 cases), intermediate (9 cases) and poor (7 cases (Fig. 4).

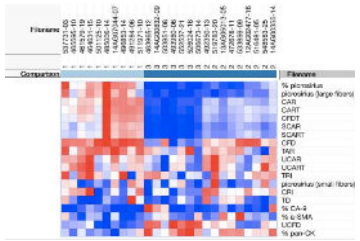


Figure 4 : Clustering of 26 cases (Morpheus®) showing 3 patterns of iCC according to the amount of fibrosis [rich (light red), poor (dark blue), and intermediate (light blue)].

•Fibrosis : A prognostic factor

Survival data demonstrated that patients with more fibrous tumors (rich) have longer time to recurrence compared to patients with less fibrous tumours (intermediate and poor) assessed by picrosirius staining. (Fig.5).

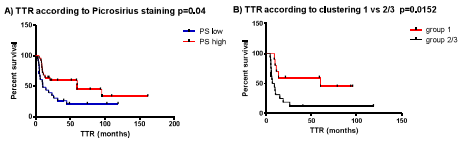
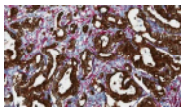


Figure 5 : Time to Recurrence (TTR) according to (A) data of 50 cases, p=0,04 and (B) clustering analysis, data of 26 cases, p=0,0152.

Fibrosis : Cellular composition

Myofibroblastic cells produce ECM components including collagen. They are characterized by cytoplasmic α-SMA marker (Fig. 6). ***In the overall series, amount of α-SMA–positive cells was not correlated with fibrosis median value 6,7% (range 1,22-18,6%)), whatever the quantitative approach used*** (CAR, Fig. 7).

Figure 6 : Double staining anti-αSMA (red) / anti-panCytokeratin (brown) x40.



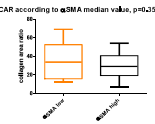


Figure 7 : Collagen Area Ratio according to the number of αSMA-positive cells

Fibrosis 3D organization

Reticulation of collagen was evaluated on TMA samples by scoring number of branchpoints in collagen fibers skeleton (HistoIndex analysis). ***Our data showed that the Collagen Reticulation Index (CRI) median value was 2,78 (2,41-3,15). The CRI was not correlated with the amount of fibrosis (Fig. 8), nor myofibroblastic cells (Fig. 9). No correlation was observed with survival nor recurrence*** (Fig. 10).

•Collagen Reticulation Index (CRI)

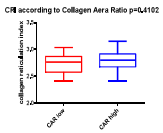


Figure 8 : CRI according to Collagen Aera Ratio, p=0.4102

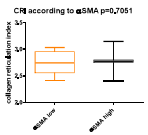


Figure 9 : CRI according to αSMA staining, p=0.7051

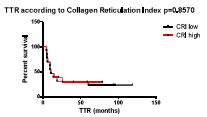
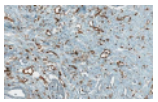


Figure 10 : TTR according to collagen reticulation index, p=0.8570

Stromal vascularization

Microvascular density (MVD) was quantified by IHC using anti-CD31 (Fig. 12), and assessed as numbers of vessels per squared micrometers. ***MVD median value was 5.57. 10^{-5} (2.8-11.9. 10^{-5}). It was inversely correlated to fibrosis amount (Fig. 13), without any impact on survival nor recurrence*** (Fig. 14).

Figure 12: CD31 immunostaining showing stromal vessels in a case of iCC (brown) x20.



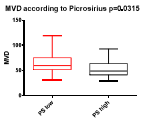


Figure 13 : MVD according to fibrosis.

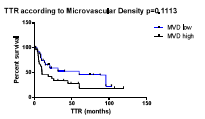


Figure 14 : TTR according to TTR.

DISCUSSION & CONCLUSION

Based on a surgical series of 50 iCC, this morphological study shows that extent of tumor stroma is not associated with amount of myofibroblastic cells, and, compared to other malignancies, is associated with a better prognosis. These results support the hypothesis that stroma may be considered as a barrier against tumoral spread and suggest that targeting myofibroblasts would not represent a relevant therapeutic strategy.

Quantitative and qualitative analysis of tumor stroma may be accurately performed on FFPE tissues, including TMA allowing the study of a large number of cases. Picrosirius staining and multiphotonic microscopy are reliable procedures and provide concordant results.

Although collagen 3D organization could have been an obstacle to vascularization and drugs delivery, collagen reticulation was not a prognostic factor in this series

ACKNOWLEDGEMENTS

Thanks to S. Lek for the multiphotonic microscopy technics.

Special thanks to technicians from the pathology department, Beaujon Hospital and Team 9 INSERM UMR1149 Beaujon Hospital, Clichy .

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DISCLOSURES

The authors have no disclosures to report.