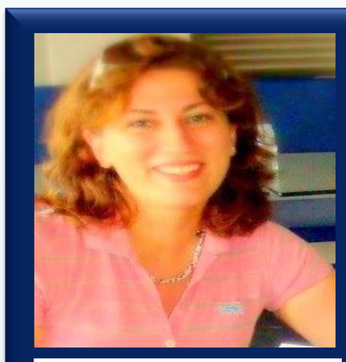

CAN FATTY LIVER LEAD TO LIVER CANCER?

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Aims

Non-alcoholic fatty liver disease (NAFLD) has been recognised as one the most common leading cause of cirrhosis and end-stage liver disease in the Western countries. It can ultimately progress to liver cancer, called hepatocellular carcinoma (HCC), **Figure 1**. Curative options are limited. In this study we have explored p62 – a marker of defective autophagy – in the progression of fatty liver disease to HCC, summarized below. Autophagy has been suggested as a therapeutic target and we wanted to see if p62 could have prognostic relevance.

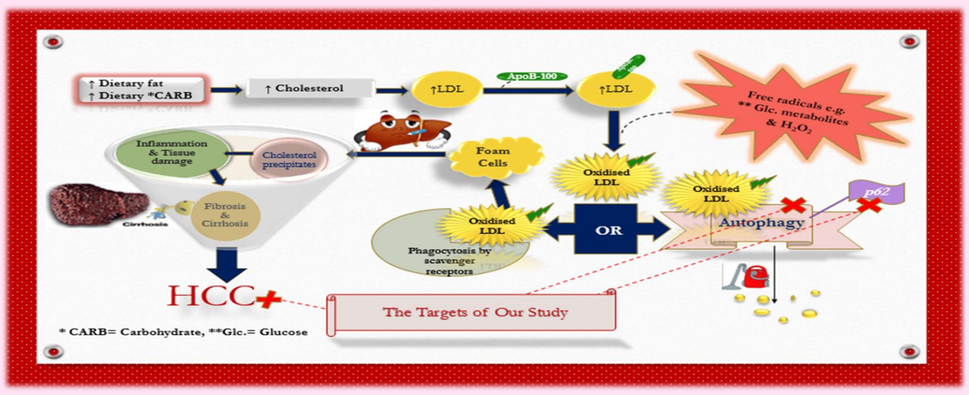

Figure 1. Showing overall cascades involved in the pathogenesis of Fatty Liver Disease and its progression to HCC.

Methods

- 87 cases of HCC with Formalin fixed paraffin embedded (FFPE) blocks were available via the ethically approved Newcastle Biobank. Clinical data retrieved for these cases included age, sex, diabetes, cirrhosis, etiology, Child-Pugh score (CPS), ascites, Encephalopathy, patients' performance score (ECOG).
- Tumour and paired non-tumour FFPE sections were stained using a p62 antibody (concentration 1 in 20) at the Department of Cellular Pathology, Royal Victoria Infirmary.
- The specimens were scanned with a digital slide scanner, (Aperio, Vista, USA).
- The scanned images were scored semi-quantitatively at X4 and X10 magnification. Examples are shown in **Figure 2**.

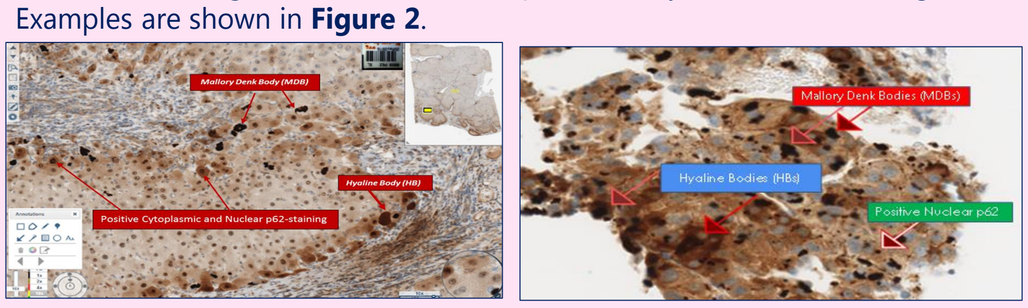

Figure 2. Images showing Positive Nuclear p62, MDBs and HBs in the lesion tissues.

- Scorable staining was available in 61 tumours (T) and 44 non tumour (NT) tissues.
- A semi-quantitative (SQ) approach was used to score the presence (1) or absence (0) of p62, the percentage of positive cells (0, 1 ,2, 3, 4 and 5 equating to 0%, 1-5%, 6-25%, 26-50%, 51-75% and >75%) and the staining intensity (0-3 equating to absent, mild, moderate and strong), in both the nucleus and cytoplasm of tumour versus NT liver. The presence or absence of the Mallory Denk Bodies (MDBs) and Hyaline Bodies (HBs) was noted. Scoring is demonstrated in **Table 1**.

P62 Case Number	Sections for p62	TUMOUR p62								Background liver p62							
		p62 status overall	Nuclear p62	p62 nuclear semiquant (0-5)	Cytoplasmic p62 intensity (0-3)	p62 cytopl semiquant- (0-5)	Total cytoplasmic p62	MDBs	HBs	p62 status overall	Nuclear p62	p62 nuclear semiquant (0-5)	Cytoplasmic p62 intensity (0-3)	p62 cytopl semiquant (0-5)	Total p62 cytoplasmic p62 (0-8)	MDBs	HBs
1	PR002686\1A lesion;2A background cirrhosis	1	1	1	3	2	5	0	0	1	1	1	2	1	3	1	1
2	PR002693\1A SH-HCC; 2A MIXED STEATOSIS	1	0	1	3	2	5	1	0	1	0	1	3	1	4	1	0
3	PR005889\1A is HCC; 2A is NT	1	1	5	3	5	8	1	1	1	0	0	1	1	2	1	1
4	PR011264\1A hcc nt ALL IN 1 CORE	1	0	1	3	2	5	1	1	1	0	1	1	3	4	1	1
5	PR021099(1A hcc & T IN ONE	1	0	0	1	3	4	0	0	-99	-99	-99	-99	-99	-99	-99	-99
6	PR022211\1A hcc & T IN ONE	1	0	1	3	3	6	1	1	-99	-99	-99	-99	-99	-99	-99	-99
7	PR026392\1A hcc & T IN ONE	-99	-99	-99	-99	-99	-198	-99	-99	-99	-99	-99	-99	-99	-198	-99	-99
8	PR027438\ 2A; 1A	1	1	1	1	4	5	1	0	1	0	0	1	4	5	1	1
9	PR027514\1A hcc & T IN ONE	1	0	0	2	5	7	1	1	-99	-99	-99	-99	-99	-99	-99	-99
10	PR029434\1A hcc & T IN ONE	1	0	0	2	3	5	0	0	-99	-99	-99	-99	-99	-198	-99	-99

Table 1. Showing part of (10 cases) semi-quantitative scorings of p62 obtained in Tumour vs. Non-Tumour tissue. [-99 denotes a case where scoring was not possible]

- Differences between p62 T and NT scores, as well as combination scores, were assessed by Chi Square, Mann Whitney or Krusakall Wallis tests.
- Associations with clinical and tumour variables were similarly explored.
- Cases were divided into two groups based on the tumour cytoplasmic p62 combined score (group 1: 0-4; group 2: 5-8) and survival compared by Kaplan Meier analysis.

Results

- Patients demographic and clinical data are summarised in **Table 2**.

Table 2. Showing Patients' Demographic and Medical Data

*TNM= T (Tumour's growth pattern and size) N (Spreading to the regional lymph nodes) and M (distant metastasis),
**OLTX= Orthotropic Liver Transplantation

Items	Variables	Number	Mean
Sex	Male	63	
	Female	18	
Age			67.99
Cirrhosis	Absent	39	
	Present	40	
Etiology	None	19	
	Alcoholic Liver Disease (ALD)	12	
	Non-Alcoholic Fatty Liver Disease	34	
	Hepatitis C (HCV)	6	
	Hepatitis B (HBV)	2	
	Haemochromatosis	2	
	Cryptogenic	0	
	Autoimmune Hepatitis	1	
	Primary Biliary Cirrhosis (PBC)	2	
	Other	2	
Diabetes	Absent	39	
	Present	40	
Body Mass Index (BMI) (18.5-30)	< 18.5 = underweight		29.97
	18.5-25= normal weight		
	25-30= overweight		
	>30=obese		
Ascites	Absent	67	
	Mild or controlled	9	
	Moderate to severe	4	
*TNM Classification	TNM 1	25	
	TNM 2	25	
	TNM 3	23	
	TNM 4	7	
TNM-stage	Stage 1 + 2	50	
	Stage 3 + 4	30	
Treatment	**OLTx	19	
	Resection	12	
	Ablation	4	
	Arterial treatment	27	
	Medical therapy	7	
	Supportive Care	12	

- P62 was present in both tumour and non-tumour tissues in the majority of cases; although, this was significantly higher in tumour cases. A summary of the mean values for each score in tumour (red) versus non-tumour tissue (blue) is shown in **Table 3**, as are the p-values comparing each tumour versus non-tumour score.

	Tumour p62 overall	Tumour nuclear p62	Tumour p62 % nuclei positive	Tumour cytoplasmic intensity	Tumour cytoplasmic % cells positive	Tumour Cytoplasmic combined score	Tumour MDBs	Tumour HBs	NT p62 overall	NT nuclear p62	NT p62 % nuclei positive	NT cytoplasmic intensity	NT cytoplasmic % cells positive	NT Cytoplasmic combined score	NT MDBs	NT HBs
Mean	1.0000	.5082	1.4754	2.4098	3.0820	5.4918	.8361	.4918	1.0000	.2500	.9773	1.4318	2.4773	3.7955	.6136	.2273
N	61	61	61	61	61	61	61	61	44	44	44	44	44	44	44	44
Std. Error of Mean	0.00000	.06454	.14335	.09460	.13690	.18646	.04779	.06454	0.00000	.06603	.11020	.10484	.19369	.23350	.07425	.06391

	NT p62 overall - Tumour p62 overall	NT nuclear p62 - Tumour nuclear p62	NT p62 % nuclei positive - Tumour p62 % nuclei positive	NT cytoplasmic intensity - Tumour cytoplasmic intensity	NT cytoplasmic % cells positive - Tumour cytoplasmic % cells positive	NT Cytoplasmic combined score - Tumour Cytoplasmic combined score	NT MDBs - Tumour MDBs	NT HBs - Tumour HBs
Asymp. Sig. (2-tailed)	1.000	.008	.006	.000	.001	.000	.020	.035

Table 3. Showing the correlation between other histological parameters and p62-expression levels are outlined

- In the tumour group, nuclear p62 was commonly present. However, etiological subgroup analyses indicated that this was significantly less often the case for NAFLD associated HCC, as shown in **Table 4**.

Histological Variables	Values	Tumour Tissue				
		NAFLD	No Chronic Liver Disease	Alcoholic Liver Disease (ALD)	HCV/HBV /others	Total
Tumour nuclear p62 (99% CI, *p= 0.19*)	Absent <5%	15	6	4	3	28
	Present >5%	6	6	7	12	31
Total		21	12	11	15	59

Table 4. Showing the comparison between the expressed p62 in the HCC tissues raised from NAFLD vs. Non-NAFLD-induced tumours.

- Notably, strong cytoplasmic intensity was common NAFLD and no chronic liver disease associated, demonstrated in **Table 5.**

Histological Variables	Values	Tumour Tissue				
		NAFLD	No Chronic Liver Disease	Alcoholic Liver Disease (ALD)	HCV/HBV /others	Total
Tumour cytoplasmic intensity (99% CI, *p=0.024*)	Mild	1	0	3	5	9
	Moderate	9	3	1	5	18
	Strong	11	9	7	5	32
Total		21	12	11	15	59

Table 5. Showing higher cytoplasmic p62-intensity in NAFLD-induced tumours vs. other tumours.

- Earlier stage cases treated with resection or transplantation are over-represented in this series (**Table 2**), where case selection was based on tissues availability for study. Consequently the overall median survival was 54 months.
- The median survival of patients with a combined score (percentage positive cytoplasmic p62 cells and intensity score) between 5-8 was greater than 150 months, compared to 31 months for those with a combined score of 0-4. Although case numbers are small (n= 15 and 45 respectively), the difference trended toward statistical significance on Kaplan Meier analysis (p=0.08), with survival curves shown in **Figure 3**.

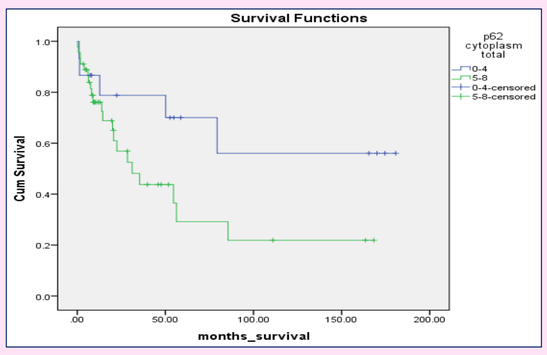

Figure 3. Kaplan Meier Curve showing estimated survival months with respect to the total cytoplasmic level of p62.

Conclusion

Returning to the hypothesis posed at the beginning of this study, it is now possible to state that:

- There was a significantly higher level of overall p62 in the tumour tissues compared to the non-tumour ones.
- With respect to the Nuclear p62, this study has found that it was generally lower in NAFLD-induced tumour tissues in comparison with the tumours arised from other etiologies.
- A comparison of the results of the cytoplasmic p62-level in both tumours, caused by NAFLD and other etiologies, revealed that the cytoplasmic levels of p62 were frequently high in the tumours associated with NAFLD. Moreover, the presence of a higher cytoplasmic p62 combined score trended toward an association with poorer survival.
- Taken together, these findings could have a number of important implications for future studies. It could support the idea of using p62 as a prognostic biomarker for HCC.