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Basic Science

Expression of hnRNPK & Claudin-4 in HCV-Induced Early HCC and Adjacent Liver Tissue

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Keywords: hnRNPK; Claudin-4; eHCC; Metavir; EMT; cirrhosis.

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Abbreviations: eHCC = Early Hepatocellular Carcinoma; hnRNPs = RNA-binding proteins; EMT= Epithelial Mesenchymal Transition; HNRNPK = Heterogeneous nuclear ribonucleoprotein K; HCV = Hepatitis C Virus; HCV = Hepatitis B Virus; VEGF = Vascular Endothelial Growth Factor; TJs = Tight Junctions; TBRI = Theodeor Bilharz Research Institute; HPFs = High Power Fields; % = Percentage; HGeHCC = High Grade Early Hepatocellular Carcinoma; LGeHCC = Low Grade Early Hepatocellular Carcinoma.

Abstract

BACKGROUND: HCC in Egypt usually occurs in HCV cirrhotic livers with poor prognosis due to late diagnosis. High hnRNPK & low Claudin-4 profiles indicate Epithelial Mesenchymal Transition (EMT), malignant transformation and high-grade tumours.

AIM: We studied the immunohistochemical expression of hnRNPK and Claudin-4 in HCV induced early HCC (eHCC) and adjacent liver tissue in Egyptian patients to improve eHCC detection in cirrhotic livers with better curative therapy options.

METHOD: We studied the immunohistochemical expression of hnRNPK and Claudin-4 in 100 Egyptian patients resection specimens of HCV induced early HCC (eHCC) and adjacent liver tissue, in order to improve eHCC detection in cirrhotic livers, thus improving their therapeutic options.

RESULTS: Early HCC grade significantly directly correlated with nuclear hnRNPK/5HPFs count and inversely correlated with Claudin-4 expression %, with a converse correlation between hnRNPK and Claudin-4. Moreover in eHCC, combined hnRNPK ≥ 30/5HPFs & Claudin-4 ≥ 40% significantly distinguished low grade eHCC (G1) from high grade eHCC (G2&G3), with sensitivity 97% & specificity 69.7% for hnRNPK ≥ 30/5HPFs, and with sensitivity 70% & specificity 94.3% for Claudin-4 ≥ 40%. Moreover in the adjacent liver, both markers expressions significantly directly correlated with each other and with METAVIR fibrosis score but not with activity. Furthermore, 58% of eHCCs showed hnRNPK ≥ 30 Claudin-4 < 40% profile, indicating EMT type 3, compared to 26% with hnRNPK ≥ 30 Claudin-4 ≤ 10% profile in adjacent cirrhotic/ precirrhotic liver, with significant use of combined hnRNPK ≥ 30/5HPFs & Claudin 4 ≤ 10% as eHCC prediction cut offs in cirrhosis ($p < 0.05$).

CONCLUSION: Combination of hnRNPK and Claudin-4 can indicate early HCC development in HCV cirrhotic livers using hnRNPK ≥ 30/5HPFs & Claudin-4 ≤ 10% cut offs. Also, combination of hnRNPK ≥ 30/5HPFs & Claudin-4 ≥ 40% can distinguish low grade eHCC (G1) from high grade eHCC (G2&G3).

Introduction

In Egypt, HCC is one of the commonest cancers [1-2]. It occurs with cirrhosis [3] since it leads to alteration of hepatocyte proliferation and promotion of tumorigenesis [4-6]. Early HCC treatment is curative [3]. Nevertheless, usually, HCCs have a poor prognosis due to late diagnosis and lack of effective therapy options [6]. Accordingly, early detection of HCC in cirrhotic patients is mandatory [3].

Heterogeneous nuclear RNA-binding proteins (hnRNPs) are crucial for RNAs control, mRNA export, turnover, localization, and translation [7-8]. Their aberrant expression is associated with cancer cell proliferation, angiogenesis, invasion, epithelial mesenchymal transition (EMT) and metastasis [8]. Heterogeneous nuclear ribonucleoprotein K (hnRNPK) is a potential tissue biomarker for early detection of HCC [8-9]. It is a DNA & RNA binding protein [10] and contributes to chromatin remodeling, transcription and translation [11]. It is distinguishable from the other hnRNPs by its capability to interact with numerous proteins through its K interactive region [12-13] that fits it in the center of a network to influence diverse cellular processes. HNRNPK is a potential tumor suppressor [10]. Its knockout results in reduced survival and increased tumorigenicity [12-13]. Its overexpression is associated with poor clinical status [10].

In hepatitis, hnRNPK is important for HCV pathogenesis [3, 8, 9]. It shows similar sequences with HCV core protein binding domain [3]. Moreover, hnRNPK is involved in the multistep process of hepatocarcinogenesis of both HBV replication and HCV pathogenesis with eventual cirrhosis and HCC [3], [8]. In this context, an increase of HCC in Egypt is due to high HCV prevalence particularly in cirrhotic patients compared to the declining incidence of HBV, since Egypt exhibits the highest HCV prevalence worldwide [14-16].

In HCC, hnRNPK overexpression is a marker for HCC [3, 11]. Positive tissue hnRNPK staining is an indicator of HCC and facilitates accurate early HCC distinction from high-grade dysplasia and other small nodules, which can be extremely challenging [3]. Moreover, hnRNPK expression in the early and late HCC is reported to be $\geq$ 3 folds higher and stronger than adjacent cirrhotic [3] or normal liver [16], due to the nuclear shift of K protein from the cytoplasm into the nucleus in tumours.

In addition, hnRNPK overexpression significantly correlates with the increased tumour size, active tumour growth, intrahepatic micrometastasis and microsatellite nodules formation [3].

In the same context**,** increased nuclear levels of K protein in cancers plays a role in the altered telomeric processes [17], exerts antiapoptotic effect on cancer cells independently of p53 status [11], activates c-*myc* promoter in hepatocytes in response to mitogens and following liver injury [17], and activates VEGF transcription by selective binding to VEGF promoter [8]. Since Epithelial Mesenchymal Transition (EMT)-angiogenesis-stem cell-like crosstalk is a key factor for HCC [18], the transformed tumour cells acquire stem cell features, show multidrug resistance, and induce local recurrence [18-19], metastasis & cancer progression [20]. Also, hnRNPK silencing significantly decreases EMT phenotype in cancer cells [8, 21].

In this regard, loss of critical junction proteins -including Claudins - leads to loss of epithelial cell adhesion, thus represents the first step of EMT [22]. Claudins are trans membrane proteins and important components of tight junctions (TJs) [23-24] that act as cell adhesion molecules, thus preserving cohesion in the tumour mass, suppressing cell proliferation & tumorigenesis, and function as cell migration barrier [24]. Moreover, Claudine expression patterns affect the tumour behavior [23]. Downregulation of several Claudins in cancer is consistent with the disruption of TJs during tumourigenesis [23] since low expression or loss of TJs is associated with malignant transformation and characterizes the highly metastatic cancers [23].

Claudin-4 is one of the most frequently dysregulated Claudins [24-26]. Its low expression indicates poor prognosis in oesophagal and colorectal cancers [24]. Nevertheless, it is upregulated in other malignancies, including breast, esophageal, gastric, pancreatic, prostate and uterine cancers [24].

In the liver, impaired Claudin-4 expression in biliary tract cancers is associated with less differentiated and more invasive phenotypes [27]. Hence it became a candidate for Claudin based targeted therapies [27-29]. More importantly, Claudin-4 protein is undetectable in HCC and normal hepatocytes compared to normal expression by normal cholangiocytes [27-28]. Nevertheless, it is expressed by severely damaged hepatocytes [30]. Hence, Claudin-4 distinguishes biliary from hepatocytic tumours [27, 30-31].

Since markers combination improves diagnosis [3], we studied the immunohistochemical expression of hnRNPK and Claudin-4 in HCV induced early HCC (eHCC) and adjacent liver tissue in Egyptian patients to improve eHCC detection in cirrhotic livers with better curative therapy options.

Material and Methods

The study was held on 100 HCC resection specimens with a history of HCV infection, obtained retrospectively from archival paraffin blocks at pathology department Theodeor Bilharz Research Institute (TBRI) (2010-2015).

A- Inflammatory activity and fibrosis in the adjacent liver tissue were evaluated using METAVIR scoring system [32] as follows:

- A for inflammatory activity: A0: No activity. A1: Mild. A2: Moderate. A3: Marked.
- F for portal fibrosis: F0: No portal fibrosis. F1: Portal fibrosis without septa. F2: Portal fibrosis with rare septa. F3: Numerous septa without cirrhosis. F4: Cirrhosis.

B- HCC graded as follows [33]:

- Well differentiated (G1): Thin plates with 1-3 hepatocytes thick, minimal nuclear atypia, doubled nuclear density and common pseudo glands.
- Moderately differentiated (G2): Trabeculae $\geq$ 4 cells thick, large cells with nucleoli, pseudo glands and bile.
- Poorly differentiated (G3): Large cells in solid sheets with hyperchromatic nuclei, marked pleomorphism and rare trabeculae or bile.
- We considered G1 as low grade (Fig. 1), while G2 & G3 as high grade [34].

C- Immunohistochemical technique:

Immunohistochemistry for hnRNPK & Claudin-4 was performed on tumours and adjacent non tumorous tissue sections cut from the paraffin blocks and stained with anti-human hnRNPK & Claudin-4 monoclonal primary antibodies (Santa Cruz Biotechnology, CA, USA) at 1:150 dilution. Slides were sectioned at 4µm onto positively charged slides (Superfrost plus, Menzel-Glaser, Germany) and the slides were stained on an automated platform the (Dako Autostainer Link 48). Heat induced antigen retrieval was used for 30 min at 97°C in the high-PH EnVision™ FLEX Target Retrieval Solution, and the primary antibody was used at a dilution of 1 in 100. The detailed histopathological assessment was done regarding confirmation of diagnosis and grading of malignant cases.

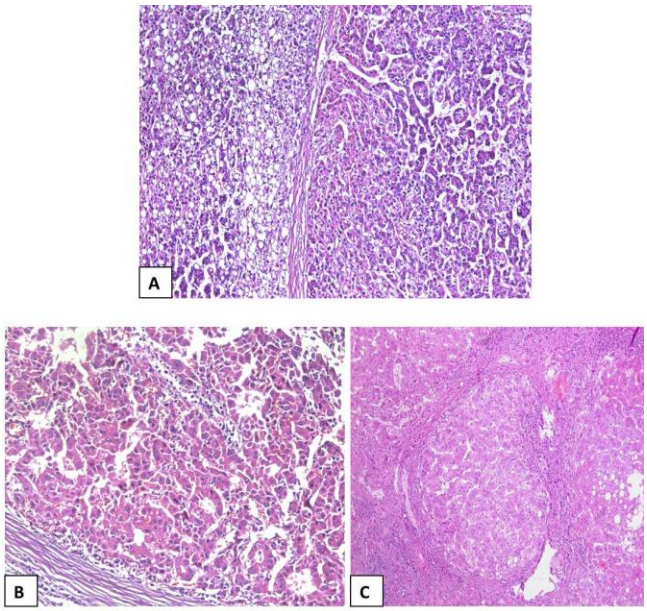

Figure 1: H&E Low (G1) & High (G2 & G3) early HCC (eHCC) and the adjacent liver. (A) High (G2-3) early HCC (eHCC) shows moderate nuclear anaplasia and pseudoglandular forms. The adjacent liver shows steatosis (H&E ×100). (B) High grade (G2-3) early HCC (eHCC), shows moderate to high nuclear anaplasia, pseudoglandular forms and bile (H&E × 200). (C) Low grade (G1) early HCC (eHCC) shows low grade nuclei and compact architecture, among a background cirrhotic liver (H&E × 100)

D- Evaluation of the immunostaining:

- HNRNPK: Only nuclear staining was counted per 5 High Power Fields (5HPFs). Positivity cut off at > 10% was established.
- Claudin-4: Semiquantitative H score for staining intensity and % of expression was used. Positivity cut off at >10% was established [35].

Statistical analysis

SPSS software version 18 was used for data management and analysis. Quantitative data were presented as mean ± SD. Qualitative data were presented as frequencies and percentages. Spearman's correlation coefficient was calculated to assess the relationship between variables. Tests were considered statistically significant when P< 0.05. Cut off values for both markers were chosen with sensitivity and specificity evaluation.

Results

I - Mean hnRNPK & Claudin-4 expression in HCC compared to the adjacent non tumorous liver:

A- Claudin-4:

All of our cases (whether HCCs or their adjacent liver) showed low Claudin-4 expression profile regarding the staining intensity -weak & negative cytoplasmic staining- compared to the moderate and strong expression in bile ducts as internal control. Non exhibited high Claudin-4 expression profiles (Fig. 2).

Table 1: Mean expression values of hnRNPK & Claudin-4 in eHCC & adjacent liver tissue

		T- test				Anova test
		N	Mean expression	Std. Deviation	Std. Error Mean	
Claudin-4 expression %	eHCC vs. adjacent liver					
	Adjacent liver	100	.0000	.00000	.00000	0.000**
	eHCC	100	31.2400	15.20043	1.52004	
	eHCC grade					
	G1	25 25%	46.8400	9.15915	1.83183	0.000**
	G2	54 54%	30.7963	6.19915	0.84360	
	G3	21 21%	25.2381	24.11204	5.26168	
	METAVIR activity in adjacent liver					
	A1	8 8%	0.000	0.000	0.000	0.637
	A2	8 8%	0.000	0.000	0.000	
	A3	84 84%	1.0714	4.46347	0.48700	
	METAVIR fibrosis in adjacent liver					
	F3	94 94%	0.000	0.000	0.000	0.000**
	F4	6 6%	15.000	8.94427	3.65148	
hnRNPK nuclear count/5HPFs	eHCC vs adjacent liver					
	Adjacent liver	100	23.8600	7.58656	.75866	0.000**
	eHCC	100	40.1400	17.05133	1.70513	
	eHCC grade					
	G1	25	24.9600	3.86738	0.77348	0.001*
	G2	54	36.6667	8.81465	1.19952	
	G3	21	67.1429	11.36348	2.47971	
	METAVIR activity					
	A1	8	29.2500	2.54951	0.90139	0.060
	A2	8	20.5000	9.03960	3.19598	
	A3	84	23.6786	7.56364	0.82526	
	METAVIR fibrosis					
	F3	94	23.2234	7.35016	0.75811	0.001*
	F4	6	34.0000	1.54919	0.63246	

**Significance differences between groups by Anova Test (p=0.001). *Significance differences between groups by Anova Test (p<0.05).

In adjacent liver, only 6 cases (6%) exhibited weak Claudin-4 in >10%. In contrast, the majority of cases (94%) significantly showed 0% - ≤ 10% Claudin-4, with mean expression value 0% compared

to 31.24% ± 15.20 in HCC (P < 0.001) (Tables 1 & 2).

B- hnRNPK:

Cytoplasmic and nuclear hnRNPK expression in eHCC & adjacent liver was noticed. Only the nuclear expression was counted (Fig. 2).

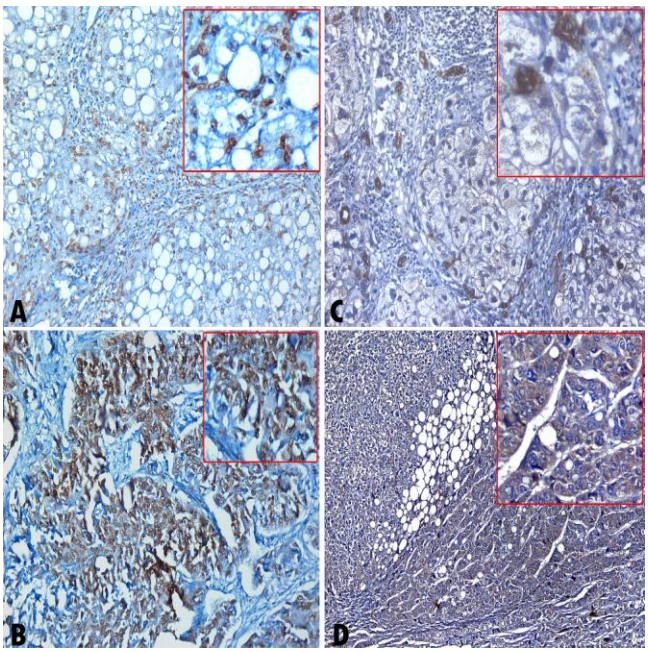

Figure 2: Immunohistochemistry expression hnRNPK & Claudin-4 in eHCC and adjacent liver. (A) Cirrhotic liver nodules are showing nuclear hnRNPK (in focus), (IHC, DAB, ×100). (B) High grade (G2-3) early HCC (eHCC), showing nuclear (in focus) and cytoplasmic hnRNPK expression, (IHC, DAB, ×200).(C) Low grade (G1) early HCC (eHCC) and cirrhotic liver nodules with foci of weak cytoplasmic Claudin-4 (in focus), compared to normal moderate Claudin-4 in proliferated bile ductules (as an internal control) (IHC, DAB, ×200). (D) High grade (G2) early HCC (eHCC) shows weak cytoplasmic Claudin-4 (in focus), (IHC, DAB, ×100)

Our study showed that all of the non tumorous liver tissue adjacent to HCC significantly exhibited increased nuclear hnRNPK from a mean nuclear expression value 23.86 ± 7.587/5HPFs to 40.14 ± 17.05/5HPFs in HCC (P < 0.001) (Tables 1 & 2; Fig. 3a).

II - Regarding eHCC grade:

Overall, nuclear hnRNPK expression significantly directly correlated with HCC grade and inversely correlated with Claudin-4 expression % (P = 0.000) (Table 3; Figs. 3a-3b) in contrast to Claudin-4 expression %.

Low grade eHCC (G1) significantly showed ≥ 40% weak cytoplasmic Claudin-4 expression in 70% of cases, with mean expression value of 46.84% ± 9.16, compared to 30.80% ± 6.20 in high grade (G2) eHCC, and to 25.24% ± 24.11 in high grade (G3) HCC.

Table 2: hnRNPK & Claudin-4 expression among the studied cases in early HCC & adjacent pre-cirrhotic/cirrhotic liver regarding the chosen cut offs

I- HCC	hnRNPK nuclear count ≥ 30/5HPFs cut off	hnRNPK nuclear count < 30/5HPFs cut off	Claudin-4 % of expression ≥ 40% cut off	Claudin-4 % of expression < 40% cut off
Low grade eHCC (G1)	3 % N = 2	69.7% N = 23	70.0% N = 21	5.7% N = 4
High grade eHCC (G2)	65.7% N = 44	30.3% N = 10	6.7% N = 2	74.3% N = 52
High grade eHCC (G3)	31.3% N = 21	0% N = 0	23.3% N = 7	20% N = 14
Significance	P = 0.000**		P = 0.000**	
II-Adjacent liver	**hnRNPK nuclear count ≥30 /5HPFs cut off**	**hnRNPK nuclear count < 30/5HPFs cut off**	**Claudin-4 % of expression ≤ 10% cut off**	**Claudin-4 % of expression > 10% cut off**
		Inflammation activity		
METAVIR activity 1	17.2% N = 5	4.2% N = 3	12.8% N = 12	0% N = 0
METAVIR activity 2	3.4% N = 1	9.9% N = 7	8.5% N = 8	0% N = 0
METAVIR activity 3	79.3% N = 23	85.9% N = 61	78.7% N = 74	100% N = 6
Significance		P = 0.063	P = 0.549	
		Fibrosis		
METAVIR fibrosis 3	79.3% N = 23	100% N = 71	96.8% N = 91	50% N = 3
METAVIR fibrosis 4	20.7% N = 6	0% N = 0	3.2% N = 3	50% N = 3
Significance		P = 0.000**	P = 0.002*	

**p, Significant differences between groups by Chi Square Test (p<0.01); *p, Significant differences between groups by Chi Square Test (p < 0.05).

In contrast, 94.3% of high grade eHCC (G2 & G3) showed 0% - < 40% of cytoplasmic Claudin-4 compared to only 5.7% of G1 (P = 0.000) (Tables 1-2; Figs. 2-3b).

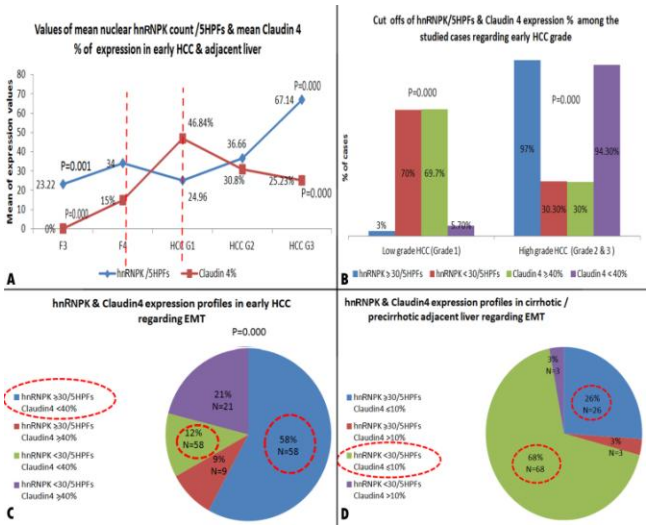

Figure 3: Statistical analysis charts for evaluation of hnRNPK & Claudin-4 expression in early HCC (eHCC) & adjacent liver. (A) Values of mean nuclear hnRNPK count /5HPFs & mean Claudin-4% of expression in early HCC (eHCC) & adjacent liver. (B) Cut offs of hnRNPK/5HPFs & Claudin-4 expression% among the studied cases regarding early HCC (eHCC) grade. (C) hnRNPK & Claudin-4 expression profiles in early HCC (eHCC) regarding Epithelial Mesenchymal Transition (EMT). (D) hnRNPK & Claudin-4 expression profiles in cirrhotic/precirrhotic adjacent liver regarding Epithelial Mesenchymal Transition (EMT)

Accordingly, we chose Claudin-4 ≥ 40% as a cut off to distinguish low grade eHCC (G1) from high

grade eHCC (G2&G3) (P = 0.000) (Tables 1-2), with sensitivity 70%, specificity 94.3%, false positive rate 5.7%, and false negative rate 30%.

On the other hand, approximately 70% of low grade eHCC (G1) significantly showed nuclear hnRNPK < 30/5HPFs, with mean nuclear expression value 24.96 ± 3.87/5HPFs compared to 36.67± 8.81/5HPFs for high grade (G2) eHCC, and to 67.14 ± 11.36/5HPFs for high grade (G3) HCC (P = 0.000). In contrast, 97% of high grade HCC (G2 & G3) significantly exhibited nuclear hnRNPK ≥ 30/5HPFs (Tables 1-2; Fig. 3b).

Accordingly, we chose nuclear count of hnRNPK ≥ 30/5HPFs as a cut off to distinguish low grade eHCC (G1) versus high grade eHCC (G2&G3) (P = 0.000) (Tables 1-2), with sensitivity 97%, specificity 69.7%, false positive rate 30.3%, and false negative rate 3%.

III - Regarding METAVIR fibrosis & activity scores in adjacent non-tumorous liver tissue:

Most of our cases showed pre-cirrhotic (F3) rather than complete cirrhotic nodules (F4) due to the clinical difficulty of obtaining liver biopsies in cirrhotic patients. Overall, both markers expressions significantly directly correlated with each other and with fibrosis score (P = 0.000) (Table 3; Fig.3a). Since 94% of cases showed Claudin-4 ≤ 10%, using Claudin-4 ≥ 40% as a cut off for adjacent non HCC liver evaluation was statistically invalid compared to hnRNPK ≥ 30/5HPFs & Claudin-4 ≤ 10% as statistically valid cut offs in this regard.

Our study showed significant direct correlation between Claudin-4 ≤ 10% and degree of liver fibrosis. About 96.8% of F3 and 3.2% of F4 (total number = 94 cases) significantly showed Claudin-4 ≤ 10% (P = 0.000). Only 6% (3 F3 & 3 F4 cases) expressed weak cytoplasmic Claudin-4 ≥ 10% in 15% ± 8.94 of hepatocytes, mostly at the periphery of the precirrhotic/cirrhotic nodules, with inconspicuous staining in nodules' centers (P < 0.000) (Tables 1-3; Figs 2-3a).

On the other hand, 29% of cirrhotic/precirrhotic nodules (23 F3 & 6 F4 cases) significantly exhibited nuclear hnRNPK in ≥ 30/5HPFs of hepatocytes (P<0.001) (Tables 1-2; Fig. 3a). Also the study showed significant increase of the mean hnRNPK nuclear expression from 23.22 ± 7.35/5HPFs in F3 up to 34 ± 1.55/5HPFs in F4 (P = 0.001) (Table 1; Fig. 3a).

Nevertheless, neither hnRNPK nor Claudin-4 showed a significant difference or correlation with inflammatory activity scores (Tables 1- 3). Furthermore, both markers neither correlated with age or gender (Table 3).

Table 3: Non-parametric correlation (Spearman's rho test) among the studied markers

	Claudin-4% of expression	hnRNPK nuclear count/ 5HPFs
	eHCC (N = 100)	
eHCC grade	-0.519-** 0.000	0.829** 0.000
hnRNPK nuclear count/ 5HPFs	-0.376-** 0.000	1
Claudin-4% of expression	1	-0.376-** 0.000
	Adjacent liver (N = 100)	
METAVIR Fibrosis	0.908** 0.000	0.368** 0.000
METAVIR activity	0.100 0.324	-0.105- 0.298
hnRNPK nuclear count/ 5HPFs	0.349** 0.000	1
Claudin-4% of expression	1	0.349** 0.000
Age	-0.032 0.753	0.003 0.975
Gender	0.160 0.122	0.023 0.821

**p: Correlation is significant at 0.01 level (2-tailed); *p: Correlation is significant at 0.05 level (2-tailed); -p: Inverse non parametric Spearman's rho test's correlation coefficient.

IV - Regarding EMT in eHCC & adjacent cirrhotic / precirrhotic liver:

In eHCC, 58% showed hnRNPK ≥ 30/5HPFs Claudin-4 < 40% profile, indicating EMT, compared to only 12% for hnRNPK< 30/5HPFs Claudin-4 < 40% profile (P = 0.000) (Fig. 3c).

On the other hand in adjacent cirrhotic / precirrhotic liver, only 26% exhibited hnRNPK ≥ 30/5HPFs Claudin-4 ≤ 10% profile, in contrast to 86% for hnRNPK < 30/5HPFs Claudin-4 ≤ 10% (Fig. 3d), however with P>0.05.

Discussion

Since HCC occurs in cirrhosis, early HCC detection is mandatory [3]. EMT physiologically or pathologically represents the conversion of an epithelial cell to a mesenchymal phenotype, and classified into three types: embryogenesis (type 1), wound healing/ fibrosis (type 2) and malignancy (type 3), [18, 36]. In cancer, EMT indicates drug resistance, local recurrence [18-19], progression and metastasis [21]. Since that cirrhosis alters hepatocyte proliferation and promotes tumorigenesis [4-6], and since hnRNPK is significantly expressed in eHCC, maintained in late HCCs [3], and contributes to HCV pathogenesis [3, 8-9], and since hnRNPK positive tissue staining is an indicator of HCC [3], we evaluated its expression in HCV induced HCCs and in their adjacent cirrhotic/precirrhotic livers.

In this study, both cytoplasmic and nuclear hnRNPK expressions in HCC as well as in the adjacent liver were noticed. Despite that cytoplasmic hnRNPK indicates its overexpression [10], nuclear hnRNPK was also reported to be higher in proliferating compared to resting hepatocytes [3, 17], which was similar to our findings. Moreover, nuclear

hnRNPK level was reported to be higher in neoplasms than in adjacent normal parenchyma in contrast to the cytoplasmic hnRNPK that remains unchanged in both neoplastic and surrounding tissues [17]. Therefore we counted only nuclear hnRNPK in our study. Furthermore, since several positivity cut offs were identified for early and late HCCs [3], and to avoid tissue variations, we used hnRNPK nuclear count > 10/5HPFs as positivity cut off.

In addition, stronger nuclear hnRNPK was reported in HCC in comparison to fainter nuclear staining in cirrhosis [3]. This is due to hnRNPK translocation into the nucleus [3, 17], which reflects its involvement in altered DNA and/or RNA in malignancy [17]. Nevertheless, our study showed rather moderate to strong nuclear hnRNPK in adjacent cirrhotic / precirrhotic liver, with a significant increase of the mean nuclear hnRNPK count in HCCs compared to adjacent cirrhotic liver, confirming the critical role of hnRNPK in hepatocytes proliferation, differentiation and tumorigenesis promotion in cirrhotic liver [10].

Regarding Claudin-4, all of our cases - whether HCC or adjacent liver- showed low Claudin-4 expression profile (weak & negative cytoplasmic staining compared to the moderate and strong expression in bile ducts) since bile ducts used as an internal control as mentioned in Holczbauer et al., 2013 [27] study. In the same context, absent Claudin-4 expression in non tumorous hepatocytes & HCC was reported, in contrast to normal cholangiocytes and cholangiocarcinomas [27]. None of our cases exhibited high Claudin-4 expression profile. Coming along with Konstantinos et al., 2014 [35], this indicates molecular down regulation and subsequent high recurrence and low disease free survival rates, hence pointing to type 3 EMT [18, 36].

Nevertheless, our study significantly showed increased Claudin-4 expression in HCC compared to the adjacent liver, in which weak cytoplasmic staining was detected. This came similar to Konstantinos et al., 2014 [35] where up regulation of Claudin-4 and other proteins in HCC was mentioned. In the same context, Holczbauer et al., 2013 [27] showed similar findings in some of their cases where Claudin-4 expressed by the apical poles of the glandular and alveolar forms of HCC. Also, Konstantinos et al., 2014 [35] reported -as other Claudins-, cytoplasmic staining pattern represented a loss of function & intracellular localization of Claudins, thus pointing to type 3 EMT [18, 36].

Moreover, Ojima et al., 2016 [34] classified eHCC into two pathologically distinct subtypes as high grade (HGeHCC) and low grade (LGeHCC). HGeHCC exhibited large tumor and nuclear sizes, high cellularity, structural atypia (including scirrhous pattern) with remarkable arterial and stromal invasions compared to LGeHCC. Similarly our study exhibited two distinct immunohistochemical profiles for the eHCC. High grade eHCC (G2&G3) significantly expressed hnRNPK $\geq$ 30/5HPFs and Claudin-4 $\geq$ 40% distinguishing it from low grade eHCC (G1), with sensitivity 97%, specificity 69.7%, false positive rate 30.3%, false negative rate 3% for hnRNPK $\geq$ 30/5HPFs cut off, and with sensitivity 70 %, specificity 94.3%, false positive rate 5.7%, and false negative rate 30% for Claudin-4 $\geq$ 40% cut off.

Furthermore, eHCC grade significantly directly correlated with nuclear hnRNPK/ 5HPFs count and inversely correlated with Claudin-4 expression %, with a converse correlation of hnRNPK with Claudin-4. In this regard, hnRNPK overexpression is considered as a marker for HCC [3, 11], besides that impaired Claudin-4 expression is associated with less differentiated and more invasive phenotype [27, 31], thus representing EMT type 3 [8, 18, 20, 36].

Despite that 50-60 years is the most frequent age range for HCC in Egypt [38], and despite association of increased Claudin-4 expression with female gender [37], our study showed no significant correlation between age, gender and both markers expression.

Regarding the adjacent liver, the majority of our cases showed F3 fibrosis. In this context, it was reported that developing HCC without advanced fibrosis (F4) may be due to other factors in the pathogenesis of HCV [38]. In Egypt, despite the high incidence of HCC in cirrhosis, HBV infection (whether occult or combined HCV HBV infection forms), diabetes and smoking have synergistic effects in HCC development [14]. Nevertheless, most of our cases showed pre-cirrhotic (F3) rather than cirrhotic F4 due to the clinical difficulty of obtaining liver biopsies from cirrhotic patients.

In our study, the mean expression of both markers significantly directly correlated with each other and with METAVIR fibrosis score but not inflammatory activity, with significant use of both of hnRNPK $\geq$ 30/5HPFs & Claudin-4 $\leq$ 10% cut offs ($P < 0.05$). The majority expressed Claudin-4 $\leq$ 10% particularly at the periphery of cirrhotic nodules. Similarly, according to Holczbaueret al., 2014 [27], it is due to bile duct proliferation [27], and according to Tsujiwaki et al., 2015 [28] is due to increased proliferation of progenitor cells that express Claudin-4, thus suggesting subsequent differentiation into mature hepatocytes.

Nevertheless, majority of adjacent cirrhotic / precirrhotic liver (68%) expressed hnRNPK < 30/5HPFs Claudin-4 $\leq$ 10% profile in contrast to 26% for hnRNPK $\geq$ 30/HPFs Claudin-4 $\leq$ 10% profile suggesting type 2 EMT as healing and regenerative process [18]. This also indicates that not all EMT in cirrhosis undergo malignant transformation into HCC particularly that the majority of those cases showed F3 pre-cirrhotic rather than F4 complete cirrhotic changes. Therefore it becomes compatible with incomplete rather than complete EMT with a chance

for reversal and healing [36] instead of malignant progression. This helps in identification of the leading factors of progression through finding of targeted therapeutic approaches to suppress nuclear hnRNPs translation [8, 11].

In contrast, since increase expression of hnRNPK is associated with EMT [8], and since that loss of Claudins is the first step of EMT [22], 58% of our eHCCs significantly exhibited hnRNPK ≥ 30/5HPFs Claudin-4<40% profile compared to only 12% for hnRNPK < 30/5HPFs Claudin-4 < 40% profile (P = 0.000), indicating EMT type 3 [18, 36] with drug resistance, local recurrence [18-19] & metastasis [21].

In conclusion, high hnRNPK and low Claudin-4 expressions indicate Epithelial Mesenchymal Transition (EMT), malignant transformation and high-grade tumours. The combination of hnRNPK and Claudin-4 in HCV cirrhotic livers can indicate eHCC development through the significant use of hnRNPK ≥ 30/5HPFs & Claudin-4 ≤ 10% as cut offs, hence helping in the identification of possible type 3 EMT that subsequently progresses to eHCC among those cirrhotic livers. Also, hnRNPK ≥ 30/5HPFs Claudin-4 ≥ 40% profile can significantly distinguish low grade eHCC (G1) from high grade eHCC (G2&G3).

References

1. GLOBOCAN: 2008 database (version 1.2). Available online: <http://globocan.iarc.fr>; 2008.

2. Ziada DH, El Sadany S, Soliman H, Abd-Elsalam S, Salama M, Hawash N, Selim A, Hamisa M, Elsabagh HM. Prevalence of hepatocellular carcinoma in chronic hepatitis C patients in Mid Delta, Egypt: A single centre study. Journal of the Egyptian National Cancer Institute. 2016; 28(4): 257–262. https://doi.org/10.1016/j.jnci.2016.06.001 PMid:27378258

3. Guo Y, Zhao J, Bi J, Wu Q, Wang X, Lai Q. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a tissue biomarker for detection of early Hepatocellular carcinoma in patients with cirrhosis. Journal of Hematology & Oncology. 2012; 5:37. https://doi.org/10.1186/1756-8722-5-37 PMid:22760167 PMCid:PMC3425156

4. Giannelli G, Bergamini E, Fransvea E, Sgarra C, Antonaci S. Laminin-5 with transforming growth factor-beta 1 induces epithelial to mesenchymal transition in hepatocellular carcinoma. Gastroenterology. 2005;129:1375–83. https://doi.org/10.1053/j.gastro.2005.09.055 PMid:16285938

5. Bataller R, Brenner DA. Liver fibrosis. J Clin Invest. 2005; 115:209–18. https://doi.org/10.1172/JCI24282 PMid:15690074 PMCid:PMC546435

6. Heerboth S, Housman G, Leary M, Longacre M, Byler S, Lapinska K, Willbanks A, Sarkar S. EMT and tumor metastasis. Clinical and Translational Medicine. 2015; 4:7. https://doi.org/10.1186/s40169-015-0048-3 PMid:25852822 PMCid:PMC4385028

7. Hogan DJ, Riordan DP, Gerber AP, Herschlag D, Brown PO. Diverse RNA-Binding Proteins Interact with Functionally Related Sets of RNAs, Suggesting an Extensive Regulatory System. PLoS Biol. 2008; 6:e255. https://doi.org/10.1371/journal.pbio.0060255 PMid:18959479 PMCid:PMC2573929

8. Han N, Li W, Zhang M. The function of the RNA-binding protein hnRNP in cancer metastasis. J Can Res Ther. 2013;9:129-34. https://doi.org/10.4103/0973-1482.122506 PMid:24516048

9. Guo YT, Zhao JM, Bi JT, Wu Q, Wang X, Lai QY. Heterogeneous nuclear ribonucleoprotein K (hnRNP K) is a tissue biomarker for detection of early Hepatocellular carcinoma in patients with cirrhosis. J Hematol Oncol. 2012; 5:37. https://doi.org/10.1186/1756-8722-5-37 PMid:22760167 PMCid:PMC3425156

10. Gallardo M, Lee HJ, Zhang X, Bueso-Ramos C, Pageon LR, McArthur M, Multani A, Nazha A, Manshouri T, Parker-Thornburg J, Rapado I. hnRNP K is a haploinsufficient tumor suppressor that regulates proliferation and differentiation programs in hematologic malignancies. Cancer cell. 2015;28(4):486-99. https://doi.org/10.1016/j.ccell.2015.09.001 PMid:26412324 PMCid:PMC4652598

11. Xiao Z, Ko HL, Goh EH, Wang B, Ren EC. hnRNP K suppresses apoptosis independent of p53 status by maintaining high levels of endogenous caspase inhibitors. Carcinogenesis. 2013; 34 (7): 1458-1467. https://doi.org/10.1093/carcin/bgt085 PMid:23455382

12. Geuens T, Bouhy D, Timmerman V. The hnRNP family: insights into their role in health and disease. Hum Genet. 2016; 135: 851. https://doi.org/10.1007/s00439-016-1683-5 PMid:27215579 PMCid:PMC4947485

13. Bomsztyk K, Denisenko O, Ostrowski J. hnRNP K: one protein multiple processes. BioEssays. 2004; 26:629–638. https://doi.org/10.1002/bies.20048 PMid:15170860

14. Atti EA. HCC Burden in Egypt. Gastroenterol Hepatol. 2015; 2(3): 00045.

15. Anwar WA, Khaled HM, Amra HA, El-Nezami H, Loffredo CA. Changing pattern of hepatocellular carcinoma (HCC) and its risk factors in Egypt: Possibilities for prevention. Mutat Res. 2008; 659(1-2): 176-184. https://doi.org/10.1016/j.mrrev.2008.01.005 PMid:18346933

16. Baghdady I, El-Kaffrawy N, Abd El-Atti E, Abd El-Bary N, Saber M. Study of the risk factors for hepatocellular carcinoma: effect of their synergism. Journal of American Science. 2013; 9(4): 211-217.

17. Ostrowski J, Bomsztyk K. Nuclear shift of hnRNP K protein in neoplasms and other states of enhanced cell proliferation. British Journal of Cancer. 2003; 89(8):1493-501. https://doi.org/10.1038/sj.bjc.6601250 PMid:14562022 PMCid:PMC2394341

18. Gurzu S, Turdean S, KovecsiA, Contac AO, Jung I. Epithelial-mesenchymal, mesenchymal-epithelial, and endothelial-mesenchymal transitions in malignant tumors: An update. World J Clin Cases. 2015; 3(5): 393–404. https://doi.org/10.12998/wjcc.v3.i5.393 PMid:25984514 PMCid:PMC4419103

19. Liu J, Shen JX, Hu JL, Dou XW, Zhang GJ. Role of epithelial-mesenchymal transition in invasion and metastasis of breast cancers. OA Cancer. 2013;1:16. https://doi.org/10.13172/2053-3918-1-2-1100

20. Brown AS, Mohanty BK, Howe PH. Identification and characterization of an hnRNP E1 translational silencing motif. Nucleic acids research. 2016;44(12):5892-907. https://doi.org/10.1093/nar/gkw241 PMid:27067543 PMCid:PMC4937310

21. Li LP, Lu CH, Chen ZP, Ge F, Wang T, Wang W, et al. Subcellular proteomics revealed the epithelial-mesenchymal transition phenotype in lung cancer. Proteomics. 201; 11:429-39.

22. Fabris L, Brivio S, Cadamuro M, Strazzabosco M. Revisiting Epithelial-to-Mesenchymal Transition in Liver Fibrosis: Clues for a Better Understanding of the "Reactive" Biliary Epithelial Phenotype. Stem Cells Int. 2016; 2016: 2953727. https://doi.org/10.1155/2016/2953727 PMid:26880950 PMCid:PMC4736590

23. Salvador E, Burek M, Förster CY. Curr Pathobiol Rep. 2016; 4: 135. https://doi.org/10.1007/s40139-016-0106-6 PMid:27547510 PMCid:PMC4978755

24. Kwon MJ. Emerging Roles of Claudins in Human Cancer. Int J Mol Sci. 2013; 14(9):18148–18180. https://doi.org/10.3390/ijms140918148 PMid:24009024 PMCid:PMC3794774

25. Morin PJ. Claudin proteins in human cancer: Promising new targets for diagnosis and therapy. Cancer Res. 2005; 65:9603–9606. https://doi.org/10.1158/0008-5472.CAN-05-2782 PMid:16266975

26. Singh AB, Sharma A, Dhawan P. Claudin family of proteins and cancer: An overview. J Oncol. 2010; 2010:541957. https://doi.org/10.1155/2010/541957 PMid:20671913 PMCid:PMC2910494

27. Holczbauer Á, Gyöngyösi B, Lotz G, Szijártó A, Kupcsulik P, Schaff Z, Kiss A. Distinct Claudin Expression Profiles of Hepatocellular Carcinoma and Metastatic Colorectal and Pancreatic Carcinomas. J Histochem Cytochem. 2013; 61(4): 294–305. https://doi.org/10.1369/0022155413479123 PMid:23385421 PMCid:PMC3636686

28. Tsujiwaki M, Murata M, Takasawa A, Hiratsuka Y, Fukuda R, Sugimoto K, Ono Y, Nojima M, Tanaka S, Hirata K, Kojima T, Sawada N. Aberrant expression of claudin-4 and -7 in hepatocytes in the cirrhotic human liver. Med Mol Morphol. 2015; 48(1):33-43. https://doi.org/10.1007/s00795-014-0074-z PMid:24737165

29. Neesse A, Griesmann H, Gress TM, Michl P. Claudin-4 as therapeutic target in cancer. Arch Biochem Biophys. 2012; 524: 64–70. https://doi.org/10.1016/j.abb.2012.01.009 PMid:22286027

30. Suzuki M, Kato-Nakano M, Kawamoto S, Furuya A, Abe Y, Misaka H, Kimoto N, Nakamura K, Ohta S, Ando H. 2009. Therapeutic antitumor efficacy of monoclonal antibody against Claudin-4 for pancreatic and ovarian cancers. Cancer Sci. 100:1623–1630. https://doi.org/10.1111/j.1349-7006.2009.01239.x PMid:19555390

31. Lódi C, Szabo E, Holczbauer A, Batmunkh E, Szijarto A, Kupcsulik P, Kovalszky I, Paku S, Illyes G, Kiss A, et al. Claudin-4 differentiates biliary tract cancers from hepatocellular carcinomas. Mod Pathol. 2006; 19:460–469. https://doi.org/10.1038/modpathol.3800549 PMid:16439986

32. Bedossa P, Poynard T. An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group. Hepatology. 1996; 24 (2):289-93. https://doi.org/10.1002/hep.510240201 PMid:8690394

33. http://www.pathologyoutlines.com/topic/livertumorHCC.html

34. Ojima H, Masugi Y, Tsujikawa H, Emoto K, Fujii-Nishimura Y, Hatano M, Kawaida M, Itano O, Kitagawa Y, Sakamoto M. Early hepatocellular carcinoma with high-grade atypia in small vaguely nodular lesions. Cancer Science. 2016; 107(4). https://doi.org/10.1111/cas.12893 PMid:26797961 PMCid:PMC4832853

35. Bouchagier KA, Assimakopoulos SF, Karavias DD, Maroulis I, Tzelepi V, Kalofonos H, Karavias DD, Kardamakis D, Scopa CD, Tsamandas AC. Expression of Claudins-1, -4, -5, -7 and Occludin in Hepatocellular Carcinoma and their Relation with Classic Clinicopathological Features and Patients' Survival. In Vivo. 2014; 28 (3) 315-326. PMid:24815833

36. Zhao Y, Zu RT, Sun YL. Epithelial-mesenchymal transition in liver fibrosis. Biomed Rep. 2016; 4(3): 269–274. https://doi.org/10.3892/br.2016.578

37. Holah NS, El-Azab DS, Aiad HA, Sweed DM. Hepatocellular carcinoma in Egypt: epidemiological and histopathological properties. Menoufia Medical Journal. 2015; 28 (3) : 718-724.

38. Mattos AA, Marcon Pdos S, Araújo FS, Coral GP, Tovo CV. Hepatocellular carcinoma in a noncirrhotic patient with sustained virological response after hepatitis C treatment. Rev Inst Med Trop Sao Paulo. 2015; 57(6): 519–22. https://doi.org/10.1590/S0036-46652015000600011 PMid:27049708 PMCid:PMC4727140