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Expression of Claudins-1, -4, -5, -7 and Occludin in Hepatocellular Carcinoma and their Relation with Classic Clinicopathological Features and Patients' Survival

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Abstract. *Background: Occludin and claudins are integral constituents of tight junction proteins and are de-regulated in various malignancies, including hepatocellular carcinoma (HCC). This study investigated whether expression of claudins 1, 4, 5, 7 and occludin may be used as prognostic markers for overall and disease-free survival in patients with HCC after hepatectomy. Patients and Methods: The study included 67 hepatectomy specimens obtained from an equal number of patients with HCC who underwent partial hepatectomy at the Patras University Hospital for therapeutic reasons. Ten normal liver tissues were used as controls. Expression of claudins 1, 4, 5, 7 and occludin in liver tissues was assessed by immunochemistry. Clinicopathological features were also available for each case. Results: Expression of claudins 1, 4, 5, 7 and occludin was significantly increased in HCC specimens compared to non-neoplastic liver tissues and normal controls (p<0.001 in each case) Moreover, there was a statistically significant association between low level of claudin-4 and advanced tumor grade (p=0.03). Down-regulation of claudin-1 was associated with low overall survival in univariate survival analysis (p=0.049) and Kaplan–Meier analysis (p=0.04). Multivariate analysis showed that the claudin-4 level was an independent factor for survival prognosis (p=0.01). In addition, down-regulation of claudin-4 expression was associated with increased recurrence rate and low disease-free survival rate in univariate analysis (p=0.038), Kaplan–Meier plot (p=0.013) and multivariate analysis (p=0.013). A low level of claudin-5 and high level of claudin-7 levels were independent negative prognostic factors according to multivariate analysis (p=0.015 and 0.009, respectively). Conclusion: The present study demonstrates that high expression of claudins 1, 4, 5 and down-regulation of claudin-7 are positive prognostic markers and are associated with good outcome and increased survival rates. Moreover, an increase in claudin-4 expression may serve as an independent positive prognostic factor for low recurrence rate after hepatectomy.*

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Tight junction proteins are multi-protein complexes composed of integral proteins that associate with cytoplasmic plaque proteins (1). To date, four groups of macromolecules are considered as bona fide integral components of tight junctions: occludins, claudins, junctional adhesion molecule (JAM) and tricellulin. Tight junctions play a crucial functional role in controlling the paracellular passage of ions, antigens and toxins (barrier function), whilst blocking the circulation of proteins and lipids between the apical and basolateral membrane, and mediate membrane polarization (fence function). In addition, tight junctions may trigger a variety of signaling pathways and communicate with the nucleus, acting as a sensor for extracellular events; they interact with cytoskeletal elements, regulate protein expression, participate in vesicle trafficking and as a multi-dynamic ligand for both homophilic and heterophilic interactions communicating with other cells (2).

Previous studies have shown that occludin and claudins are de-regulated in various types of cancers and these alterations have been correlated with the differentiation, invasiveness and metastatic potential of cancerous cells, thus affecting patients' prognosis in terms of disease recurrence and survival. Only a limited number of studies have examined the role of the expression of tight junction proteins in hepatocellular carcinogenesis and their potential

usefulness as prognostic markers or therapeutic targets. It has been shown that increased levels of claudin-10 enhance activity of MT1-matrix metalloproteinase (MT1-MMP) which strongly promotes invasion, intrahepatic metastasis and aggressive characteristics of hepatocellular carcinoma (HCC) (3). Claudin-1 expression has also been correlated with tumorigenesis in hepatitis C virus-related liver cirrhosis, whilst reduced expression of claudin-1 has been associated with poorly-differentiated HCC with enhanced metastatic dynamic and increased incidence of portal invasion(4). In addition, decreased levels of endothelial claudin-5 in HCCs have been associated with portal invasion by tumor cells and enhanced invasive and metastatic potential (5). The present study was undertaken to evaluate, through immunohistochemistry, the expressions of claudins 1, 4, 5, 7 and occludin in patients with HCC who underwent partial hepatectomy for therapeutic reasons and their possible role as prognostic markers for overall survival and disease-free survival, after surgical intervention.

Patients and Methods

Patients. The study included 67 hepatectomy specimens obtained from an equal number of patients with hepatocellular carcinoma who underwent partial hepatectomy from 1993 until 2011 at the University Hospital of Patras for therapeutic reasons. They were 54 males and 13 females ranging from 32-84 (mean±SD=66.98±9.57) years in age. Patients were followed-up from 2.13 months (min) to 228.80 months (max) (mean=27.96±35.91 month, median=15 months) until 28/2/2012, which was the cutoff date for all information concerning overall and disease-free survival times. Normal liver tissues obtained from 10 patients who underwent cholecystectomy for cholelithiasis but without co-existent choledocholithiasis were used as normal controls, after informed consent had been given.

Routine pathology. For each case, hematoxyline and eosin (H&E) slides were retrieved from the archives of the Department of Pathology, University of Patras School of Medicine. For each slide, several tumor features (number of tumor nodules, size of the largest tumor nodule, tumor grade, infiltration or not of Gleason capsule and presence or absence of microscopic vascular invasion) were evaluated. Furthermore co-existence or not of features, suggestive of chronic hepatitis or cirrhosis in the surrounding non-neoplastic liver was recorded. Finally, all tumors were graded according to Edmondson-Steiner system(6) and staged according to TNM system seventh edition(7).

Immunohistochemistry. Immunohistochemistry was performed on 4-μm thick formalin-fixed, paraffin-embedded tissue sections mounted on gelatin-coated glass slides. De-paraffinization, rehydration and antigen retrieval were performed in an electric pressure cooker using Trilogy retrieval solution (Cell Marque, Hot Springs, AR, USA) for 30 min. Polyclonal antibodies against occludin (1:80), claudin-1 (1:40) and claudin-7 (1:60) and monoclonal antibodies against claudin-4 (1:60) and claudin-5 (1:50) were used for the primary reaction. All antibodies were purchased from Zymed (Carlsbad, CA, USA). The sections were incubated with primary antibodies for 1 (occludin and claudin-1) or 2 (claudin-4-5 and -7) h at room temperature, followed by sequential 30-min incubation with Dako EnVision Labelled Polymer (Dako, Carpinteria CA, USA). Diaminobenzidine (Dako, Carpinteria CA, USA) was used as chromogen. Sections were counterstained with Harris hematoxylin (Vector, Burlingame, CA, USA). Sections from breast carcinoma were used as positive control for occludin. Normal skin was used as positive control for claudin-1 and normal colon for claudin-4 and -7. Sinusoidal endothelial cells were used as positive control for claudin-5. For negative control slides, the same method was performed and the primary antibody was substituted by 1% Tris-buffer solution.

Morphometric analysis. Microscopic evaluation of the slides was carried out independently by two observers (KAB and ACT). When discrepancies occurred, a consensus was achieved using a double-headed microscope. For morphometric analysis, and in order to quantify the cells that displayed positive staining, we used a method described in details elsewhere (8-10). Briefly: All tissue sections were scanned at low magnification and areas with positive stain were chosen. Thereafter, a 10×10 microscope grid at ×400 magnification was applied to the sections and both the number of positively stained cells and the total number of cells (at least 500) was determined by visual inspection of six different fields per section. For each field, a percentage value for each marker was obtained by dividing the number of positively stained cells by the total number of cells included in the grid. It should be noted that

Table I. *Clinicopathological features of all 67 patients included in the study.*

Gender, male/female	54/13
Mean±SD (range) age, years	66.98±9.57 (32-84)
Number of tumor nodules	
Single	62 (92.5%)
Multiple	5 (7.5%)
Size of tumor nodules	
≤5 cm	40 (59.7%)
>5 cm	27 (40.3%)
Tumor grade	
I	20 (29.85%)
II	22 (32.84%)
III	24 (35.82%)
IV	1 (1.49%)
Tumor stage	
I	9 (13.43%)
II	25 (37.11%)
III	31 (46.26%)
IV	2 (2.98%)
Infiltration of Gleason capsule	No=35 (52.24%)
	Yes=32 (47.76%)
Presence of microscopic vascular invasion	No=34 (50.75%)
	Yes=33 (49.25%)
Co-existent hepatitis	No=32 (47.76%)
	Yes=35 (52.24%)
Co-existent cirrhosis	No=48 (71.64%)
	Yes=19 (28.36%)

Table II. *Results of immunohistochemical staining for occludin and claudins 1, 4, 5 and 7 in controls, non-neoplastic liver tissue (NNLT) and hepatocellular carcinoma (HCC).*

	Controls (n=10)				NNLT (n=67)				HCC (n=67)				*p*-Value			
	N	W	M	S	N	W	M	S	N	W	M	S	A	B	C	D
Occludin	9	1	0	0	40	23	4	0	13	44	9	1	<0.001	0.173	<0.001	<0.001
Claudin-1	8	2	0	0	23	22	20	2	1	7	49	10	<0.001	0.041	<0.001	<0.001
Claudin-4	10	0	0	0	37	25	5	0	9	45	12	1	<0.001	0.026	<0.001	<0.001
Claudin-5	10	0	0	0	35	17	15	0	0	25	38	4	<0.001	0.017	<0.001	<0.001
Claudin-7	10	0	0	0	34	26	7	0	6	40	17	4	<0.001	0.013	<0.001	<0.001

N: Negative, W: weak, M: moderate, S: strong. A: when all three groups compared; B: controls *vs*. NNLT; C: controls *vs*. HCC; D: NNLT *vs*. HCC. Pearson-X^2 test (with Yates correction).

the difference in cell counts from field to field in the same section was <10%. For purposes of statistical analysis, the scoring for all the markers studied was as follows: i) we used a 3-point scale: 0: 0%, 1: 1-25%, 2:25-50%. 3: >50% for the percentage of positively stained tumor cells; ii) we used another 3-point scale depicting the intensity of the expression: 0: no intensity, 1: weak intensity, 2: moderate intensity, 3: strong intensity; iii) we then multiplied the two 3-point scales for each case, giving scores ranging from 0-9. A new scale was then adopted: N: score 0, weak expression: score 1-2, moderate expression: score 3-6, strong expression: score 7-9. However, for statistical analysis reasons further categorization was performed as a) high expression profile group, which includes strong and moderate expression; and b) low expression profile group, which includes weak and no expression.

Statistical analysis. Associations between discrete (categorical) attributes were examined using Pearson's χ^2-test of independence. For the variable age, Mann–Whitney nonparametric test was used after checking normality plots. In addition, we performed the more sensitive Jonckheere-Terpstra test for ordinal categorical variables Such as grade and stage to evaluate their association with claudin expression. Claudins 1, 4, 5 and 7, occludin and various clinicopathological parameters of potential prognostic value were analyzed for their effect on the duration of overall and disease-free survival, using Cox regression univariate and multivariate analyses. Furthermore, analysis of overall survival and disease-free survival was accomplished with Kaplan–Meier plots, where the differences in survivals between the groups were compared using log-rank test. In all statistical tests, the significance level was defined as $p<0.05$. Statistical analysis was implemented using the SPSS statistical tool (SPSS®. 20.0; SPSS, Chicago, IL, USA).

Results

At follow-up, 37/67 patients (55.22%) were dead, and 30/67 patients (44.78%) were alive, whereas 25/67 patients (37.31%) had developed tumor recurrence and 42/67 (62.68%) not. The mean±SD for the total survival time was 34.58±40.7 months and the median 23.6 months, whereas the mean±SD for the disease-free survival time was 27.96±35.91 months and the median 15 months. All clinicoepidemiological data are displayed in Table I.

Immunohistochemistry. Table II shows that overall, intensity of expression of tight junction proteins in HCCs was statistically significant increased compared to non-neoplastic liver tissues and healthy controls ($p<0.001$ for each category). All claudins examined were expressed at the basolateral membrane surfaces in a linear fashion. However, a cytoplasmic dot-like pattern was quite often noted and occasionally, immunostaining, in the form of small vesicles, was located at the membrane. Figure 1 shows a representative section including neoplastic and non-neoplastic liver tissue. Figures 2-6 show representative results from the immunostaining for claudins 1, 4, 5 and 7 and occludin, respectively. Positive controls displayed strong-positive staining for the four claudins and occludin.

Statistical analysis. As seen in Figure 7, claudin-1 was expressed in 66/67 (98.5%) cases whereas 58/67 cases (86.57%) displayed high expression (strong and moderate) and 9/67 cases (13.43%) displayed low expression (weak and no expression). Respectively, claudin-4 was expressed in 58/67 (86.56%) cases and more specifically, 13 cases (19.4%) demonstrated a high expression profile whereas 54 cases (80.6%) demonstrated low expression profile. Claudin -5 was expressed in 56/67 (83.58%) cases. A high expression profile was detected in 42 (62.69%) cases while a low expression profile was detected in 25 (37.31%) cases. As far as claudin-7 is concerned, positive staining was recorded in 61/67 (91.04%) cases, with high expression profile in 21 (31.34%) cases and low expression profile in 46 (68.66%) cases. Finally, occludin was expressed in 54/67(80.5%) cases with high expression profile being found in 11 (16.42%) cases and low expression profile in 56 (83.58%) cases. Table III reports the association between expression of claudins and occludin and various clinicopathological parameters. There was a borderline statistical association between claudin-1 expression and the number of tumor nodules ($p=0.07$), implying that single nodules may be associated with an increased level of claudin-1. Moreover, the study also found a borderline

Table III. *Association of claudins 1, 4, 5 and 7 and occludin expression with clinicopathological variables of all 67 cases with hepatocellular carcinoma included in the study.*

	Occludin		*p*-Value	Claudin-1		*p*-Value	Claudin-4		*p*-Value
	Low	High		Low	High		Low	High	
Gender									
Male	46 (85.19%)	8 (14.81%)	0.470	8(14.81%)	46 (85.19%)	0.498	43 (79.63%)	11 (20.37%)	0.683
Female	10 (76.92%)	3 (23.08%)		1(7.69%)	12 (92.31%)		11 (84.62%)	2 (15.38%)	
Tumor size									
≤5 cm	34 (85%)	6 (15%)	0.703	3 (7.5%)	37 (92.5%)	0.08	31 (77.5%)	9 (22.5%)	0.435
>5 cm	22 (81.5%)	5 (18.5%)		6 (22.2%)	21 (77.8%)		23 (85.2%)	4 (14.8%)	
No. of tumor nodules									
Single	52 (83.87%)	10 (16.13%)	0.822	7 (11.29%)	55 (88.71%)	0.07	50 (80.65%)	12 (19.35%)	0.972
Multiple	4 (80%)	1(20%)		2 (40%)	3 (60%)		4 (80%)	1 (20%)	
Tumor grade									
I	16 (76.19%)	5 (23.81%)		2 (9.52%)	19 (90.48%)		14 (66.67%)	7 (33.33%)	0.192
II	18 (85.71%)	3 (14.29%)	0.712	3 (14.29%)	18 (85.71%)	0.883	17 (80.95%)	4 (19.05%)	0.03*
III	21 (87.5%)	3 (12.5%)	0.280*	4 (16.67%)	20 (83.33%)	0.559	22 (91.67%)	2 (8.33%)	
IV	1 (100%)	0 (0%)		0 (0%)	1 (100%)		1 (100%)	0 (0%)	
Tumor stage									
I	6 (66.67%)	3 (33.33%)		1 (11.11%)	8 (88.89%)		6 (66.67%)	3 (33.33%)	
II	23 (92%)	2 (8%)	0.186	1 (4%)	24 (96%)	0.158	20 (80%)	5 (20%)	0.611
III	26 (83.87%)	5 (16.13%)	0.978	6 (19.35%)	25 (80.65%)	0.08	26 (83.87%)	5 (16.13%)	0.248*
IV	1 (50%)	1 (50%)		1 (50%)	1 (50%)		2 (100%)	0 (0%)	
Infiltration of Gleason capsule									
Yes	28 (87.5%)	4 (12.5%)	0.407	7 (21.88%)	25 (78.13%)	0.052	26 (81.25%)	6 (18.75%)	0.897
No	28 (80%)	7 (20%)		2 (5.71%)	33 (94.29%)		28 (80%)	7 (20%)	
Infiltration of vessels									
Yes	29 (87.88%)	4 (12.12%)	0.349	5 (15.15%)	28 (84.85%)	0.684	27 (81.82%)	6 (18.18%)	0.803
No	27 (79.41%)	7 (20.59%)		4 (11.76%)	30 (88.24%)		27 (79.41%)	7 (20.59%)	
Coexistent hepatitis									
Yes	31 (88.57%)	4 (11.43%)	0.249	3 (8.57%)	32 (91.43%)	0.222	28 (80%)	7 (20%)	0.897
No	25 (78.12%)	7 (21.875%)		6 (18.75%)	26 (81.25%)		26 (81.25%)	6 (18.75%)	
Coexistent cirrhosis									
Yes	16 (84.21%)	3 (15.79%)	0.930	2 (10.53%)	17 (89.47%)	0.660	14 (73.68%)	5 (26.32%)	0.368
No	40 (83.33%)	8 (16.67%)		7 (14.58%)	41 (85.42%)		40 (83.33%)	8 (16.67%)	

*Jonckheere-Terpstra test.

	Claudin-5		*p*-Value	Claudin-7		*p*-Value
	Low	High		Low	High	
Gender						
M	21 (38.89%)	33 (61.11%)	0.586	38 (70.37%)	16 (29.63%)	0.537
F	4 (30.77%)	9 (69.23%)		8 (61.54%)	5 (38.46%)	
Size						
≤5 cm	14 (35%)	26 (65%)	0.634	28 (70%)	12 (30%)	0.773
>5 cm	11 (40.7%)	16 (59.3%)		18 (66.7%)	9 (33.3%)	
Number of tumor nodules						
Single	24 (38.71%)	38 (61.29%)	0.405	44 (70.97%)	18 (29.03%)	0.151
Multiple	1 (20%)	4 (80%)		2 (40%)	3 (60%)	
Tumor grade						
I		13 (61.9%)		14 (66.67%)	7 (33.33%)	
II	10 (47.62%)	11 (52.38%)	0.241	13 (61.9%)	8 (38.1%)	0.708
III	6 (25%)	18 (75%)	*0.510	18 (75%)	6 (25%)	*0.440
IV	1 (100%)	0 (0%)		1 (100%)	0 (0%)	

Table III. *continued*

Table III. *continued*

	Claudin-5		*p*-Value	Claudin-7		*p*-Value
	Low	High		Low	High	
Tumor stage						
I	3 (33.33%)	6 (66.67%)		5 (55.56%)	4 (44.44%)	
II	11 (44%)	14 (56%)	0.621	19 (76%)	6 (24%)	0.641
III	11 (35.48%)	20 (64.52%)	*0.545	21 (67.74%)	10 (32.26%)	*0.947
IV	0 (0%)	2 (100%)		1 (50%)	1 (50%)	
Infiltration of Gleason capsule						
Yes	15 (46.88%)	17 (53.13%)	0.121	21 (65.63%)	11 (34.38%)	0.608
No	10 (28.57%)	25 (71.43%)		25 (71.43%)	10 (28.57%)	
Infiltration of vessels						
Yes	14 (42.42%)	19 (57.58%)	0.394	23 (69.7%)	10 (30.3%)	0.856
No	11 (32.35%)	23 (67.65%)		23 (67.65%)	11 (32.35%)	
Co-existent hepatitis						
Yes	12 (34.29%)	23 (65.71%)	0.592	25 (71.43%)	10 (28.57%)	0.609
No	13 (40.625%)	19 (59.375%)		21 (65.625%)	11 (34.375%)	
Co-existent cirrhosis						
Yes	5 (26.32%)	14 (73.68%)	0.241	12 (63.16%)	7 (36.84%)	0.541
No	20 (41.67%)	28 (58.33%)		34 (70.83%)	14 (29.17%)	

*Jonckheere-Terpstra test.

statistical significance between claudin-1 expression and tumor size (p=0.08), revealing a tendency of small tumors to overexpress claudin-1 protein. Furthermore, Jonckheere-Terpstra test showed a borderline significant statistical association between increased expression of claudin-1 and low-stage tumors (I and II); in contrast, claudin-1 tended to be downregulated in high-stage tumors (p=0.08). The Jonckheere-Terpstra test also showed a statistically significant association between a low claudin-4 level and high tumor grade (p=0.03), indicating that down-regulation claudin-4 is associated with poorly-differentiated tumors. No other statistical correlation was recorded between clinicopathological features and expression of tight junction proteins.

Survival analysis. Table IV shows the results of univariate Cox regression survival analysis. Thus, increased overall survival was found to be significantly associated with increased claudin-1 level (p=0.049), female gender (p=0.011), low tumor grade (p<0.0001), single nodule existence (p=0.032), tumor size ≤5 cm (p=0.002), low tumor stage (p<0.0001), no infiltration of Gleason capsule (p=0.015) and no co-existent cirrhosis (p=0.002). Furthermore, increased disease-free survival was significantly associated with a high level of claudin-4 expression (p=0.038), female gender (p=0.038), low tumor grade (p=0.001), single nodule presence (p=0.016), low tumor stage (p=0.019) and no cirrhosis (p<0.0001).

Table V shows the results of multivariate Cox regression survival analysis. Overexpression of claudin-4 (p=0.01) and claudin-5 (p=0.015), down-regulation of claudin-7 (p=0.009), low tumor grade (p<0.0001), small tumor size (p=0.031), low tumor stage (p=0.004), hepatitis (p=0.008) and absence of cirrhosis (p=0.008) were found to be independent prognostic factors for increased overall survival and good prognosis. Furthermore, decreased claudin-4 expression (p=0.013) and presence of cirrhosis (p=0.019) were independent prognostic factors for increased disease recurrence.

Figures 8 and 9 display Kaplan–Meier survival plots. Log-rank test showed increased overall survival to be significantly associated with high levels of claudin-1 expression (p=0.04), female gender (p=0.007), low tumor grade (p<0.0001), presence of single tumor nodule (p=0.025), tumor size ≤5 cm (p=0.001), low tumor stage (p<0.0001), no infiltration of Gleason capsule (p=0.013) and no underlying cirrhosis (p=0.002). The effect of correlation of high levels of claudin-4 and increased survival duration was of limited statistical significance (p=0.052). Furthermore, increased disease-free survival was significantly associated with a high claudin-4 level (p=0.013), female gender (p=0.013), low tumor grade (p=0.001), single tumor nodule (p=0.008), low tumor stage (p=0.017) and no co-existent cirrhosis (p<0.0001).

Discussion

The building blocks of tight junctions, namely occludin and numerous forms of claudins, serve tissue-specific and cell type-specific needs, combining strict structural requirements and the need for quick and versatile adaptive requirements.

Table IV. *Univariate analysis of overall and disease-free survival in all 67 cases with hepatocellular carcinoma included in the study.*

	Overall survival rate (%) Mean±SD HR (95% CI)	*p*-Value	Disease-free survival rate (%) Mean±SD HR (95% CI)	*p*-Value
Age	HR=0.982 CI=0.947-1.019	0.334	HR=0.961 CI=0.920-1.003	0.07
Gender				
M	Mean±SD=25.67±19.61		Mean±SD=19.79±17.33	
F	Mean±SD=71.58±74.85		Mean±SD=61.88±64.96	
	HR=0.237 CI=0.079-0.718	0.011	HR=0.118 CI=0.016-0.886	0.038
Tumor grade				
I	Mean±SD=55.78±62.2		Mean±SD=48.15±54.25	
II	Mean±SD=30.2±23.87		Mean±SD=24.17±21.57	
III	Mean±SD=20.35±16.25		Mean±SD=13.83±11.93	
IV	Mean±SD=22.8±0.2		Mean±SD=22.8±0	
HR=3.091 CI=1.956-4.884		<0.0001	HR=2.319 CI=1.406-3.824	0.001
Number of tumor nodules				
Single	Mean±SD=35.76±41.77		Mean±SD=29.55±36.87	
Multiple	Mean±SD=19.95±23.09	0.032	Mean±SD=8.17±4.26	0.016
	HR=2.850 CI=1.095-7.420		HR=4.847 CI=1.344-17.483	
Size of tumor nodules				
≤5 cm	Mean±SD=43.42±48.03		Mean±SD=34.99±42.23	
>5 cm	Mean±SD=21.48±21.42	0.002	Mean±SD=17.53±20.24	0.126
	HR=2.844 CI=1.454-5.561		HR=1.912 CI=0.833-4.389	
Tumor stage				
I	Mean±SD=66.81±68.55		Mean±SD=61.53±70.91	
II	Mean±SD=37.57±42.93		Mean±SD=28.37±28.09	
III	Mean±SD=23.07±20.61		Mean±SD=17.71±19.25	
IV	Mean±SD=30.52±37.59		Mean±SD=30.52±37.59	
	HR=2.418 CI=1.540-3.797	<0.0001	HR=1.877 CI=1.107-3.181	0.019
Infiltration of Gleason capsule				
Yes	Mean±SD=22.47±23.5		Mean±SD=17.47±18.23	
No	Mean±SD=45.65±49.59	0.015	Mean±SD=37.55±44.75	0.302
	HR=2.308 CI=1.176-4.530		HR=1.536 CI=0.680-3.470	
Infiltration of vessels				
Yes	Mean±SD=27.16±36.44		Mean±SD=22.01±26.19	
No	Mean±SD=41.78±43.91	0.340	Mean±SD=33.73±42.95	0.942
	HR=1.374 CI=0.715-2.641		HR=0.970 CI=0.434-2.169	
Co-existent hepatitis				
Yes	Mean±SD=38.13±37.85		Mean±SD=29.55±29.48	
No	Mean±SD=30.69±44.01	0.454	Mean±SD=26.21±42.27	*p*=0.564
	HR=0.780 CI=0.406-1.496		HR=1.273 CI=0.561-2.889	
Co-existent cirrhosis				
Yes	Mean±SD=17.88±17.14		Mean±SD=10.69±10.36	
No	Mean±SD=41.19±45.43	0.002	Mean±SD=34.8±40.02	<0.0001
	HR=2.979 CI=1.470-6.035		HR=4.777 CI=1.990-11.465	
Occludin				
High	Mean±SD=52.78±68.99		Mean±SD=51.06±69.55	
Low	Mean±SD=31±32.4	0.125	Mean±SD=23.42±23.24	0.136
	HR=2.285 CI=0.796-6561		HR=3.071 CI=0.703-13.410	
Claudin-1				
High	Mean±SD:37.68±42.73		Mean±SD:30.27±37.75	
Low	Mean±SD:14.62±13.73	0.049	Mean±SD:13.03±14	0.890
	HR=2.687 CI=1.006-7.180		HR=1.108 CI=0.258-4.758	
Claudin-4				
High	Mean±SD:43.64±58.78		Mean±SD:43.19±58.96	
Low	Mean±SD:32.4±35.53	0.06	Mean±SD:24.29±27.36	0.038
	HR=3.044 CI=0.933-9927		HR=8.337 CI=1.120-62.051	
Claudin-5				
High	Mean±SD:35.04±36.08		Mean±SD:26.5±26.43	
Low	Mean±SD:33.81±48.43	0.574	Mean±SD:30.4±48.41	0.241
	HR=1.212 CI=0.620-2372		HR=0.576 CI=0.229-1.449	
Claudin-7				
High	Mean±SD:32.06±49.37		Mean±SD:29.94±48.9	
Low	Mean±SD:35.73±36.74	0.461	Mean±SD:27.05±28.74	0.283
	HR=0.771 CI=0.386-1.540		HR=1.715 CI=0.640-4.595	

CI: Confidence interval; HR: Hazard ratio; SD: standard deviation.

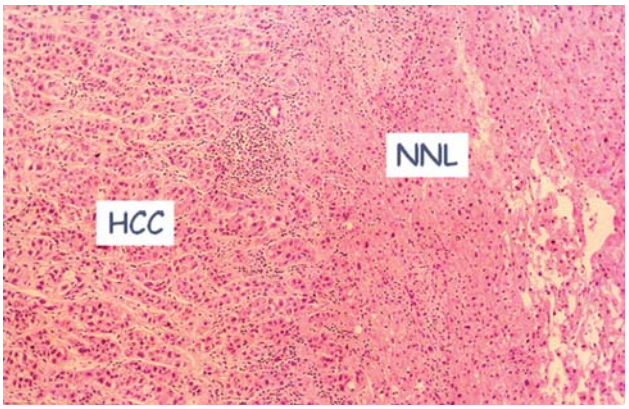

Figure 1. *Photomicrograph from a section of hepatocellular carcinoma (HCC) case. NNL: Non-neoplastic liver (H&E staining, magnification ×250).*

Diverse combination ratios of these integral tight junction protein constituents determine the epithelial tissue barrier function, controlling the size and number of molecules passing through the paracellular route (1, 11-13). In malignant epithelial tissues, the loss, augmentation or altered cellular localization of tight junction proteins leads to disruption of tight junction integrity and increased influx of nutrients, growth factors, and other tumor-promoting molecules. This improved nutrient supply adds selection advantage for the development, survival and, in more advanced stages, invasion of malignant cells (14, 15). Moreover, loss of claudin expression results in loss of cell-to-cell adhesion, thereby preventing the cells from forming organized epithelial structures and leading to an undifferentiated phenotype. Therefore, claudins and occludin seem to play an important role in development of the malignant phenotype.

Previous studies regarding expression of tight junction proteins in HCC and normal liver tissues have shown that increased levels of claudin-10 are associated with poor survival and increased recurrence rates (3, 16-19), and that claudin-3 is highly expressed in hepatocellular carcinoma cells, as well as in colorectal liver metastases and brain metastases (19). *ZO-1* tight junction protein mRNA was significant down-regulated in HCCs in comparison to normal liver tissues (20).

The present study examined the expression profiles of tight junction proteins claudin 1, 4, 5, 7, and occludin in cases of HCC. We found there to be a statistically significant increased expression of the five proteins studied in HCC specimens compared to non-neoplastic liver tissues adjacent to the tumor. Furthermore, the expression in non-neoplastic liver tissues was significantly higher in comparison with that of the normal control group. Thus, we could speculate that there is up-regulation of these proteins in cases of HCC.

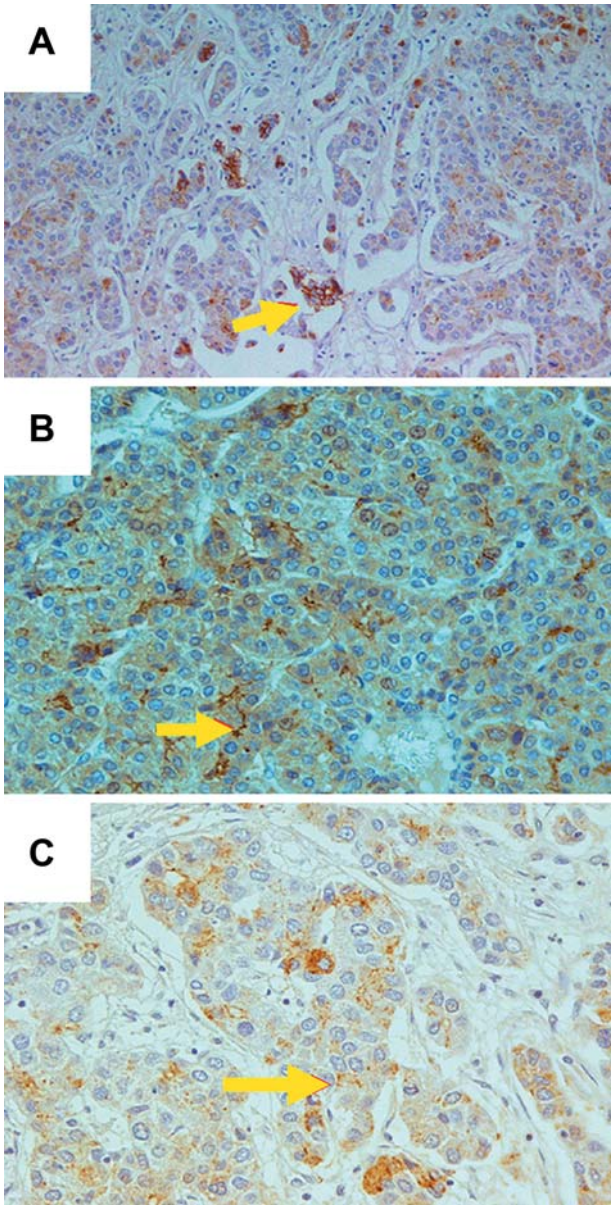

Figure 2. *Photomicrographs showing claudin-1 expression in a bile duct (membranous stain, yellow arrow) (A), in tumor cells (membranous stain, yellow arrow) (B) and in canaliculus (yellow arrow) (C). Streptavidin biotin peroxidase; A: magnification ×100 ; B, C: magnification ×250.*

The findings of the present study show that there is a slight statistical association between high claudin-1 expression and low tumor stage, presence of single tumor nodule and small size tumors (≤5 cm). In addition, according to Kaplan–Meier plot and Cox regression univariate analysis, increased levels of claudin-1 were associated with better prognosis and higher overall survival. These results are in accordance with previous studies showing that low levels of claudin-1 are associated

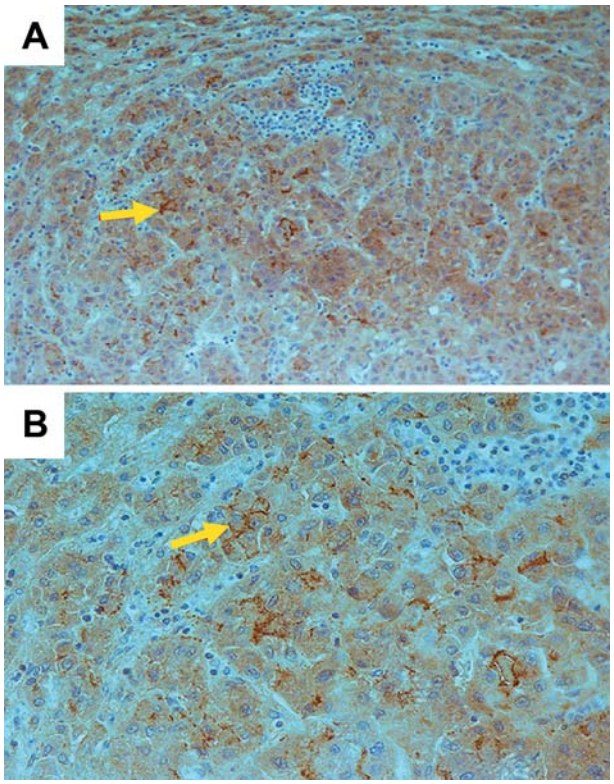

Figure 3. *Photomicrographs showing claudin-4 expression in tumor cells (membranous stain, yellow arrow). Streptavidin biotin peroxidase; A: magnification ×100 and B: magnification ×250).*

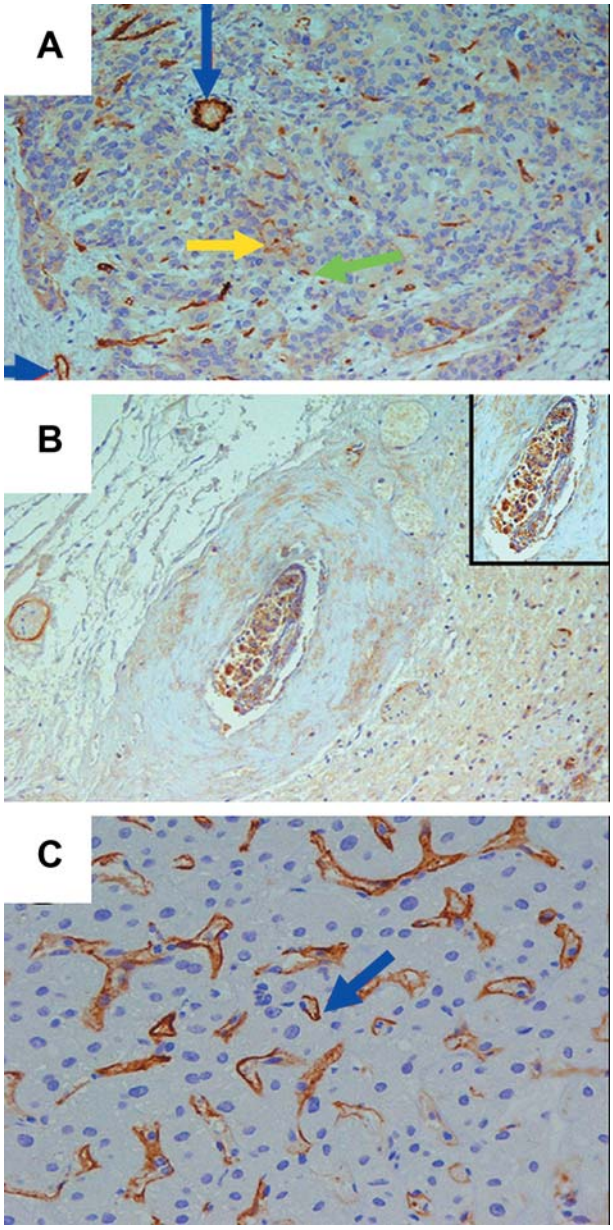

Figure 4. *A: Photomicrograph showing claudin-5 expression in vessels (blue arrows), tumor cells (membranous stain-yellow arrow) and canaliculus (internal positive control, green arrow). Streptavidin, biotin peroxidase; ×100. B: Photomicrograph showing neoplastic vascular embolus with membranous expression of claudin-5. Streptavidin-biotin peroxidase; ×250, (inset ×400). C: Photomicrograph showing claudin-5 expression in canaliculus (internal positive control, blue arrow). Streptavidin-biotin peroxidase, ×400.*

with lower survival rates after hepatectomy (4). Thus, increased claudin-1 expression in HCC may well serve as a potential marker for good prognosis.

Regarding claudin-4, previous studies have shown that claudin-4 levels are generally low, through immunochemistry, in HCC in contrast to colorectal liver metastases (21-23). In the present study we found that decreased expression of claudin-4 was associated with high-grade tumors (grade III and IV). This means that there is a down-regulation of the molecule with increasing de-differentiation of the tumor. Moreover, we found that the level of claudin-4 is an independent prognostic factor for survival prognosis in multivariate analysis and a decrease of claudin-4 is associated with increased recurrence rates and low disease-free survival rates in univariate analysis, Kaplan–Meier plot and multivariate analysis. Thus, we may speculate that claudin-4 expression may also be useful as a prognostic marker for overall survival and recurrence rates.

As for claudin-5 expression, previous studies have shown that it is decreased in poorly differentiated HCCs compared to well- or moderately-differentiated ones (5). It is detected in vascular endothelial cells (14) and in the normal liver, is expressed in sinusoidal endothelial cells, being down-regulated in conjunction with the increase of hepatitis or fibrotic change (5). The present study showed that claudin-5 is expressed in a linear fashion in tumor cells and its up-regulation is an independent prognostic factor for increased overall survival. These results are in accordance with a

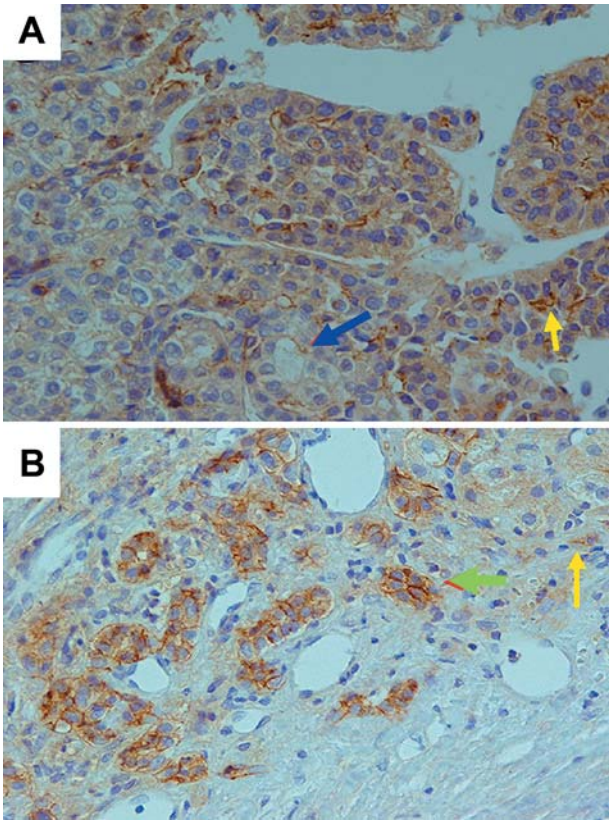

Figure 5. *Photomicrographs showing claudin-7 expression in tumor cells (membranous stain, blue arrow) and in canaliculus (yellow arrow) (A) and in bile duct (membranous stain-green arrow) and in canaliculus (yellow arrow) (B). Streptavidin-biotin peroxidase; magnification ×250.*

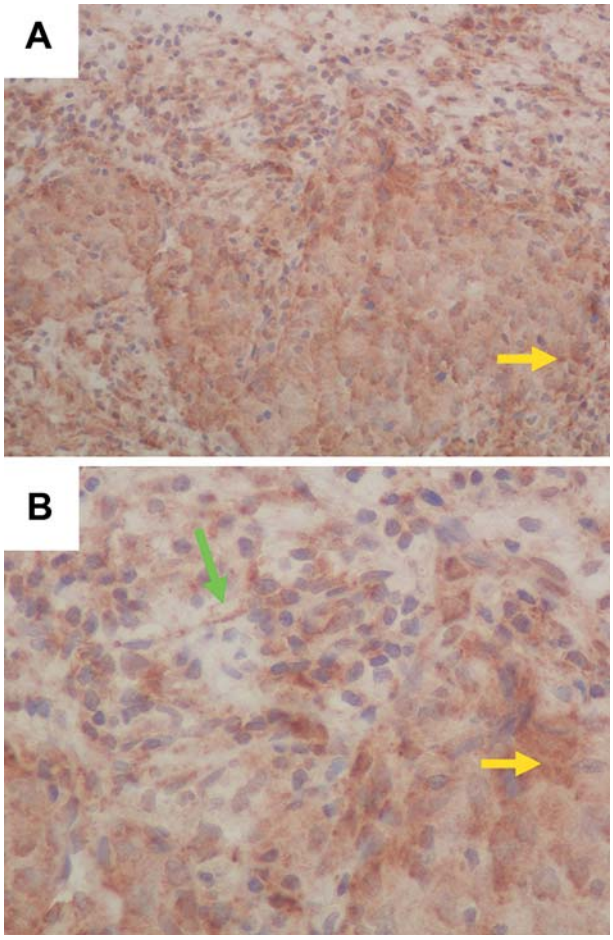

Figure 6. *Photomicrographs showing occludin expression in a dot-like fashion in the cytoplasm (yellow arrow) and membrane (green arrow) of tumor cells. Streptavidin-biotin peroxidase; A: magnification ×250, B: magnification×400.*

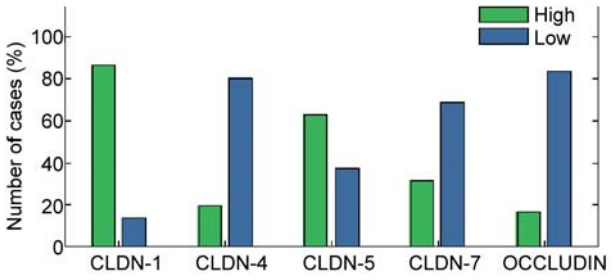

Figure 7. *Expression of claudins (CLDN) 1, 4, 5 and 7 and occludin according to low and high intensity scale.*

previous study where down-regulation of claudin-5 was associated with poor overall survival (5).

Lodi *et al*., considered claudin-7 as a possible marker for hepatic stem cells since claudin-7 expression is significantly strong in regenerating liver (24). In this study, multivariate analysis showed that down-regulation of claudin-7 is an independent prognostic factor for increased overall survival. This might be explained by the fact that claudin-7 is a marker of undifferentiated stem cells, which means that decreased expression of claudin-7 is associated with good prognosis because hepatic tumor cells might be well differentiated, not having similarities to undifferentiated multipotent stem cells. However, our study showed no correlation between claudin-7 levels and tumor grade. These results are in contrast with those of a previous study which showed that there was a trend towards a better survival among patients with overexpression of claudin-7 compared to those with down-regulation of claudin-7 in tumor tissues (25).

In this study, in the majority of the cases, occludin expression was localized only in the cytoplasm of the tumor cells, in contrast to the claudins studied, which exhibited a more variable staining pattern varying from membranous to cytoplasmic pattern or both. We may assume that this is due to loss of classic molecular structure of occludin proteins with subsequent loss of function and intracellular localization. Tzelepi *et al*. (15), in their study of expression

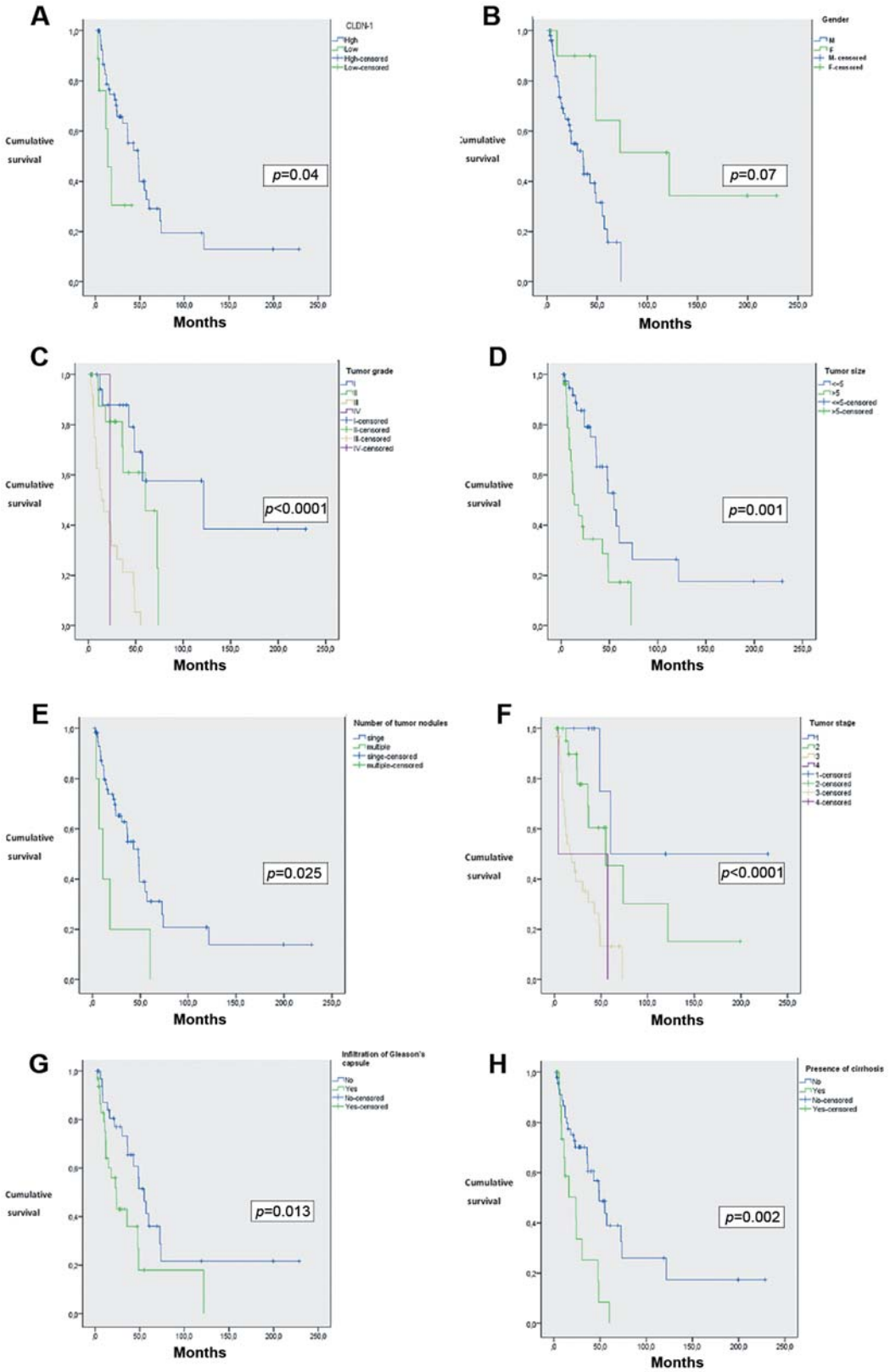

Figure 8. *Kaplan-Meier plot of overall survival according to claudin-1 level (A), gender (B), tumor grade (C), tumor size (D), number of tumor nodules (E), tumor stage (F), presence of Gleason capsule infiltration (G) and presence of cirrhosis (H).*

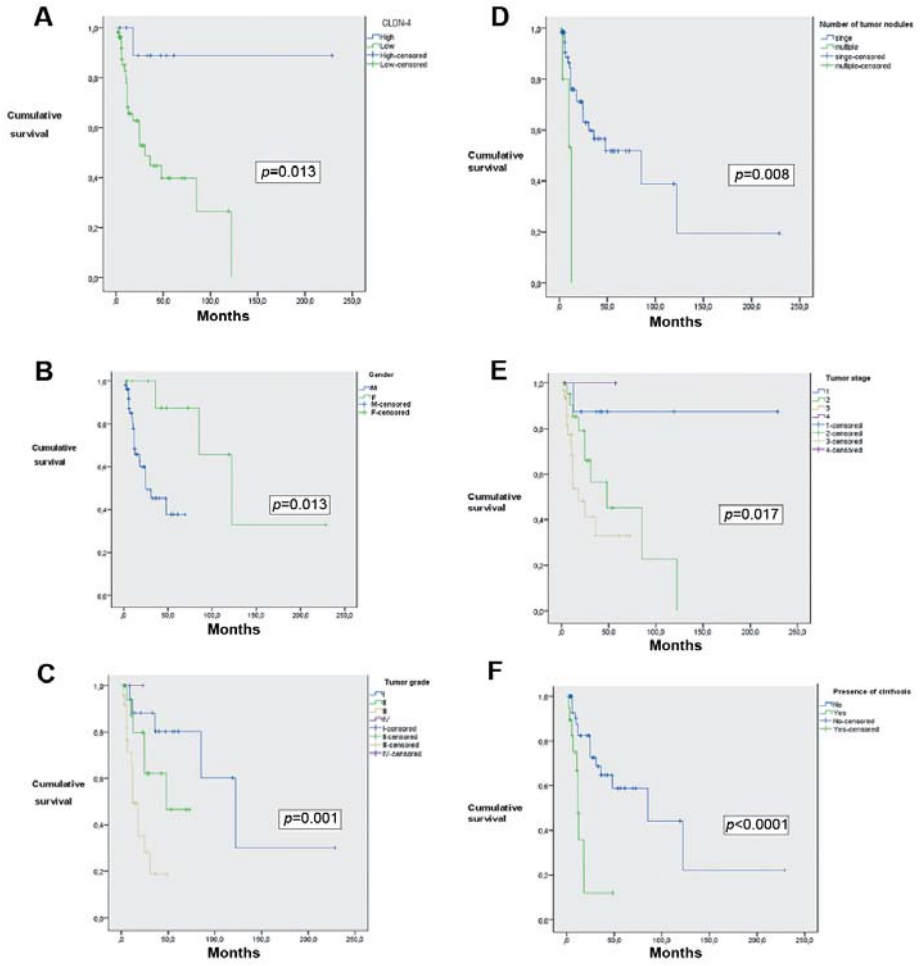

Figure 9. *Kaplan Mayer disease-free survival plot. Association between: A: Claudin-4 levels and disease free survival. B: Gender and disease free survival. C: Tumor grade and disease free survival. D: Kaplan Mayer disease free survival plot. Number of tumor nodules and disease free survival. E: tumor stage and disease free survival. F: Presence of cirrhosis and disease-free survival.*

Table V. *Multivariate analysis of overall survival and disease-free survival in all 67 cases with hepatocellular carcinoma included in the study.*

	Overall survival rate (%) HR (95% CI)	*p*-Value	Disease-free survival rate (%) HR (95% CI)	*p*-Value
Age	1.030 (0.971-1.093)	0.327	0.935 (0.863-1.013)	0.102
Gender (Male→Female)	0.634 (0.095-4.243)	0.638	0.141 (0.013-1.520	0.106
Tumor grade (I→IV)	3.969 (1.852-8.505)	0.000	1.321 (0.628-2.777)	0.463
Number of nodules (Single→Multiple)	6.188 (0.524-73.025)	0.148	3.305 (0.238-45.932)	0.373
Tumor size (≤5→>5cm)	4.870 (1.159-20.458)	0.031	3.073 (0.561-16.818)	0.196
Tumor stage (I→IV)	3.668 (1.520-8.849)	0.004	1.680 (0.728-3.881)	0.224
Infiltration of Gleason capsule (Yes→No)	2.432 (0.591-10.012)	0.218	1.353 (0.326-5.613)	0.677
Infiltration of vessels (Yes→No)	0.607 (0.187-1.966)	0.405	0.742 (0.192-2.864)	0.665
Co-existent hepatitis (Yes→No)	0.150 (0.037-0.606)	0.008	1.167 (0.320-4.253)	0.815
Co-existent cirrhosis (Yes→No)	5.806 (1.589-21.217)	0.008	5.925 (1.345-26.093)	0.019
Occludin(High→Low)	1.999 (0.547-7.298)	0.295	0.791 (0.120-5.226)	0.808
Claudin-1 (High→Low)	0.158 (0.024-1.047)	0.056	0.889 (0.116-6.817)	0.910
Claudin-4 (High→Low)	7.473 (1.614-34.606)	0.010	19.162 (1.871-196.226)	0.013
Claudin-5 (High→Low)	5.833 (1.416-24.023)	0.015	1.710 (0.393-7.440)	0.474
Claudin-7 (High→Low)	0.206 (0.063-0.674)	0.009	0.846 (0.206-3.467)	0.816

CI: Confidence interval; HR: hazard ratio; SD: standard deviation.

of tight junction proteins in thyroid neoplasms described the same localization of occludin expression mainly in poorly-differentiated thyroid carcinomas, implying that these intracellularly-stained vesicles are non-functioning occludin stereotypes lost from tight junction proteins. In the present study, occludin expression was not associated with overall survival and disease-free periods.

In conclusion, our study demonstrates that up-regulation of claudins 1, 4, and 5, and down-regulation of claudin-7 are positive prognostic markers associated with good outcome and high survival rates. Moreover, an increased level of claudin-4 may serve as an independent positive prognostic factor for low recurrence rates after hepatectomy. These markers could be used in clinical practice for prognosis models concerning patients with HCC.

Conflicts of Interest

The Authors have no conflict of interest to declare.

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