Novel Prognostic Biomarkers for HCC Progression in Egyptian Patients

Abstract

Objectives: To evaluate values of Cyclin D and Cdk4 in HCC, hepatitis diseases and healthy controls, their clinicoradiological correlations and prognosis of HCC.

Methods: Group 1:50 patients with HCC, Group 2: 50 patients with hepatitis diseases and Group 3: 30 healthy controls were enrolled. All patients were positive for hepatitis C virus (HCV) antibody and confirmed by HCV RNA. Calculation of Barcelona-Clinic Liver Cancer (BCLC) staging system, MELD and Child-Pugh scores. mRNA for cyclin D1 and Cdk4 were analyzed by quantitative RT-PCR.

Results: The mean Cyclin D1 and Cdk4 values were higher in HCC group compared with the other two groups (p value= 0.001). In HCC group, the mean Cdk4 and cyclin D1values were significantly higher among patients with multiple hepatic focal lesion (HFL) (p value= 0.0001, 0.003 respectively) compared with those with single lesion. A significant correlation between size of (HFL), alpha-Fetoprotein (AFP) and mean Cdk4 value (p value= 0.028, 0.0001 respectively).

Conclusion: Significant values of cyclin D1 and Cdk4 in HCC, compared to normal and hepatitis diseases, correlated to the number, size of HFL and AFP level. Thus, the assessment of cyclin D1 and Cdk4 may provide a novel strategy for prognostication and targeted therapy of HCC.

Keywords: Hepatocellular carcinoma; Cyclin D1; Cdk4

Research Article

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Abbreviations: HCC: Hepatocellular Carcinoma; BCLC: Barcelona Clinic Liver Cancer; CLIP: Cancer of the Liver Italian Program; GETCH: Groupe d'Etude Et De Traitement Du Carcinome Hepatocellulaire; JIS: Japan Integrated Staging

Introduction

Hepatocellular carcinoma (HCC) is ranked as the fifth most common cancer worldwide [1], and the third most common cause of cancer related death worldwide [2]. Prognosis and survival estimation for HCC patients is a challenging issue not only because the extent of liver dysfunction has a key impact on survival more than the tumor itself but also due to the unique geographic characteristics of the disease. Therefore, over the years multidimensional staging systems including both the extension of tumor and liver function parameters (with general health variables included) have been developed: Okuda[3], Barcelona Clinic Liver Cancer (BCLC) [4], Cancer of the Liver Italian Program (CLIP) [5], Groupe d'Etude et de Traitement du Carcinome Hepatocellulaire (GETCH) [6] and Japan Integrated Staging (JIS) [7].

Cyclin D1 and its catalytic partner CDK4 are known to play an important role in the G1/S checkpoint of the cell cycle. The activity of CDKs is negatively regulated by the cyclin-dependent kinase inhibitors (CDKIs) p16 and p27 which avert excessive cell replication [8-10]. CDK4 and cyclin D1 complex has been strongly implicated in the control of cell proliferation and prognoses in human malignancies. Overexpression of CDK4 and cyclin D1 has been reported in many cancers. Correlations between cyclin D1 overexpression and the prognosis of colorectal cancer [11,12], cell cancers of the urinary bladder [13] varian cancers [14], breast cancer [15], non-small cell lung cancer [16,17], and oesophageal cancer [18] have been reported. Moreover, an association between cyclin D1 status and the recurrence of head and neck squamous cell carcinoma [19-21], and survival has also been reported [22-26].

Since malignant transformations may occur as a result of alterations in the genes that directly control the cell cycle and mitosis, identification of new useful prognostic molecular markers (cell-cycle regulators) for HCC is required to improve the accuracy of predicting tumor behavior. The aims of this study is to determine the serum level of Cyclin D1 and cdk4 in HCC patients and in benign liver conditions, to correlate them with some clinical and radiological HCC criteria and to investigate the prognostic importance of these markers by the potential correlation between them and the BCLC prognostic system.

Patients and Methods

This is a prospective cross sectional study including three age-matched groups; Group 1: 50 with HCC, Group 2: 50 patients with liver diseases (25 liver cirrhosis and 25 chronic hepatitis C). Patients were randomly recruited from the inpatient section and outpatient clinic of tropical medicine, gastroenterology and internal medicine departments, Assiut University. Group 3: included 30 healthy controls (volunteers).

Histological and/or AASLD radiological criteria [27] or the diagnosis of HCC was used for inclusion of HCC patients. Patients who had undergone any interventional treatment for HCC were excluded. The diagnosis of chronic hepatitis C was based on standard serological assay results (positive HCV antibody by the third generation enzyme-linked immuno sorbent assay, with infection confirmed by detection of circulating HCV RNA by polymerase chain reaction using the Amplicor HCV assay) and abnormal serum aminotransferase level for at least 6 months. The diagnosis of liver cirrhosis was made dependent on typical clinical signs, laboratory and on unequivocal radiological signs.

All baseline clinical and tumor parameters necessary to calculate the BCLC staging system were obtained

Cyclin D1 and Cdk4 mRNA analysis by quantitative real-time RT-PCR

Total RNA isolation: Total RNA was isolated with RNA easy Mini Kit (Qiagen) and further analyzed for quantity and quality with Beckman dual spectrophotometer (USA). The RNA integrity and the GAPDH- RNA (house keeping gene) ratio were used as the quality control.

Quantitative Reverse transcriptase PCR (QRT–PCR): The mRNA expression level was quantified by qRT–PCR (Real time PCR). 1000 ng of the total RNA from each sample were used for cDNA synthesis by reverse transcription using High capacity cDNA Reverse Transcriptase kit (Applied Biosystem, USA). The cDNA was subsequently amplified with the Syber Green I PCR Master Kit (Fermentas) in a 48-well plate using the Step One instrument (Applied Biosystem, USA) as follows: 10 minutes at 95 ˚C for enzyme activation followed by 40 cycles of 15 seconds at 95 ˚C, 20 seconds at 55 ˚C and 30 second at 72 ˚C for the amplification step. Changes in the expression of each target gene were normalized relative to the mean critical threshold (CT) values of GAPDH housekeeping gene by the ΔΔCt method. We used 1 μM of both primers specific for each target gene. Primers sequence and annealing temperature specific for each gene demonstrated in Table 1.

Table 1: Primers sequence and annealing temperature specific for each gene.

Target Gene	Primer Sequence: 5`- 3`	Gene Bank Accession Number
Cyclin D1	Forward: CCTCCTCTCCGGAGCATTT Reverse: CTGTAGCACAACCCTCCTCC	XM_006718653.1
CDK4	Forward: TCCAGTGCGTGCAAAAGGA Reverse: CCGTCCGGGCCTTACCT	XM_011531810.1
GAPDH	Forward: CCTCTACTGGCGCTGCCAAGGCT Reverse: GTCCACCACTGACACGTTGG	NT_009759.16

Statistical Analysis

Patients' data were analyzed using SPSS 17.0 for windows 7 (SPSS Inc, Chicago, IL, USA). Quantitative variables were expressed by mean and SD (Standard deviation) and the Qualitative variables were expressed by numbers (Frequency) and percent. Students T test and ANOVA Test were used to compare numerical variable. Pearson and Spearman's correlation were used to assess the correlation between each of CDK4 and cyclin D1 and other studied variables. Univariate and multivariate linear regression were used to investigate possible associations between each of Cdk4 and cyclin D1 and other studied variable. P value was considered to be significant if < 0.05.

Ethical Considerations

The study steps were explained to all participants and an informed consent was obtained. Patients' data was considered confidential. The research was conducted only by scientifically qualified and trained personnel. The project was submitted to and approved by the ethical committee of the faculty of medicine, Assuit University.

Results

The three studied groups;

Group 1: included 41(82%) males and 9(18%) females with mean ± SD age of 59.1±7.7 and

Group 2: included 38(76%) males and 12(24%) females with mean ± SD age of 57.3± 5.7and

Group 3: included 24(80%) males and 6 (20%) females with mean ± SD age of 55.8± 6.3 with no statistical significant difference between them as regards both the age and sex.

The baseline demographic, clinical, and radiological data of HCC group are shown in Table 2. The mean± SD of AFP and MELD score were 153.2±131.7 IU/l and 13.4±4.9 respectively.

Table 2: Demographic, clinical and radiological data in HCC group.

		Mean	±SD / Frequency
Age		59.1	7.7
Sex	Male	41	83.7%
	Female	9	18%
Performance	0	37	75.5%
	1	10	20.4%
	2	3	6%
Portal HTN	Yes	40	81.6%
	No	10	20%
Encephalopathy	Yes	9	18%
	No	41	83.7%
Ascites	Yes	20	40%
	No	30	61.2%
PVT	Yes	13	26%
	No	37	75.5%
Type of PVT	Benign	2	16.7%
	Malignant	10	83.3%
Number of HFL	Single	27	55.1%
	Multiple	23	46%
Size of HFL	Small	12	24.5%
	Medium	13	26.5%
	Large	25	50%
BCLC Stage	Stage 0	7	14.3%
	Stage A	8	16.3%
	Stage B	13	26.5%
	Stage C	14	28.6%
	Stage D	8	16.3%
Child Grade	Child A	17	34.7%
	Child B	25	51.0%
	Child C	8	16.3%

Cyclin D1 and Cdk4 mean serum level results in the studied groups

a. In group 1, HCC patients, the mean cyclin D1value was 1.06± 0.35 and Cdk4 mean value was 2.63± 1.2.

b. In group 2: in chronic hepatitis C patients, the mean cyclin D1 value was 0.284± 0.042 and Cdk4 mean value was 0.945± 0.524. In patients with liver cirrhosis the mean cyclin D1 value was 0.547± 0.26 and Cdk4 mean value was1.186± 0.95 with statistical significance difference (p value= 0.001).

c. In group 3, the control group, the mean cyclin D1 value was 0.026± 0.014 and mean Cdk4 value was 0.513± 0.367. In the HCC group there was a direct correlation between cyclin D1 mean value and Cdk4 mean value however it was not significant (*r*: 0.063, *p* value= 0.666).

Relation between the demographic, clinical, radiological data and mean cyclin D1 and Cdk4 in HCC group (Table 3)

There was no significant difference as regard mean Cdk4 or cyclin D1 level between males and females patients (p value=0

.299, 0.937 respectively), different BCLC stage (p value=0 .162, 0 .928 respectively), different Child grade (p value= 0.186, 0.604 respectively) . While there was a highly significant difference as regards mean Cdk4 and cyclin D1 values among patients with multiple lesions compared to patients with a single lesion (mean Cdk4 3.3 ± 1.2 Vs 2.0 ± 0.9, p value= 0.0001) and (mean cyclin D1 1.2 ± 0.4 Vs 0.9 ± 0.19, p value= 0.003).

Correlation between demographic, clinical and radiological data and mean cyclin D1 and Cdk4 in HCC group (Table 4)

There was a direct significant correlation between the size of HFL, alpha-Fetoprotein and Cdk4 level (p value= 0.028 and 0.0001 respectively); otherwise no significant correlation was detected.

Univariate analysis of factors affecting mean cyclin D1 and cdk4 in HCC group (Table 5)

Univariate analysis showed that the number of HFL was the significant factor affecting Cyclin D1 and Cdk4 levels, while alpha-Fetoprotein, BCLC Stage3 were significant factors affecting Cdk4 level.

Multivariate analysis of factors affecting mean cyclin D1 and Cdk4 in HCC group (Table 6)

After adjustment to other variables, multivariate analysis showed that the number of HFL was the significant factor affecting cyclin D1 and Cdk4 levels while alpha-Fetoprotein, and large size of HFL were significant factors affecting Cdk4 level.

Table 3: Relation between some clinical and radiological data and CDK4 and cyclin D1 in HCC group.

		CDK4		*p* value	Cyclin D1		*p* value
		Mean	±SD		Mean	±SD	
Sex	Male	2.54	1.20	.299*	1.06	.35	.937*
	Female	3.04	1.34		1.07	.38	
BCLC	Stage 0	1.85	.51	.162**	1.00	.18	.928**
	Stage A	2.32	.99		1.01	.21	
	Stage B	2.96	1.30		1.06	.41	
	Stage C	3.04	1.43		1.13	.43	
	Stage D	2.29	1.03		1.08	.40	
Child Grade	Child A	2.30	1.05	.186**	1.00	.17	.604**
	Child B	2.94	1.33		1.11	.43	
	Child C	2.29	1.03		1.08	.40	
Number of HFL	Single	2.07	.91	.0001*	.93	.19	.003*
	Multiple	3.31	1.22		1.22	.44	
Size of HFL	Small	2.12	.90	.145**	1.00	.17	.338**
	Medium	2.50	1.28		1.19	.45	
	Large	2.95	1.27		1.03	.36	

*student test , **ANOVA

Table 4: Correlations between some clinical, radiological and laboratory data and CDK4 and cyclin D1 in HCC group.

		CDK4	Cyclin D1
Age	Correlation coefficient*	.157	-.208
	P	.280	.152
Size of HFL	Correlation coefficient**	**.314**	-.088
	P	**.028**	.547
BCLC Stage	Correlation coefficient**	.179	.040
	P	.218	.782
Child Grade	Correlation coefficient**	.063	.040
	P	.669	.783
Alpha-Fetoprotein	Correlation coefficient**	**.639**	.022
	P	**.0001**	.882
MELD Score	Correlation coefficient*	-.113	.157
	P	.440	.282

*Pearson correlation, **Spearman's correlation

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Table 5: Univariate analysis of factors affecting CDK4 and Cyclin D1.

	CDK4				Cyclin D1			
	Regression coefficient	P Value	95% CI for Regression Coefficients		Regression Coefficient	*P* Value	95% CI for Regression Coefficients	
Age	.025	.280	-.021	.071	-.010	.152	-.023	.004
Sex(female)	.494	.299	-.453	1.441	.011	.937	-.266	.288
alpha-Fetoprotein	.006	**.0001**	-1.602	3.891	.000	.574	-.001	.001
MELD Score	-.028	.440	.004	.008	.011	.282	-.009	.032
BCLC Stage*								
Stage1	.466	.451	-.769	1.701	.009	.963	-.372	.389
Stage2	1.111	.052	-.008	2.230	.064	.711	-.281	.408
Stage3	1.189	**.036**	.084	2.293	.129	.448	-.211	.470
Stage4	.433	.498	-.843	1.708	.085	.666	-.308	.478
Child Grade**								
Grade B	.636	.099	-.123	1.396	.112	.322	-.113	.338
Grade C	-.017	.975	-1.102	1.068	.087	.589	-.235	.409
Number of HFL (multiple)	1.241	**.0001**	.630	1.853	.290	**.003**	.102	.477
Size of HFL ***								
Medium	.382	.428	-.580	1.345	.189	.187	-.095	.472
Large	.828	.056	-.022	1.677	.032	.799	-.219	.282

*stage 0 reference, **Child A reference, ***small size is reference

Table 6: Multivariate analysis of factors affecting CDK4 and Cyclin D1.

	CDK4				Cyclin D1			
	Regression Coefficient	P value	95% CI for Regression Coefficients		Regression Coefficient	P Value	95% CI for Regression Coefficients	
Age	.005	.785	-.033	.043	-.011	.111	-.026	.003
Sex(female)	.002	.997	-.863	.867	-.075	.639	-.398	.247
alpha-etoprotein	**.006**	**.0001**	**.003**	**.009**	.001	.254	.000	.002
MELD Score	-.082	.056	-.165	.002	.002	.921	-.030	.033
BCLC Stage*								
Stage1	.552	.399	-.761	1.864	-.076	.752	-.557	.406
Stage2	1.043	.178	-.496	2.582	.041	.883	-.523	.605
Stage3	.850	.301	-.792	2.492	-.052	.861	-.651	.547
Stage4	.679	.581	-1.796	3.155	.467	.295	-.425	1.359
Child Grade**								
Grade B	.093	.824	-.751	.937	-.102	.513	-.417	.212
Grade C	1.212	.111	-.291	2.714	-.056	.842	-.616	.504
Number of HFL (multiple)	1.001	**.003**	.352	1.649	**.364**	**.004**	.122	.606
Size of HFL ***								
Medium	-.440	.400	-1.488	.608	.189	.333	-.202	.579
Large	**-1.340**	**.028**	**-2.530**	**-.150**	-.242	.275	-.686	.201

*stage 0 reference, **Child A reference, ***small size is reference

Discussion

Deregulation in cell cycle-related kinase activities, specifically cyclin D1 and cyclin-Cdk4/Cdk6 complexes, is one of the most significant alterations in cancer cells. Although there is an increasing evidence that perturbation of cell cycle regulation is one of the important factors contributing to cancer, there have been few studies showing the relationship between cyclins and human HCC [28,29], Here we investigated biochemical differences of mean cell cyclin D1 and Cdk4 mRNA values in controls, benign (chronic hepatitis C and liver cirrhosis) and malignant (HCC) liver diseases. We also investigated the relationship between these proteins and the clinicoradiological characteristics and BCLC staging of HCC.

In this study, cyclin D1 and Cdk4 mean serum levels were higher in HCC compared to healthy controls and benign liver diseases suggesting that an increase of cyclin D1/Cdk4 may be associated with the process of transition from hepatitis C–induced cirrhosis to HCC. These data appear to be consistent with the results of previous studies showing that cyclin D1 and cdk4 increased proportionally with the development of HCC, especially in the transition process from chronic hepatitis to HCC [30]. Moreover, overexpression of cyclin D1 and Cdk4 also has been also shown in human cancers other than HCC [11-19].

HCC usually develops on top of chronic liver diseases such as chronic hepatitis and liver cirrhosis in which the hepatocytes are persistently regenerating following hepatic injury. This process helps to select cell clones with growth advantage that harbor disorder of cell cycle genes resulting in hepatocarcinogenesis.

However, this contradicts reports by Shian-Yang Peng & colleagues [31] who showed that cyclin D1 is downregulated in HCC. Moreover, Zhang and his colleagues reported that cyclin D1 genotype frequencies were similar in HCCs and controls and were not associated with susceptibility to develop HCC probably owing to the small sample size (97 HCCs and 35 controls) [32].

In consistence with [33], our study showed that the high mean level of Cyclin D1 correlated with a high mean level of Cdk4 in HCC patients, supporting the notion that Cyclin D1 and Cdk4 form a complex to promote HCC proliferation. Moreover, the co-overexpression of both cyclin D1 and Cdk4 was also reported in 65% liver tumors other than HCC [34]. Contradicting reports by Lu and his team demonstrated that the mRNA level of Cdk4 was up-regulated, while that of Cyclin D1 was down-regulated [35].

This study also revealed significantly higher mean Cdk4 and cyclin D1 levels among patients with multiple hepatic focal lesions compared to patients with single lesion. Thus, the enhancement of cyclin D1 and Cdk4 activities may play an important role in the growth of HCC. These cyclin and cyclin-dependent kinase (Cdk4) complexes are known to play a critical role in cell proliferation and differentiation [36].

In our study, direct significant correlations between the size of HFL, alpha-Fetoprotein level and Cdk4 value were detected which may reflect some prognostic importance. These findings were in concordance with other studies which reported high Cdk4 protein levels and kinase activities in poorly differentiated HCC [37] and tumor size and stage [35].

Univariate analysis of the level of cyclin D1 and Cdk4 with clinicoradiological parameters, and BCLC staging demonstrated that the high serum level of both correlates only with the multiple number of HFL (p =0.003 and 0.000 respectively) while alpha-Fetoprotein, BCLC Stage3 and large size HFL were significant factors correlating with Cdk4 level.

Finally, we want to emphasize that the activity of cell cycle–related kinases, namely cyclin D1 and Cdk4, were high in cirrhosis (preneoplastic stage) before the development of HCC, this activation appears to be an early event in hepatocarcinogenesis. These data suggest that hepatitis C virus-induced cirrhosis is a precancerous condition. In addition, the high serum levels of cyclin D1 and Cdk4 was shown to be related to the progression of HCC. Thus, the assessment of cyclin D1 and Cdk4 activities may provide a novel strategy for prognostication and targeted therapy of HCC.

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