Research Article

A Powerful Prognostic Tool Indicator for the Risk of Hepatocellular Carcinoma in a Cohort of Chronic Hepatitis-B Patients in Southern West-Bank: Genetic Variants and lamivudine Resistance

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ABSTRACT

Palestine is Hepatitis B moderate endemic country. Hepatitis B carrier stage ranges from 2-6%. Liver cancer incidence rates are 2.6 and 0.7 per 100,000 in males and females, respectively. Several genetic mutations within HB virus have been identified lately in Palestine. Annual liver ultrasonography for detection of HCC in addition to monthly Nucleos(t)ide therapy to all Hepatitis B patients is highly costly. Therefore, using a powerful prognostic tool for the risk of hepatocellular carcinoma is a crucial step. The objective of the study is for exploring available HCC risk prediction tools in order to determine the best available tool for our patients. To identify major risk factors associated with HCC among our patients. The study proceeded in 2 directions; first, we carried a retrospective review of all registered hepatitis patients` profiles in the 3 primary health care units in Hebron district, in order to identify Chronic Hepatitis-B patients who might be candidates for this study. Out of 750 profiles, 145 Chronic Hepatitis-B patients were identified and included in the study. Second, we searched all HCC risk estimation tools on line where we identified four well-validated and previously used tools; CU-HCC, REACH-B, PAGE-B and GAG. We applied 3 of these tools on our subjects to determine risk of HCC. Descriptive data analysis and multi-regression models were utilized in order to determine the major risk factors associated with HCC using SPSS version 20S. Predicted risk scores among subjects in this study were 5.6 ±9.75 (intermediate risk) and 4.8 ±5.6 (low risk) on CU-HCC and PAGE-B, respectively. REACH-B average risk score was 6.3 ± 3.4. The 3-, 5-, and 10-year risk of HCC among our patients, using REACH-B tool, were; (0.1 to 0.2%), (0.3 to 0.5%), and (0.7 to 1.2%), respectively. On REACH-B, it was found that HBeAg positive subjects had higher risk of HCC comparing to HBeAg negative subjects, 9.1 ± 2.5 vs. 5.6 ± 3.3, respectively. 43% of subjects in this study have high DNA load despite being on Lamivudine treatment for an average of 5.4 years. Multiple regression analyses indicated that PAGE-B was the most predictive tool for HCC risk in our patients. PAGE-B is a cost-effective prognostic indicator tool that precisely predicts HCC risk among hepatitis B patients. However, finding further prediction tools that relies on targeting ccc-DNA in kupfers cells would be a future better approach.

Keywords: HCC, CHB, Lamivudine, Viral DNA load, HBeAg, PAGE-B, REACH-B, CU-HCC.

INTRODUCTION

Palestine is categorized as HB moderate endemic area. HB carrier stage ranges from 2-6%. Transmission of infection occurs mainly by needle stick injury, surgery, dental procedures, and vertically from mother to child.[1-3] Hepatocellular Carcinoma (HCC) is the most devastating end stage liver disease associated with Chronic Hepatitis B (CHB) infection[4,5] among other severe complications [6-9] with heavy economic and clinical burden.

A set of lab tests have been used for diagnosis and/ or monitoring of prognosis of HB-Viral infection. They include Hepatitis-B surface antigen (HBs-Ag), Hepatitis-B Envelope antigen (HBe-Ag), HB-Viral DNA load (copies/ml), Alanine Amino-transferase (ALT), Platelets (PLT), Albumin, and Bilirubin (indirect and direct). In fact, neither of these tests can predict severity or time of prognosis of HCC[10-14]. Different combinations of these tests were incorporated into risk calculator tools in order to estimate risk of HCC in CHB patients. It is a heavy financial burden in most low income countries to issue Antiviral therapy (AV) and to do HCC surveillance for all CHB patients.[15,16] Only small proportion of CHB patients develop HCC. Therefore, it is important to determine who are at higher risk of developing HCC.[17]

Four risk estimation tools were identified on line for prediction of risk of HCC in CHB patients worldwide. These are; Risk estimation for HCC guided by age and gender (GAG), risk estimation for HCC in chronic hepatitis B patients (REACH-B), Chinese University-HCC (CU-HCC), and HCC based only on baseline patient's age, gender and PLT (PAGE-B). Ambiguous results were obtained when applying these tools to different ethnic groups [15, 18, 21].

New guidelines for management of CHB patients depend on the effect of the AV therapy, such as viral load determination, ALT reduction, and HBe-Ag seroconversion. These guidelines didn`t provide a clear insight into the actual HCC risk development in patients [20, 21]. In addition to that, it was found that inter-individual genetic polymorphism at the level of cytochrome P450 might play a major role in HCC risk development [30-35]. There has been a genetic shift of HB Virus in Palestine and the world. Two studies in Palestine [36, 37] found several variants of HB virus

and several genetic mutations within the same virus among studies subjects. A third study [38] showed that anti-HB titer started to decline by age 5 and 6 from 69.2% to 66.7%, then down to an average of 39.8% between ages 7 and 19. This should lead to review of diagnostic procedures and test, antiviral therapy, and vaccination protocols and follow up tests.

The aim of this study was to predict HCC risk in a cohort of Chronic Hepatitis B patients in Southern West Bank.

MATERIALS AND METHODS

A retrospective cohort study utilizing the descriptive analysis as well as multiple regression models were conducted to determine the best HCC risk estimation tool among these subjects. We reviewed retrospectively the records of 750 Hepatitis cases in Hebron district collected from patients` medical records at Ministry of Health (MOH) 4- Primary Governmental Health Care Directories. 145 active CHB cases were identified according to WHO criteria for active Chronic Hepatitis B cases which were adopted by the 4-directorates. Each individual whose medical record included all the necessary lab values indicated in the 3 risk estimation tools was included in the study. Any subject with liver carcinoma of any type, or liver precancerous conditions, or unknown liver pathology was excluded from the study. Our focus was on the isolated risk of HCC in subjects who acquainted Hepatitis-B. As such, patients with hepatic carcinoma of any kind or HCC, liver fibrosis, Hepatitis C or who didn't complete their Hepatitis B specific lab work for the past 6 months were excluded from the study.

Informed consent was taken from the ministry of health main office in Ramallah to conduct the study and to get an access to patients' profiles in the 3 governorate primary preventive health care units in Hebron district. The risk of developing HCC was calculated using CU-HCC and PAGE-B risk scores. Then we measured the year-specific risk of developing HCC in 3, 5, and 10 years using the REACH-B test. These tools were validated worldwide but not in Arab communities. In order to determine the most predictive tool among our subjects, three multiple regression analyses were estimated and t-test scores were calculated. The specification of the three regression models are as follows:

$$\text{PAGE-B}_i = \beta_0^1 + \beta_1^1 \text{Age}_i + \beta_2^1 Gen_i + \beta_3^1 \text{DNA}_i + \beta_4^1 \text{PLT}_i + u_i^1 \quad (1)$$

$$\text{REACH-B}_i = \alpha_0^2 + \alpha_1^2 \text{Age}_i + \alpha_2^2 Gen_i + \alpha_3^2 \text{DNA}_i + \alpha_4^2 \text{ALT}_i + \alpha_5^2 \text{HBe\_Ag}_i + u_i^2 \quad (2)$$

$$\text{CU-HCC}_i = \gamma_0^3 + \gamma_1^3 \text{Age}_i + \gamma_2^3 Gen_i + \gamma_3^3 \text{DNA}_i + \gamma_4^3 \text{ALB}_i + \gamma_5^3 BIL_i + \gamma_6^3 CIR_i + u_i^3 \quad (3)$$

The first regression model (1) is initially estimated using the age, gender, DNA and PLT variables. The second model in addition to the previous parameters, it also included ALT and HBeAg where it tested their effect on REACH-B. In addition to the first three variables included in the first regression, the third regression model includes Albumin (ALB), Bilirubin (BIL) and Cirrhosis (CIR) variables. Variable u represents the stochastic disturbance term and i is the ith observation. The results of the regression showed major risk factors in our cohort.

RESULTS

Baseline demographic and laboratory test results from October, 2015 to January, 2016 are shown in Table 1. Risk of developing HCC for study subjects was ranging from low to intermediate. Specifically, the average risk score ± standard deviation (SD) followed by estimated risk for the three risk tools were: 5.6 ± 9.75 for CU-HCC which corresponds to intermediate risk. REACH-B results were as follows: (6.3 ± 3.4), 3-year risk (0.1 to 0.2%), 5-year risk (0.3 to 0.5%), and 10-year risk (0.7 to 1.2%). For PAGE-B, results were; (4.8 ± 5.6) which correspond to low risk. The table also shows difference between HBe-Ag positive and HBe-Ag negative subjects in terms of HCC risk.

CU-HCC and PAGE-B risk scores were close to each other, 5.6 (9.75) and 4.8 (5.6), respectively, whereas REACH-B risk score was higher than both but closer to CU-HCC, 6.3 (3.4). When stratifying for HBeAg, it was found that HBeAg positive subjects had higher risk comparing to HBeAg negative, 9.1 ± 2.5 vs. 5.6 ± 3.3, respectively using REACH-B.

When comparing subjects in Nucleotide Analogous (NA) therapy group to subjects in non-NA therapy group, patients in second group showed better results in all 3 risk tools. Further analysis for data showed that 11% and 21% of our patients had high risk scores on CU-HCC and PAGE-B, respectively. Data in table (1) represents average risk scores, so we should interpret results carefully, as shown also in figures (1) and (2).

Further analysis of the three risk estimation tools revealed that PAGE-B precisely predicted risk of HCC among subjects in this study. Age, gender and platelet count were the most significant contributing factors. Results of multiple regression models are presented in table 3. As previously mentioned, the first regression shows the impact of age, gender, DNA load, and platelet count on PAGE-B. Results suggest a significant positive relationship between age and PAGE-B (0.466) and a negative relationship between platelet number and PAGE-B. In other words, the lower the platelet count, the higher the PAGE-B score and henceforth the higher the risk for HCC. The result showed a gender deviation among patients. PAGE-B risk scores for males in average were higher than those for females. In addition to that viral DNA load had no significant effect on risk prediction using PAGE-B tool. REACH-B regression suggests that males in average were found to have higher REACH-B scores than females. It was also found that REACH-B values for HBe-Ag positive subjects were higher than values for HBe-Ag negative subjects. DNA does not have an effect on REACH-B results. There was also a significant positive relationship between Age, ALT and REACH-B values. For CU-HCC equation, age and bilirubin had positive relation with CU-HCC. CU-HCC values in average for females were higher than in males.

Further, CU-HCC results for cirrhotic patients were higher than non-cirrhotic patients. There was a significant negative relationship between CU-HCC values and Albumin. Finally, DNA viral load values have no effect on CU-HCC results. Table 3 showed that PAGE-B equation was the most predictive tool for HCC development in our patients with R^2 value of 86.5 %. Age, gender and platelet count are the most predictive variables of this tool.

Table 1: Demographic, laboratory data and HCC risk estimation results as measured by the 3-risk estimation tools; CU-HCC, REACH-B, and PAGE-B

Characteristics		M±S.D N=145	HBe-Ag Positive N=31	HBe-Ag Negative N=114	NA¤ N=126	Non-NA N=19
Gender	Male	N= 99 (68%)	10	78	89	10
	Female	N= 46 (32%)	21	36	37	9
Age		37.35 (12.4)	38.6 (12.5)	32.9 (10.9)	37.84 (12.67)	34.11 (9.74)
HBe-Ag	Positive	N= 31 (21%)			29	2
	Negative	N= 114 (79%)			97	17
Cirrhosis	Yes	N= 15 (10%)	2	13	15	0
	No	N= 130 (90%)	29	101	111	19
Albumin		4.7(4.13)	4.2 (0.5)	4.8 (4.6)	4.79 (4.42)	4.11 (0.71)
Bilirubin		0.90 (0.93)	1.1 (1.5)	0.84 (0.7)	0.93 (0.97)	0.68 (0.40)
ALT		55 (85.6)	46.3 (32.7)	57.4 (95)	58.56 (90.97)	31.57 (22.4)
DNA¥		1.5 (5.9) 8 $\log_{10}$	4.8 (10) 8 $\log_{10}$	6.0 (3.5) 8 $\log_{10}$	1.46 (5.6) 8 $\log_{10}$	1.8 (7.8) 8 $\log_{10}$
PLT		211.8 (70)	187.9 (62.3)	218.3 (70.8)	204.5 (68.18)	260.16 (64.13)
CU-HCC*		5.6 (9.75)	5.4 (8.6)	5.6 (10.1)	5.98 (10.1)	3 (6.5)
REACH-B**		6.3 (3.4)	9.1 (2.5)	5.6 (3.3)	6.7 (3.35)	4.2 (3.4)
PAGE- B§		4.8 (5.6)	5.1 (5.7)	4.7 (5.6)	5.3 (5.6)	1.2 (4.4)

¤ NA (Nucleo (s) tide Analogue Therapy). Risk score: *CU-HCC; low < (5), intermediate (5-20), high > (20).

**REACH-B; a 17-point risk score, see reference (19) for REACH-B scoring system and risk estimation table and Table 2 below. §PAGE-B: A 27- point risk scale (- 4 to 22); low (< 6), intermediate (6-10), and high (> 10).

¥ An arbitrary value of 16 was assigned to undetectable HBV DNA values for statistical analysis purposes.

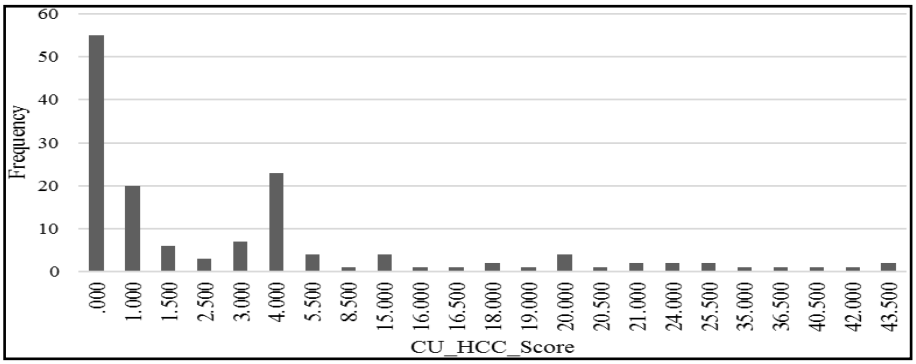

Figure 1: CU-HCC scoring system and the corresponding spread of patients according to their CU-HCC scores. See footnote of Table 1 for risk estimation.

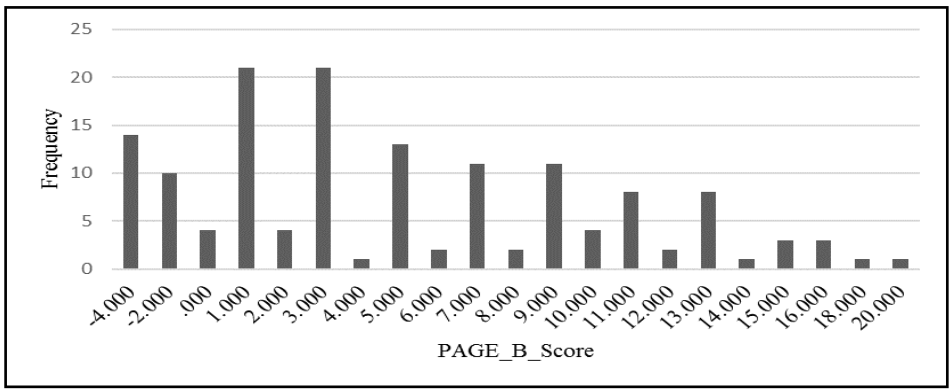

Figure 2: Score estimation for patients using PAGE-B and the corresponding frequency for each score.

Further analysis of subjects for 3, 5 and 10-year risk on REACH-B risk estimator showed that some patients have high risk of developing HCC as shown in table 2 and figure 3.

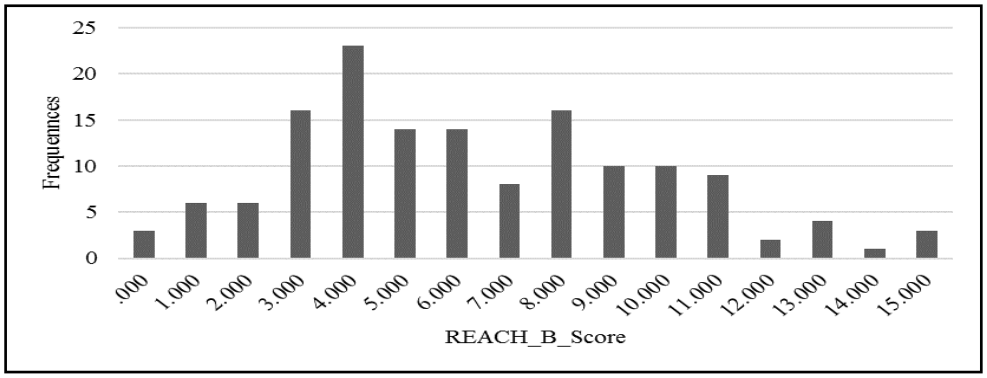

Figure 3: Patient distribution and corresponding scores on REACH-B, for risk estimation. See footnote of Table 1 and REACH-B risk estimation Table 2.

There were at least 3 patients whose score was 15 on REACH-B scale which corresponds to a 10-year-risk of 46.8 %, see Table 2. The average REACH-B score ± SD for all patients was 6.3 ± 3.4 which correspond to a 10-year risk of only 0.7% to 1.2 %, Table (1).

Table 2: Patient scores on REACH-B and estimated 3, 5, and 10-year risk of developing HCC.

*REACH-B score	Frequency N=145	Percentage	3 year risk %	5 year risk %	10 year risk %
1	6	4.1	0.0	0.0	0.1
2	6	4.1	0.0	0.0	0.1
3	16	11.0	0.0	0.1	0.2
4	23	15.9	0.0	0.1	0.3
5	14	9.7	0.1	0.2	0.5
6	14	9.7	0.1	0.3	0.7
7	8	5.5	0.2	0.5	1.2
8	16	11.0	0.3	0.8	2.0
9	10	6.9	0.5	1.2	3.2
10	10	6.9	0.9	2.0	5.2
11	9	6.2	1.4	3.3	8.4
12	2	1.4	2.3	5.3	13.4
13	4	2.8	3.7	8.5	21.0
14	1	0.7	6.0	13.6	32.0
15	3	2.1	9.6	21.3	46.8
Total	145	100.0			

* REACH-B scale is from 1-17, there were no patients with risk score of 16 or 17.

Table 3: T-test results for our patients using the 3-risk calculator tools against lab tests and other relevant socio-demographic variables.

	PAGE-B equation	REACH-B equation	CU-HCC equation
Age	0.466** (13.682)[a]	0.645** (12.314)	0.138** (2.423)
Male	0.357** (11.232)	0.193** (3.753)	-0.190** (-3.584)
DNA	0.037 (1.177)	0.091 (1.650)	-.005 (-0.088)
Platelet	-0.547** (-16.162)		
Albumin			-0.097* (-1.822)
Bilirubin			0.174** (3.038)
ALT		0.233** (4.478)	
HBeAg		0.527** (9.739)	
Cirrhosis			0.632** (11.115)
R Square	0.865	0.640	0.620
P-value	0.000	0.000	0.000

Notes: [a] Numbers in parentheses are t-values. *(**) denotes statistical significance at the 10(5) percent level.

DISCUSSION

In this study, cirrhosis was not an important factor in explaining PAGE-B or REACH-B scores in our patients. However, it was important factor for CU-HCC tool. This tool was originally developed in a cohort of subjects who were 30 % cirrhotic. Having this said, cirrhosis is still one of the most important risk factors for HCC. Overall, we have low percentage of cirrhotic subjects in this study, perhaps due to genetic traits among the Palestinian population.

We found that average risk of HCC, calculated by the three risk tools, was lower in non-NA therapy group comparing to NA-therapy group. Average HBV DNA load for NA- therapy group was comparable to that for non-NA therapy group as shown in table 1. This comparison is limited by the small number of patients in the non-NA group (19 patients only). It is also limited by the fact that there is a non-homogeneous distribution of the considered variables among the two groups (NA and non-NA groups). We know that the incidence was higher in males than in females which might be contributed to by genetics. However, male to female ratio in non-NA therapy group was 1:1 comparing to 2:3 ratio in NA group. A close look at non-NA therapy group profiles showed that they had recent HBV profiles which means they might have less years in disease. Subjects in this category were younger, have better ALT, Bilirubin, platelet and albumin test results, and might be in immune control stage. It is quite challenging to explain these results.

However, it was found that Lamivudine therapy itself was a risk factor for HCC development in more than one study as shown in the introduction. Treatment failure could be one of the reasons for the worsening of subjects in the NA-therapy group. This could be in turn attributed to drug resistance to Lamivudine since it is an old drug with a low genetic barrier. It could be also due to genotype mutation or BCP region mutation in the HBV virus itself [5,18]. It was shown that Lamivudine monotherapy was associated with AV resistance to 15% - 60% of patients.[10] For the non-NA therapy group , high DNA values could reflect low immunity for HBV, recent seroconversion, or simply the absence of viral suppression due to lack of therapy. On the other hand, high DNA value for NA therapy group could reflect acute over chronic infection, relapse of infection, immune-tolerance or drug- resistance. Papatheodoridis et al. (2015) demonstrated that even under AV treatment and after achieving Viral Remission (VR), older age and gender are still associated with HCC development. On the other hand, available antivirals are not able to eradicate HB neither they are able to decrease the risk of developing HCC to a measurable level[18].In some Asian studies, but not Caucasian studies, Nucleotide Analogues (NAs) therapy resulted in 30% to 80 % HCC risk reduction in cirrhotic vs. non-cirrhotic patients, respectively [22,23].

Host, virus, and environment factors[24-29] play a role in determining the severity of CHB infection and its prognosis to HCC. Chronically infected patients with HBV could have anywhere from (0 - 10^{10}) DNA copies/ml.[10] In the same context, 43% of study subjects have DNA level between 1.0 x10^4 - 3.4 x 10^9. The study found that 83 patients (57%) had DNA levels ≤ 10^4. This value represents the cut off value for HCC risk development.[5] No data was available on persistence of viral suppression. Viral suppression defined as persistent VR for 6 months or more of a DNA level ≤ 2500 copies/ml which could be one of the drawbacks of our study since we didn`t determine VR period in subjects of this study. Thirty five (42%) out of the 83 patients in this study showed undetected DNA levels in their sera. Wong et al. (2013) reported that achieving VR had no impact on HCC rate in overall patient population (8.7% vs. 10.7%, $p = 0.33$), but it was associated with significant impact on cirrhotic subgroup *(p = 0.02).* We were unable to find any information about vaccination history in the records. In that case we were unable to determine whether the patients were already vaccinated by 3 doses. That would also contribute to the finding that the majority of patients who were able to develop a DNA level below ≤ 2500 copies/ml are non- responders for the 3 doses of the vaccine since they were unable to produce anti- Hbs but were able to produce antibodies for

Hbc Ag, and Hbe Ag. This would indicate that these groups are in a good and favorable outcome for the progression of the disease or developing HCC.

VIRGIL, a large European study, showed 71% reduction in HCC risk, other liver-related clinical events, and death for patients who had VR on *entecavir* treatment (HR = 0.29; $p = 0.05$). Three multi-regression analyses were applied in this study and found that DNA levels did not have a significant impact on risk estimation in our patients in the 3 risk tools we applied, table 3. It is the DNA level inside tissues that might predict the prognosis to HCC. DNA integration into host cell sensitive genome locations, such as Retinoic Acid Receptor Alpha Gene or within human Cyclin Gene are important in HCC development. These mechanisms are involved in cell growth regulation and could lead to carcinogenic events. This process is random and in most cases viral DNA integration site is not critical.[5] Hepatitis B x gene (HBx) product, a transcriptional activator of various cell growth genes, is implicated in HCC development.

HBx integration into host genome activates RAS-Raf-MAP kinase pathway and interacts with p53 (human tumor growth suppressor protein.)

Up to date, there is no single test that measures DNA levels inside tissues or infers such a value from serum DNA levels. However relying on future consideration as ccc-DNA, free circulating tumor DNA, and/or enhanced liver fibrosis (ELF) tests, can be employed for that purpose of measuring DNA levels inside the tissue and serum.

In regard to HBe-Ag status, there was a significant difference in risk prediction using REACH-B compared to the other 2 risk tools, 9.1 ± 2.5, 5.4 ± 8.6 and 5.1 ± 5.7 for REACH-B, CU-HCC, PAGE-B, respectively. This difference could be attributed to the fact that REACH-B was the only test that integrates HBe-Ag positivity as a risk factor for

HCC, Table 1. In the same table if you look at the risk for HBe-Ag negative group using REACH-B, it was comparable to other values estimated using CU-HCC and PAGE-B, 5.6 ± 3.3, 5.6 ± 10.1 and 4.7 ± 5.6, respectively. 79% of subjects in our study were HBe-Ag negative with low to intermediate HCC risk average. Traditionally, seroconversion to anti-HBe-Ag-positive and loss of HBe-Ag from patients sera have been used to indicate therapy response and were associated with low morbidity and mortality.[10]

HBV genotypes associated with precore mutations may not have detectable HBe-Ag in their sera. Therefore, for these patients we monitor response for treatment by DNA levels.[10] 90% of our patients supposed to be HBV D1 genotype, a pro-mutation genotype, as shown in a study done up north of West Bank. [36] We know that 43% of our patients had DNA levels between $1.0 \times 10^4 – 3.4 \times 10^9$ which considered high for patients on NA therapy. This comes in concordance with a recent epidemiologic study by Bissinger et al. (2015)[30].

In our sample, ALT values range from 7.3 - 664 U/L, normal values are (male:0-43 U/L, Female: 0-32 U/L). We have 9 males and 4 females (9%) had ALT results over 1.5 X Upper Limit Normal (ULN), which means liver inflammation is still ongoing in these patients. Multi-regression analysis proved that PAGE-B is the most valid tool for HCC risk estimation in our patients where most of patients showed low risk of HCC. Two main suggested reasons for such a result. First; the multi-regression analysis proved that DNA load was of no value as a risk factor in our patients when incorporated in this tool.

It could have been the opposite if it was really identified as a risk factor since most of our patients had high levels of DNA despite the antiviral therapy. Second; the presence of some ethnicity and/or genetic unrevealed risk factors that played a role in determining the risk in Palestinian people which weren`t incorporated in PAGE-B. Of notice the subjects who were in the non-NA therapy group, had the lowest risk on PAGE-B. This could be attributed to young age, better levels of PLT comparing to the NA therapy group and being 50% females. The risk was 1.2 ± (4.4) in non-NA therapy group comparing to 5.3 ± (5.6) in NA therapy group, see, Table 1.

CONCLUSION

As a conclusion, this original work formally predict a powerful tool and prognostic indicator of HCC in a cohort of CHB patients in Southern West Bank. PAGE-B is the best model for predicting HCC risk in our patients. Practitioners should consider including this tool and other risk estimation tools, as pertinent to the case, in a comprehensive clinical-decision making process. Palestinians- as a Middle eastern people- are the nearest to Europe with history of cultural and people migration and exchange. So no wonder our results came in align with the results of this tool (PAGE-B) when tried in Caucasians.

NA therapy could help prevent progression of HBV to HCC by reducing inflammation, regeneration and growth, and fibrosis. This will ultimately lead to reducing HCC risk. They may also reduce HBx protein levels. They might also act on preventing HBV DNA integration into host chromosomes or affecting its malignant potential.[18]

We were unable to determine on treatment failure in this study. On the light of this conclusion, we cannot deprive our patients from AV therapy. Having this said, we have to decide which NA to use and what therapy regimen should be adopted. We should also employ PAGE-B to predict HCC among CHB patients in order to decide on yearly liver ultrasonography.

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