

Original Research Article

Delta bilirubin: a sensitive and predictive marker for acute rejection in liver transplant recipients

Deepa Gupta*, Aditi Gupta, Arun Raizada

Department of Biochemistry, Medanta the Medicity, Gurgaon, Haryana, India

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***Correspondence:**

Dr. Deepa Gupta,

E-mail: guptadeepa003@gmail.com

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ABSTRACT

Background: This study was aimed to investigate the diagnostic utility of delta bilirubin for acute rejection in liver transplant recipients.

Methods: The present study was conducted on 80 patients (56 men and 24 women) who were admitted for a major operation of liver transplantation at super-speciality hospital, Medanta-The Medicity, Gurgaon. The average age of the patients was 43 ± 19 years. Data was analyzed as mean, standard deviation; student t test by using statistical package for social sciences (SPSS) software. Sensitivity, specificity, positive predictive value and negative predictive value were calculated in percentage.

Results: The result from the present study indicates that delta bilirubin had highest sensitivities of 93% whereas conjugated bilirubin has 43% while AST, ALT, GGT and ALKP had sensitivities of 61%, 81%, 80% and 31% respectively. There was a significant difference of delta bilirubin between rejection and non rejection transplant recipients.

Conclusions: Our findings supported that the serial measurement of delta bilirubin would be a reliable marker for recognizing early rejection in liver transplant recipients.

Keywords: Acute rejection, Delta bilirubin, Liver transplantation

INTRODUCTION

End stage liver disease is the fourth leading cause of death worldwide. Liver transplantation was recognized as an acceptable treatment for many forms of potentially fatal liver diseases since 1983.¹ In spite of this, acute rejection is one of the most common causes of graft dysfunction during the first three months of post operative periods, although it can occur at any time.² Evidences of rejection could be suspected on the basis of commonly used liver function test (LFT) markers. Abnormal LFT patterns often but not always indicate liver injury. Liver biopsy at this stage may help to diagnose rejection.³ Despite the value of biopsy, it is too invasive technique for routine use, also non-definitive,

costly procedure with potential serious side effect, risks and also not always accepted by patients.

Delta bilirubin (DB) was first identified as bilirubin fraction covalently linked to albumin protein. It is third form of under normal conditions. Bilirubin, nontoxic, neither excreted in urine nor in bile.⁴ DB was formed in liver, when hepatic excretion of bilirubin glucuronide was impaired. As a result, DB represents a significant fraction of total serum bilirubin in patients with cholestasis and hepatobiliary disorder.⁵ In obstructive jaundice, conjugated bilirubin (CB) was not excreted into the bile and consequently re-enters the blood stream. Once in circulation, it slowly binds with albumin forming DB.⁶ DB had attracted the attention of scientist towards

complementary marker of early rejection in liver transplant recipients.⁷

To date there was no non-invasive test for early identification of acute rejection. With the acknowledgement of clinical utility of DB, the present study was undertaken to find out the diagnostic utility of delta bilirubin for acute rejection in liver transplantation.

METHODS

Study population

We studied 80 patients (56 males and 24 females) who were admitted for a major operation of liver transplantation in Medanta-The Medicity, Gurgaon. The average age of the patients was 43±19 years. Patients were admitted in the hospital with complication of chronic liver diseases: chronic hepatitis (n=31), alcoholic liver disorder (n=11), cholestatic liver diseases (n=11), cryptogenic cirrhosis (n=7), liver malignancy (n=5), acute liver failure (n=4), extra hepatic biliary atresia (n=2), NAFALD (n=4), alagille syndrome (n=1), Budd-chiari syndrome (n=1), hemochromatosis (n=1), tyrosineamia type I (n=1), Wilson's diseases (n=1) (Number of patients aside in bracket).

Sample collection and assay

Under all aseptic condition, 5ml of blood samples was drawn daily by trained nursing staff and collected in serum separating tubes (SST) vacuainers by using standardized procedures.⁸ Centrifuge specimens at 5000rpm for 15min and removed the serum from the cellular material within 4hours of collection. Sera were analyzed within 1-3hrs of acquisition and were preserved at 4°C.

All serum samples were assayed for total bilirubin (TBIL), indirect bilirubin (IB), conjugated bilirubin (CB), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALKP), gamma glutamyl transferase (GGT) by using VITROS 5600 clinical chemistry analyzer based on dry slides techniques and were analyzed in same laboratory to prevent the variation in measurement. Delta bilirubin was calculated by using specific formula i.e. (TBIL- (CB+ Unconjugated Bilirubin)). A comprehensive internal quality control programme was followed and results were released after calibrating values between mean ±1SD. This internal quality control analysis was performed daily.

Assessment of rejection

Immediately a day after transplantation, DB was determined in blinded fashion along with other liver function test markers. According to our study criteria, patients undergoing suspected rejection, if DB fraction remained were lesser than 40% of TBIL while CB should

increase rapidly along with abnormal LFT patterns i.e. if LFT markers were increased 1.5 times the upper limit of normal ranges and 10% increase within two consecutive days. Suspected rejection on the basis of our study criteria was confirmed by using gold standard test for the diagnosis of graft rejection i.e. liver biopsy. Liver biopsy showing bile duct damage mixed portal inflammation infiltration, eosinophils and endophlebitis. Banff's criteria and rejection activity index (RAI) scores the severity of rejection.⁹

Data analysis

Statistical analysis was done using SPSS, Version, 22.0, IBM, USA software. Student t test was used to analyze the significant difference between parameters of liver transplant patients those experiencing rejection and non-rejection ($p < 0.05$). Data were analyzed using chi square test (χ^2) test; $p < 0.05$ was considered as significant. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) was determined by using Haynes formulae.¹⁰

RESULTS

The present study was done on 80 liver transplant recipients. Table 1 showing the Mean ±SD and range of all the studied parameters of the transplant recipients.

Table 2 shows student t test was applied to show the significant difference between parameters of liver transplant patients those experiencing rejection and non rejection ($p < 0.05$) respectively.

Table 1: Overall trend of biochemical data of given parameters for study population (n = 80).

Variables	Range (in patients) min-max	Mean ±SD
Sex (male/female)	56/24 (n = 80)	
Age (years)	19-68	43±19
Post-transplant biopsy (days)	6-177 (n = 80)	47±39
Total bilirubin (mg/dl)	0.3-24.7	3.7±4.9
Indirect bilirubin (mg/dl)	0.2-5.9	0.9±0.7
Conjugated bilirubin (mg/dl)	0-18.5	2.1±3.9
Delta bilirubin (mg/dl)	0-5.5	1.0±1.2
Aspartate aminotransferase (IU/L)	20-804	150±125
Alanine aminotransferase (IU/L)	27-881	234±168
Gamma glutamyl transferase (IU/L)	34-914	335±209
Alkaline phosphatase (IU/L)	44-887	210±161

Liver biopsies were done in patients at the time of suspected rejection. Patients were classified in rejection

category only when acute rejection was confirmed by histological evidences. In 54 of these 80 biopsies were

classified in rejection category due to histological confirmation and 26 were in non rejection.

Table 2: Demographic and various biochemical parameters in stable liver transplant recipients and those experiencing rejection.

Parameters	Non rejection ⁺ (n =26)	Rejection ⁺ (n = 54)	Student t test	P value [#]
Total bilirubin (mg/dl)	1.9 ± 1.6	4.6 ± 5.8	2.32	0.023
Indirect bilirubin (mg/dl)	0.6 ± 0.5	1.0 ± 0.8	2.34	0.022
Conjugated bilirubin (mg/dl)	0.7 ± 1.7	2.7 ± 4.4	2.23	0.028
Delta bilirubin (mg/dl)	0.6 ± 0.5	1.3 ± 1.1	3.09	0.002
Aspartate aminotransferase (IU/L)	99 ± 45	175 ± 143	2.64	0.010
Alanine aminotransferase (IU/L)	170 ± 112	265 ± 182	2.45	0.017
Gamma glutamyl transferase (IU/L)	254 ± 145	374 ± 224	2.49	0.015
Alkaline phosphatase (IU/L)	272 ± 206	180 ± 126	2.47	0.015

In 50 of 54 rejection cases, there was either a decline in the DB fraction or DB fraction remain consistently low (<40% of TBIL). Whereas the presumption of CB fraction (> 50% of TBIL) for rejection category was only 23. On the other hand, in 21 of 26 non rejection cases,

there was a decline in CB fraction with a concomitant increase in DB fraction. There was a total of 48 patients having increases in AST, 60 patients of increased ALT, 65 having increased GGT and 31 having increased ALKP.

Table 3: Association between changes in Liver Function Markers in liver transplant recipients with early rejection & non rejection.

Parameters	Analysis	Liver biopsy (rejection)		Total (80)	X ²	P value
		Yes (54 cases)	No (26 cases)			
Delta bilirubin (mg/dl)	↓ %DB	50	05	55	43.95	<0.001
	No ↓ (or ↑) %DB	04	21	25		
Conjugated bilirubin (mg/dl)	↑ %CB	23	05	28	4.209	<0.05
	No ↑ (or ↓) %CB	31	21	52		
AST (IU/L)	↑AST	33	15	48	1.08	>0.05
	No ↑ AST	21	11	32		
ALT (IU/L)	↑ALT	44	16	60	3.72	>0.05
	No ↑ALT	10	10	20		
GGT (IU/L)	↑GGT	43	22	65	2.28	>0.05
	No ↑ GGT	11	04	15		
ALKP (IU/L)	↑ALKP	17	14	31	3.69	>0.05
	No ↑ ALKP	37	12	49		

p < 0.001= highly significant; p< 0.05= significant; p>0.05= non significant

Table 4: Sensitivity, Specificity, positive predictive value (PPV) and negative predictive value (NPV) of all the given parameters.

Parameters	Sensitivity	Specificity	PPV	NPV
DB	93%	81%	91%	84%
CB	43%	81%	82%	40%
AST	61%	42%	69%	34%
ALT	81%	38%	73%	50%
GGT	80%	15%	66%	27%
ALKP	31%	46%	55%	24%

The sensitivity of ALT and GGT were 81% and 80%. This is because liver enzymes ALT and GGT can pick 44 and 43 true positive rejection. But also, can pick 16 and 22 false positive of 26 non-rejection patients. Thus, relative to DB, liver enzymes possess nominal specificity for identifying rejection (Table 3).

According to Haynes method, our data revealed that DB had highest sensitivities of 93% ($p < 0.001$) whereas CB has 43% while AST, ALT, GGT and ALKP have sensitivities of 61%, 81%, 80% and 31% respectively. In our sampling, the calculated specificity for both DB and CB were 81%. The corresponding specificities for AST, ALT, GGT and ALKP were 42%, 38%, 15% and 46% respectively (Table 4). Positive Predictive Value and Negative Predictive Value were also calculated in which DB had the highest values as compared to the other parameters.

DISCUSSION

With the development of liver transplantation in India, our understanding of the pathology of liver transplants is making rapid progress. Despite the progress, the life expectancy and quality of life for patients remains poor due to several advanced post operative complications. If the rate of survival after liver transplantation is to be enhanced, then early recognition and treatment of complications like rejection and infection are essential.

Allograft rejection is one of the major barriers for the prognosis after liver transplantation.² During past decades, the incidence of acute cellular rejection can be made based on serial abnormalities in clinical manifestation, radiological and some immunological examinations. Besides this none has proven sufficiently sensitive and specific test.¹¹ Evidences from liver allograft biopsy have been considered as gold standard for monitoring and diagnosis acute rejection after liver transplantation.¹² Even though it was not totally definitive and also too invasive test for routine use of liver transplant recipients.¹³

The present study shows that determination of DB might be used as sensitive measure of early rejection in liver transplant recipients. Our data revealed that DB would possess highest sensitivity as compared with CB and other liver enzymes. Statistically significant differences ($p < 0.05$) occur between patients undergoing rejection and non rejection shows that our study for determining sensitivity and specificity of DB, CB and other liver enzymes are also significant.

Wu et al 1990, found that the DB determination along with CB, act as a sensitive marker of rejection in the setting of orthotopic liver transplantation particularly in advanced liver failure and also suggest that both are complementary marker of graft rejection, their combined sensitivity approaching 100%. Their study that relative to DB and CB, AST and also entailed ALT had a lower

nominal sensitivity for rejection. This study had supported the fact of the clinical diagnostic utility of DB along with CB for early rejection in liver transplant recipients.⁷

In our study, 54 patients had rejection that have been proven by histological examination i.e. liver biopsy, out of which 50 true positive rejection cases had been picked by DB. Thus, DB can encompass sensitivity at the peak of 93%. CB and other liver enzymes would possess lesser sensitivity as compared with DB. The sensitivity and specificity of ALT and GGT are 81% and 80% respectively. But as a marker for rejection, ALT and GGT lack some specificity 38% and 15% because they are also found in many tissues. Levels of these enzymes can rise to several times normal after severe muscular exertion or in other conditions.

Cox et al 1987 found that CB had a sensitivity of 78% and specificity of 77% for identifying rejection in patients between 30 and 154 days after liver transplantation. This supported the clinical diagnostic utility of CB for rejection in transplant recipients.¹⁴ In our study, CB had a nominal sensitivity i.e. 43% but specificity was quite higher 81% for identifying early rejection. CB can be directly measured by using a dry film method.

Gautam et al (1984) reported that at least a portion of DB was formed non-enzymatically in-vivo, probably via an O to N transfer of bilirubin from CB to a nucleophilic site on albumin.^{15,16} The formation of both albumin and CB, the precursor of DB and the excretion of nascently formed CB into the bile are energy expensive processes that require intact hepatic synthesis. Therefore, any impairment of one or more of these events (including ATP generation) could lead to a decrease in the DB fraction or an accumulation in CB or both. Presumably, such functional changes can occur with acute rejection before hepato-cellular membrane injury and the subsequent increase in AST.

Wu et al 1989 demonstrated that DB isolated from serum, in concentrations as low as 16 $\mu\text{mol/L}$, protected cultured rat hepatocytes effectively against oxy-radical damage and salvaged by 55% the ischemic rat liver upon reperfusion.¹⁷

These two lines of evidences suggest the probable mechanistic reason behind that DB should be such a promising marker of liver rejection and had strong clinical utility. All these observations recommended strongly a possibly beneficial role of the relatively long-lasting DB in promoting the survival of the newly transplanted liver in the recipient. But still further verification of this above mechanistic reason is needed.

Limitations of our study is that DB was not estimated directly rather it was calculated by using specific formula and the sample size was quite small to consider its a marker.

CONCLUSION

In summary, the present study indicates that there was a significant difference of DB between non-rejection transplant recipients and those experiencing rejection. This finding supports the DB would be a reliable marker for recognizing early rejection in liver transplant recipients than LFT markers. Our study suggested that the serial measurement of DB after liver transplantation may leads to earlier diagnostic and therapy of rejection. Further detailed study in this regard on a larger cohort of transplant patients is needed to fully evaluate this observation.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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