Increase of Serum Kallikrein-8 Level After Long-term Telbivudine Treatment

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Abstract. Background/Aim: Our previous cDNA microarray study revealed increased cellular mRNA levels of a panel of genes, including kallikrein-8 (KLK8), after long-term telbivudine treatment in chronic hepatitis B patients. The aim of this study was to verify whether serum protein levels of KLK8, a cancer-related enzyme, are indeed increased after telbivudine treatment. Patients and Methods: A total of 83 chronic hepatitis B patients receiving telbivudine for >2 years were retrospectively analyzed. Serum KLK8 protein and estimated glomerular filtration rate (eGFR) changes were compared before and after treatment. Results: Both serum KLK8 protein and eGFR increased significantly after long-term telbivudine treatment (paired t-test: KLK8, p<0.001; eGFR, p=0.001). No direct correlation was found between KLK8 increase and eGFR change. However, eGFR change was positively associated with post-treatment KLK8 levels following adjustment for body height (p<0.001). Conclusion: Telbivudine treatment resulted in increased levels of serum KLK8 protein. Furthermore, eGFR increase was associated with body height-adjusted, post-treatment KLK8 levels.

Hepatitis B is a major global health problem. According to the Global hepatitis report, 2017 of the World Health Organization report, it chronically infects approximately 240 million people worldwide. Patients with chronic hepatitis B virus (HBV) infection have increased risk of developing hepatic decompensation, liver cirrhosis and hepatocellular carcinoma. Oral antiviral agents are the main choice of chronic hepatitis B infection in the present days. Approved oral antiviral agents include entecavir, tenofovir, telbivudine, lamivudine, and adefovir. These nucleos(t)ide analogues are safe and effective for long-term suppression of hepatitis B virus replication if no drug resistance is developed. Telbivudine is one of the choices for treatment of chronic hepatitis B (1). Besides its antiviral effect, an increase of estimated glomerular filtration rate (eGFR) has been observed after long-term telbivudine treatment (2). Additionally, increased or decreased mRNA levels in a panel of genes have been found by peripheral blood mononuclear cell-derived cDNA microarray study (3). Of those, decreased serum levels of angiotensin-converting enzyme upon long-term telbivudine treatment have been verified (3). However, of the list of dysregulated genes, up-regulation of kallikrein-8 (KLK8) gene expression appeared to statistically, be the most significant change.

KLK8 belongs to a group of serine proteases – kallikreins, carrying diverse physiological functions. Many kallikreins are implicated in the development or the regulation of cancers (4, 5). KLK8 is one of the 15 kallikrein subfamily members. Different isoforms of KLK8 mRNA have been reported in the GenBank. The greatest abundance of KLK8 is found in esophagus and skin. Elevated tissue or serum levels of KLK8 have been found in ovarian cancer (6-8), salivary gland tumors (9), endometrial carcinoma (10), and cervical cancer (11). Decreased KLK8 levels have been observed in breast cancer (12). KLK8 serves as a biomarker for poor prognosis in colorectal cancer (13), lung cancer (14), and oral squamous cell carcinoma (15). Conversely, it serves as a biomarker for favorable prognosis in non-small cell lung cancer (16) and ovarian cancer (17, 18). Despite these clinical correlations, the actual molecular mechanisms remain unknown.
Although increased \textit{KLK8} mRNA levels after long-term telbivudine treatment were discovered by cDNA microarray analysis, further verification has never been performed. The correlation between the changes in \textit{KLK8} levels and eGFR is also unknown. The goal of this study is to verify whether serum \textit{KLK8} protein levels indeed increase after telbivudine treatment and if this increase is associated with eGFR changes.

\section*{Materials and Methods}

\textbf{Patients.} This study was conducted under the approval of institutional review board, Chang Gung Memorial Hospital, Taiwan. A total of 83 chronic hepatitis B patients receiving long-term telbivudine were analyzed retrospectively. These patients were under regular out-patient follow-ups at Chang Gung Memorial Hospital, Liver Clinic. The duration of telbivudine treatment was >2 years for all patients enrolled. The clinical data was collected by reviewing medical records. The \textit{KLK8} protein levels and eGFR changes before and after >2 years of treatment were assayed and compared.

\textbf{Measurement of \textit{KLK8} protein levels.} \textit{KLK8} protein levels were assayed using human plasma samples derived from patients before or after telbivudine treatment. Plasma samples were retrospectively retrieved from the serum bank, Liver Research Center, Chang Gung Memorial Hospital. Protein concentration was measured using the Bradford Protein Assay Kit (AMRESCO, Inc., Solon, OH, USA). Equal amounts of protein were loaded on gels. Proteins were separated with 10\% SDS-PAGE and transferred to a PVDF membrane. Western blot analysis was performed using \textit{KLK8} antibody (Bioss Inc., Woburn, MA, USA) overnight at 4\degree C. Blots were washed and incubated with HRP-labeled secondary antibody. Enhanced chemiluminescence was applied for signal detection. The density of Western-blot bands was quantified using GeneTools Image Software. All experiments were technically repeated more than twice. Representative blots are shown in Figure 1A.

\section*{Results}

There were totally 83 patients receiving telbivudine treatment for chronic hepatitis B for at least 2 years. The baseline characteristics of these patient are listed in Table I. Of these 83 patients, all had paired \textit{KLK8} data before and after treatment, whereas only 53 patients had paired eGFR data. The \textit{KLK8} and eGFR levels in each patient were compared before and after treatment. Both serum \textit{KLK8} and eGFR levels were increased significantly after >2-years of telbivudine therapy (paired \textit{t}-test before and after treatment: \textit{KLK8}, \textit{p}=0.001; eGFR, \textit{p}=0.001) (Figure 1B and C).

The \textit{KLK8} protein levels were analyzed by linear regression in relation to age, gender, duration of treatment, aspartate transaminase (AST), alanine transaminase (ALT), HBV e antigen (HBeAg), antibody against HBeAg (anti-HBe), initial HBV DNA levels, serum total bilirubin, liver cirrhosis, albumin, body height (BH), body weight (BW) and body mass index (BMI) (Table II). Univariate analysis revealed that the change of \textit{KLK8} protein levels was significantly correlated to AST (\textit{p}=0.012), ALT (\textit{p}=0.017), BH (\textit{p}=0.030), and BW (\textit{p}=0.018). Multivariate analysis including variables with \textit{p}<0.1 revealed increase of \textit{KLK8} protein levels was positively associated with AST (\textit{p}=0.004) and BH (\textit{p}=0.008).

Accordingly, the change of eGFR levels was analyzed by linear regression in relation to age, gender, duration of treatment, AST, ALT, HBeAg, anti-HBe, HBV DNA levels, serum total bilirubin, liver cirrhosis, albumin, initial \textit{KLK8} levels, final \textit{KLK8} levels, changes of \textit{KLK8} levels, BH, BW and BMI (Table III). Univariate analysis revealed that the change of eGFR levels was significantly correlated with gender (\textit{p}=0.009), BH (\textit{p}=0.002), and BW (\textit{p}=0.006). Multivariate analysis including variables with \textit{p}<0.1 revealed that the increase of eGFR levels was positively associated with final \textit{KLK8} levels (\textit{p}=0.017) and negatively associated with BH (\textit{p}<0.001).

According to these data, no direct correlation between the increase in \textit{KLK8} levels and the increase in eGFR levels was found. However, eGFR change was positively associated with the post treatment \textit{KLK8} levels following adjustment for BH (Linear regression, \textit{p}<0.001) (Figure 2). We further analyzed the relation between initial the \textit{KLK8} value (before telbivudine treatment) and BH. It was found that \textit{KLK8} initial value was negatively correlated with BH (Figure 3).

\section*{Discussion}

In this study, serum \textit{KLK8} levels were found to be significantly increased after prolonged telbivudine treatment. However, despite that both \textit{KLK8} and eGFR were increased after treatment, there was no simple linear

| Variables | Value |
|-----------|-------|
| Age, years, mean±SD | 50.5±12.6 |
| Gender, male, % | 60 (72.3\%) |
| Duration of treatment, months, Median (range) | 39.1 (14.6, 100.3) |
| AST, U/L, mean±SD | 88.6±89.3 |
| ALT, U/L, mean±SD | 149.5±173.9 |
| Bilirubin, mg/dL, mean±SD | 1.0±0.5 |
| HBeAg, positive, % | 18 (21.7\%) |
| Anti-HBe, positive, % | 69 (83.1\%) |
| HBV-DNA, Meq/ml, Median (range) | 2.83 (>640, <0.0002) |
| Initial eGFR, mean±SD | 91.2±19.5 |
| Cirrhosis, Yes, % | 31 (38.6\%) |
| BH, cm, mean±SD | 163.7±8.3 |
| BW, kg, mean±SD | 66.1±11.6 |
| BMI, kg/m2, mean±SD | 24.5±3.1 |

SD: Standard deviation; AST: aspartate transaminase; ALT: alanine transaminase; eGFR: estimated glomerular filtration rate; BH: body height; BW: body weight; BMI: body mass index.
correlation between the two changes. Instead, correlation was found between BH-adjusted, post-treatment KLK8 levels and eGFR changes. The relation between the initial KLK8 value (before telbivudine treatment) and BH was further examined. It was found that KLK8 initial value was also correlated with BH (Figure 3). Therefore, KLK8 levels are a BH-dependent factor and adjustment by BH is needed when correlation with other clinical factors is assessed. The present results suggested that after telbivudine treatment, KLK8 levels had to increase to a certain level (post-treatment KLK8 level, adjusted by BH), before significant eGFR increase could occur, while a simple increase of KLK8 levels, if will not reach a sufficient degree, will not associate with eGFR change.

Additionally, in the mostly used equation for eGFR, the MDRD equation, BH is not an independent predictor for eGFR (19). Therefore, the finding that the eGFR change was positively associated with the post-treatment KLK8 levels, following adjustment for BH, was not biased by the association between eGFR and BH.

KLK8 is a biomarker for prognosis of several cancers (4, 5). And, the results of the present study indicate that an increase of KLK8 protein levels occurs after long term telbivudine treatment. Assuming that KLK8 plays a role in pathogenesis of cancers, the telbivudine-induced increase of KLK8 levels can influence the prognosis or associate with progression/regression of the related cancers. Alternatively, telbivudine usage could interfere with the predictive value of KLK8 as a prognostic marker. In the treatment of several cancers, chemotherapy is associated with hepatitis B reactivation (20) and antiviral therapy is recommended in all therapeutic guidelines. The present study suggests that
telbivudine might be a better choice for this purpose in certain type of cancers, such as non-small cell lung cancer (16) and ovarian cancer (17, 18), where the increase of KLK8 has been associated with a better prognosis.
Table III. Linear regression analysis for eGFR changes after treatment (n=53).

| Variables                      | Beta   | SE    | 95% CI          | p-Value |
|--------------------------------|--------|-------|-----------------|---------|
| Age                            | 0.256  | 0.199 | -0.144, 0.655   | 0.204   |
| Male                           | -15.275| 5.621 | -26.560, -3.989 | 0.009   |
| Duration of treatment          | 0.023  | 0.138 | -0.255, 0.301   | 0.869   |
| AST                            | 0.022  | 0.028 | -0.034, 0.077   | 0.440   |
| ALT                            | 0.003  | 0.014 | -0.025, 0.032   | 0.807   |
| HBcAg                          | 8.394  | 6.604 | -4.865, 21.652  | 0.210   |
| Anti-HBe                       | -1.715 | 7.171 | -16.112, 12.683 | 0.812   |
| HBV-DNA >2.83 Meq/ml           | 7.967  | 5.553 | -3.182, 19.116  | 0.157   |
| Bilirubin                      | -9.588 | 5.555 | -20.739, 1.564  | 0.090   |
| Cirrhosis                      | 0.684  | 5.927 | -11.215, 12.584 | 0.909   |
| Albumin                        | 2.264  | 10.788| -20.315, 24.842 | 0.836   |
| Initial KLK8                   | 0.773  | 1.781 | -2.803, 4.350   | 0.666   |
| Final KLK8                     | 3.132  | 1.917 | -0.717, 6.980   | 0.108   |
| KLK8 changes                   | 0.005  | 0.008 | -0.011, 0.022   | 0.509   |
| BH                             | -0.989 | 0.306 | -1.602, -0.375  | 0.002   |
| BW                             | -0.727 | 0.252 | -1.232, -0.222  | 0.006   |
| BMI                            | -1.087 | 0.971 | -3.037, 0.863   | 0.268   |

Multivariate (Stepwise mode; including variables with univariate p<0.2)

|                | Beta   | SE    | 95% CI          | p-Value |
|----------------|--------|-------|-----------------|---------|
| BH             | -1.116 | 0.296 | -1.711, -0.521  | <0.001  |
| Final KLK8     | 4.274  | 1.735 | 0.788, 7.759    | 0.017   |

SE: Standard error; AST: aspartate transaminase; ALT: alanine transaminase; eGFR: estimated glomerular filtration rate; BH: body height; BW: body weight; BMI: body mass index.

Conclusion

Long-term (>2 years) telbivudine treatment results in an increase of KLK8 protein levels, besides the increase of eGFR levels. The increase of eGFR was associated with post-treatment KLK8 levels following adjustment for BH. Usage of telbivudine for prevention of hepatitis B reactivation in cancer patients receiving chemotherapy might also affect the prognosis of cancers through the increase in KLK8 levels.

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