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Evaluation of EZH2 expression in hepatitis B patients

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ABSTRACT:

— ***Introduction:*** Hepatitis B virus (HBV) is one of the major global health problems, being one of the leading causes of acute and chronic Hepatitis B, Hepatocellular Carcinoma (HCC) and Liver Cirrhosis (LC). According to the World Health Organization (WHO), more than one-third of the entire world population has been infected with HBV; about 5% are chronic carriers of this infection and approximately 25% of infected people develop a liver disease such as LC and HCC.

— ***Patients and Methods:*** Identifying the molecular mechanisms of cell-virus interaction is critical to understand and predict the risk factors for cancer. To accomplish this goal, this study focuses on Enhancer of Zeste Homologue 2 (EZH2). EZH2 is a catalytic subunit of Polycomb Repressive Complex 2, which catalyzes the addition of methyl groups to lysine 27 of the N-tail of histone H3. Furthermore, EZH2 can be considered as a tumor marker in breast and prostate cancers. High levels of EZH2 are correlated with metastatic cells and unfavorable diagnosis. In this work, we evaluate the gene expression of EZH2 from blood samples of HBV patients.

— ***Results:*** Preliminary results showed an increase of gene expression of EZH2 in HBV patients treated pharmacologically with nucleotide analogues.

— ***Conclusions:*** These encouraging preliminary results are of importance to further study the molecular mechanisms of HBV-related liver disease, up to HCC. The potential role of EZH2 in liver tissue damage, remodeling and regeneration need to be explored in further investigations.

— ***Keywords:*** HBV, HCC, Epigenetic, PcG, EZH2, Liver.

INTRODUCTION

Nowadays one of the biggest global health problems is Hepatitis B Virus (HBV). Indeed, it can be considered as one of the leading cause of acute and chronic Hepatitis B, Hepatocellular Carcinoma (HCC) and Liver Cirrhosis (LC).

Recently, epigenetic has become one of the most studied subjects in research. DNA methylation is an important epigenetic modification involved in different events in biology. During this process, an enzyme of the DNA methyltransferase family transfers a methyl group from the donor S-adenyl methionine (SAM) to the carbon in the fifth position of a residue of cytosine forming 5 methylcytosines (5mC)[1].

The methylation of histone tails can happen in different lysine residues causing either chromatin compaction and inhibition of transcription or an opening

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of chromatin which takes to an activation[2]. Moreover, DNA methylation is involved in fundamental processes such as X chromosome inactivation and genomic imprinting[3,4]. In mammalian, methylation of DNA is essential for the development of the organism and for outliving[5]. All these reasons have led scientists to focus their attention on the study of epigenetic modifications.

One of the most studied methyltransferase is the Enhancer of Zeste Homolog 2 (EZH2). This protein belongs to a well-defined class of proteins called Polycomb Group Proteins (PcG), which were firstly identified in *Drosophila Melanogaster*. These proteins are responsible for the regulation of HOX genes in fruit fly[6], and several studies showed their strong conservation in different kinds of eukaryotes[7].

According to their incredible adjustment, PcG has been widely studied in mammalian. Indeed, it has been found that they are able to organize themselves onto two different complexes: Polycomb Repressive Complex 1 (PRC1) and Polycomb Repressive Complex 2 (PRC2). PRC1 is made up of the chromobox (CBX), RING1A/1B, the B lymphoma Mo-MLV insertion region 1 (BMI1) and PHC. Instead, PRC2 is formed by embryonic ectoderm development (EED) and retinoblastoma-binding protein 4/7 (RBBP4/7), SUZ12 and EZH2. Both complexes play a crucial role in the control of cell development and differentiation, acting together. Firstly, PRC2 has a histone methyltransferase activity on lysine-27 of histone H3 (H3K27me3). Indeed, EZH2 catalyzes the adding of methyl groups on H3K27 and the trimethylation occurs when at least three components of the complex are participating[8-10]. Once H3K27me3 trimethylates, the recruitment of PRC1 happens. After this, PRC1 is able to induce transcriptional repression of target genes because of the catalyzing of a mono-ubiquitilation of lysine 119 of histone H2[11-14].

The fundamental role of EZH2 in transcriptional events has opened the door to several studies in cancer research about its behavior in cancer appearance and progression. It was already known that the changes in chromatin structure are able to cause modifications in gene expression which can cause several pathologies such as cancer[15]. EZH2 is involved in different types of cancer and its overexpression is often correlated with metastasis and poor prognosis. The first studies were performed in the most common malignancies such as prostate, breast and lung cancers[16,17]. Furthermore, recent findings showed the involvement of EZH2 in other types of cancer such as rhabdomyosarcoma (RMS). In RMS, EZH2 is overexpressed and it does not allow the commitment of differentiation program in cells, uncovering its important role in stem cell[18]. For this reason, EZH2 can be considered as a stemness marker[19]. Furthermore, several studies are focusing their attention on targeting EZH2 for cancer treatment[20].

EZH2 is involved in the pathogenesis of liver Cancer (LC), which is the ninth most common cancer in female and the fifth most common cancer in the male. Worldwide, 782,000 are the new cases in 2012. About 83% of the cases occurred in less developed regions. HCC is often diagnosed at its advanced stage, and for this reason, it ranks as the second leading cause of mortality in the world[21]. The high incidence of mortality is associated with metastasis, which is able to settle not only in different part of liver but also in other sites in the body.

Multistep processes are responsible for the development of HCC. Recent studies uncovered the crucial role of epigenetic deregulations in HCC and the involvement of EZH2 and its important methylation activity on chromatin[22].

PATIENTS AND METHODS

Samples collection

All blood samples were collected from the *Unit of Infectious Diseases of the University of Sassari Hospital*. The study was approved by the local Bioethics Committee. All patients signed an informed consent to participate in the study.

We consecutively enrolled HBV-infected patients either receiving nucleoside analogues or never treated. Patients with concomitant infection with HCV or HIV were excluded as well as patients with decompensated cirrhosis. Data regarding age, stage of disease, current treatment, treatment history and HBV-DNA levels were taken from clinical records. In each patient whole blood samples were withdrawn by venipuncture.

Once collected, blood samples have been analyzed following the manufacturer protocol from Roche (11 814 389 001) using the Red Blood Cell Lysis Buffer. Successively, pellets were collected and either stored at -80°C or used for RNA extraction.

RNA Extraction, quantification and reverse transcription

Cell pellets have been used for the total extraction of RNA, which has been performed using High Pure RNA Isolation Kit from Roche (11828665001). To understand the purity of samples, they have been quantified using Nanodrop (Thermo Scientific, Waltham, MA, USA). 1 µg of RNA has been used for reverse transcription. Firstly, samples have withstood a denaturation at 38°C for 2 minutes and maintained at 4°C. After that, a mix of random primers, M-MLV Reverse Transcriptase (Life Technologies 28025-013) has been added to the samples and reverse transcription occurred using Thermal Cycle (GeneAmp PCR System 2400, Applied Biosystems). The conditions of reverse transcription have been: 37°C for 90'; 95°C for 5' and samples have been maintained at 4°C. After that, the obtained cDNA has been stored at 20°C or forthwith used for Real-Time PCR.

Real-time PCR

Real Time PCR assays were performed using Fast-Start Universal SYBR Green Master (ROX) from Roche

Table 1. Primers used during Real-time PCR.

GENE	For	Rev
GAPDH	5'-GAA GGT GAA GGT CGG AGT-3'	5'-CAT GGG TGG AAT CAT ATT GGA-3'
EZH2	5'-GCG GGA CGA AGA ATA ATC ATG	5'-CCA AAA TTT TCT GAC GAT TGG AAC-3'

(04913850001) and the fluorescence released were analyzed using iQ5 Real Time (BioRad, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is the housekeeping gene, which allowed the study of the variations of EZH2 gene expression. ΔΔCT method has been used in order to understand the differences between the two genes.

Primers used during Real-time PCR are listed in Table 1.

We described variables as mean and standard deviation or median and interquartile range, as appropriate. To test for difference between continuous variables *t*-test of Mann-Whitney U test were used depending on the distribution of the variable. Correlations between variables were evaluated calculating Spearman ρ and its significance.

RESULTS

We enrolled a total of 29 HBV-infected patients (14 receiving NAs and 15 naïves), with a mean age of 55 ± 12, 25 (86.2%) of them were male and 4 (13.8%) were female. Among NA-treated, 6 patients were receiving a combination of Adefovir + Lamivudine, 6 Tenofovir + Lamivudine and 2 Entecavir monotherapy. All patients receiving NAs had an undetectable HBV-DNA.

Patients receiving NAs had significantly higher ($p = 0.029$) levels of EZH2 expression when compared to untreated subjects, as shown in Figure 1. No difference was observed in terms of age and known infection duration. EZH2 levels strongly ($p = 0.45$; $p < 0.05$) correlated with cumulative NA-therapy duration (Figure 2) whereas no correlation with age and HBV-DNA was found.

In order to understand the expression of EZH2 in HBV patients treated pharmacologically with nucleos(t)ide analogues, we performed Real-Time PCR assays.

CONCLUSIONS

The present preliminary study demonstrates that EZH2 may be involved in HBV infection. EZH2 could be part of the regeneration mechanisms taking place during the course of HBV infection and treatment. Since EZH2 might work at several levels at different stages of the HBV diseases, further investigations are needed to characterize its role in the natural history of HBV-related tissue damage and regeneration, up to HCC. Studying EZH2 regulation in patients affected by HCC will be critical to understand whether EZH2 is a useful marker in a clinical setting.

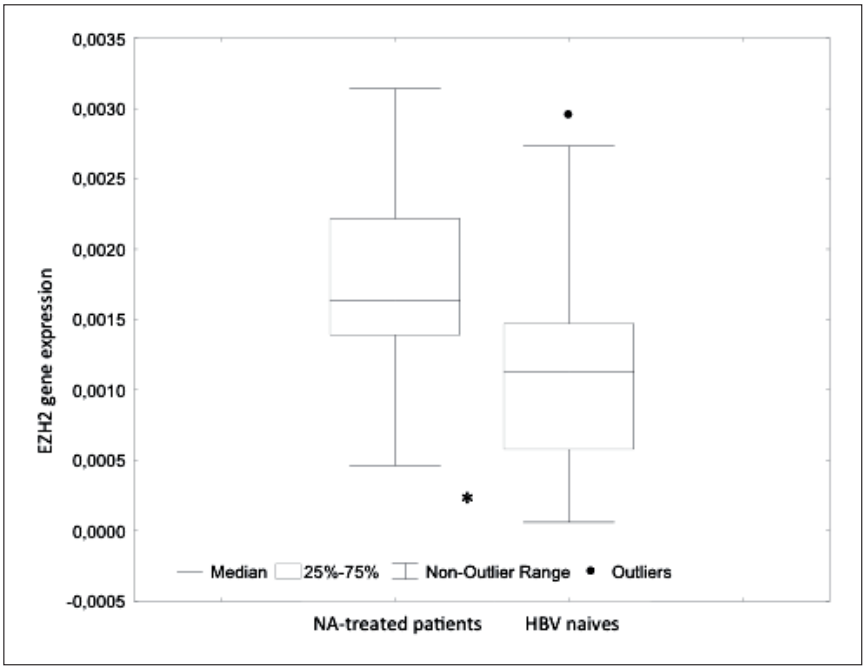

Figure 1. Comparison of Enhancer of Zeste Homologue 2 (EZH2) expression between HBV-infected patients receiving nucleos(t)ide (NA) therapy and HBV-infected naïves. *p=0.029.

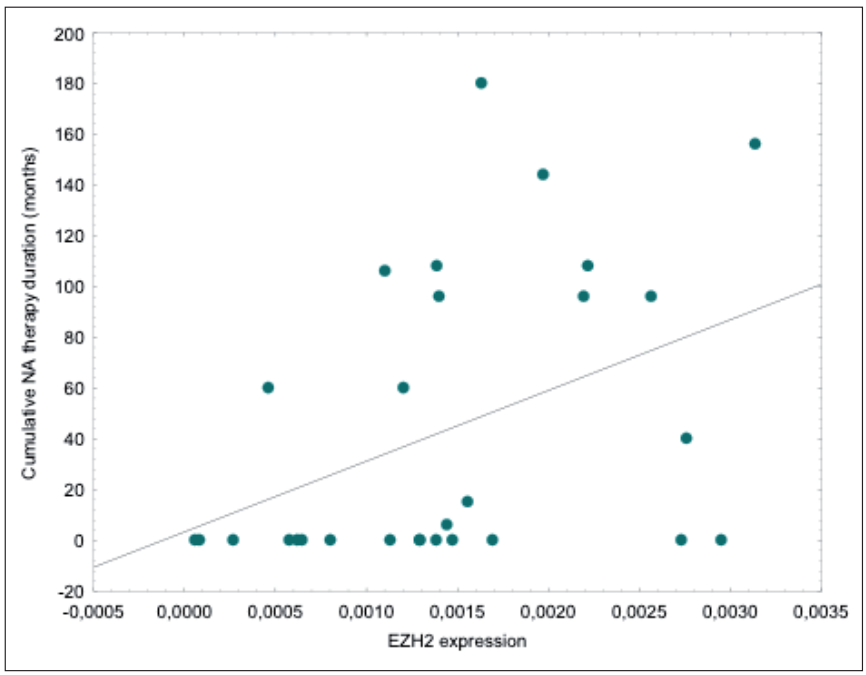

Figure 2. Correlation between cumulative nucleos(t)ide therapy duration and Enhancer of Zeste Homologue 2 (EZH2) gene expression in HBV-infected patients.

In the last decades, a non-coding small class of RNAs which contains ~22 nucleotides, called microRNA (miRNA), has been widely studied. miRNA are endogenously expressed and growing studies suggest that they are implicated in a multitude of human pathologies, such as cancer[24,25].

Recent studies showed the expression profile of miRNA in human HCC tissues, and one of them miR-101, showed a decreased expression[26]. Furthermore, the correlation between miR-101 expression and clinical pathological factors and prognosis in HCC was studied. Specifically, the level of miR-101 was deeply analyzed in the plasma of HCC patients and was found to be substantially down-regulated, compared to others miRNA. The down-regulation of miR-101 is correlated with distant metastasis and poor diagnoses in HCC patients[27]. Different miRNAs are able to regulate the activity of EZH2, but miR-101 appears to have a powerful negative association with cancer progression. Indeed, several studies showed that a loss of miR-101 causes over expression of EZH2 leading to cancer progression, metastasis, and poor prognosis. In parallel, EZH2 protein expression could be suppressed by up-regulation of miR-101 contributing to maintain the homeostasis condition[28].

The above mentioned molecular complex mechanisms need further investigations to clearly understand at which level EZH2 is involved in HBV-related liver disease and whether it could be used as a marker of HCC. Evaluating the expression of EZH2 and miRNAs in HBV infection will be critical to dissect the exact biological implications.

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Conflict of Interests:

The Authors declare that they have no conflict of interests.

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