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Case Control Study

Previous hepatitis B viral infection – underestimated cause of pancreatic cancer.

Previous HBV infection in PDAC development

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Abstract

BACKGROUND

Etiology of pancreatic cancer (PC) remains unclear. This limits possibility of the prevention and effective treatment. Hepatitis B virus (HBV) is responsible for the development of different types of cancer, but its role in PC is still being discussed.

AIM

To assess the prevalence of previous HBV infection (PBI) and to identify viral biomarkers in patients with pancreatic ductal adenocarcinoma (PDAC) to support the role of the virus in etiology of this cancer

METHODS

The data of 130 HBsAg-negative subjects were available for the final analysis, including 60 patients with PDAC confirmed by cytology or histology, and 70 sex- and age-matched controls. All the participants were tested for HBV biomarkers in blood (anti-HBc, anti-HBs, HBV DNA), and those with PDAC — the biomarkers in resected pancreatic tissues (HBV DNA, HBV pregenomic RNA and cccDNA). We performed immunohistochemistry staining of pancreatic tissues for HBxAg and Ki-67 protein. Non-parametric statistics was used for the analysis.

RESULTS

Anti-HBc were detected in 18\60 (30%) patients with PDAC and in 9\70 (13%) participants in the control group (p=0.029). Accordingly, the odds of PDAC in anti-HBc-positive subjects were higher compared to those with no PBI: Odds ratio (95% confidence interval) 2.905 (1.191-7.084), Standard Error 0.455. HBV DNA was detected in eight cases of PDAC, and in six of them — in the pancreatic tumor tissue samples only (all patients were anti-HBc positive). Blood HBV DNA was negative in all subjects of the control group with positive result of serum anti-HBc test.

Among nine patients with PDAC, in five we revealed signs of replicative competence of the virus (cccDNA with or without pgRNA) in the pancreatic tumor tissue samples. HBxAg expression and active cells proliferation was revealed by immunohistochemistry in four participants with PDAC in the pancreatic tumor tissue samples.

CONCLUSION

We found significantly higher risks of PDAC in anti-HBc-positive patients. Detection of viral replication and HBx protein expression in the tumor tissue prove involvement of HBV infection in pancreatic cancer development.

Key Words: Hepatitis B virus; previous hepatitis B; Occult hepatitis B virus infection; Pancreatic cancer; Pancreatic ductal adenocarcinoma

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Core Tip: Hepatitis B viral (HBV) infection is responsible for different types of cancer. However, its role in pancreatic ductal adenocarcinoma (PDAC) remains unclear. This study aimed to assess the prevalence of previous HBV infection and to identify viral biomarkers in patients with PDAC to support the role of the virus in etiology of this cancer. We found almost three-fold risks of PDAC in anti-HBc-positive patients. Detection of viral replication and HBx protein expression in the tumor tissue prove a possible involvement of HBV infection in pancreatic cancer development. Previous HBV infection is currently an underestimated cause of PDAC.

INTRODUCTION

Pancreatic cancer (PC) is aggressive gastrointestinal malignancy with low rate of early detection, poor survival and limited number of therapeutic options. It causes more than 430,000 deaths yearly worldwide[1]. This makes PC the third leading cause of cancer-related deaths in the US, and fourth in the EU[2, 3]. The incidence rate of PC is growing, while the improvement in the survival rates is negligible[4]. Pancreatic ductal adenocarcinoma (PDAC) is a most prevalent type of PC, found in 85% of cases[5]. The etiology of PC remains unclear, which limits the possibility for the prevention and effective treatment. Early detection of PC remains a challenge. Therefore, it is still relevant to explore etiological factors of PDAC further and to identify subjects at the risk of the disease. Numerous risk factors for the disease have been identified (smoking, excessive alcohol intake, history of chronic pancreatitis, obesity and diabetes, *etc*)[6]. Some viruses, including hepatitis B virus (HBV), are responsible for the development of different types of cancer, but their role in PC is still being discussed[7].

In endemic regions, like South-East Asia, the blood markers of current HBV infection are commonly found in subjects with PC [8]. Some authors from endemic regions report that HBV infection is not associated with the risk of developing PC after adjusting for age, sex, diabetes, and smoking[9].

Cohort studies from Northern Europe (Denmark, Sweden), where HBV infection is not that widespread, showed conflicting results and made an association of the PC and HBV infection questionable[10-12]. In most of these studies, association of PC with previous HBV infection was not considered. However, it may be important, as the risk of liver cancer development preserves even after HBsAg loss[13-15]

Cancerogenic mechanisms of HBV infection may be explained by the integration of viral DNA fragments into genome of host cells, persistence of the viral genome as a covalently closed circular DNA (cccDNA), which plays the role of viral reservoir and template for life-long synthesis of new virions. Both are responsible for preserved expression of viral proteins (especially HBx), that can lead to potentially oncogenic mutations[16-18].

Thus, not only active infection, but also the resolved one may contribute to pancreatic cancer development. Detection of HBV DNA and viral antigens in the pancreatic tumor tissues may provide direct evidence of the involvement of the virus in the etiology of this cancer. However, only a few studies demonstrated the presence of HBV DNA (cccDNA) and / or viral antigens in pancreatic tumor tissue.

Therefore, the aim of our study was to assess the prevalence of previous HBV infection (PBI) and to identify viral biomarkers in patients with pancreatic ductal adenocarcinoma to support the role of the virus in etiology of this cancer.

MATERIALS AND METHODS

Study population

The study was based on the data of complex examination of patients that applied for pancreatic cancer treatment to Moscow Clinical Scientific Center named after A.S. Loginov from January 2019 to November 2020 and subjects of the control group. The study (registered AAAA-A18-118021590196-1, AAAA-A20-120051990006-1 at www.rosrid.ru) was approved by Local Ethics Committee and was conducted in accordance with the Declaration of Helsinki (1968) and its consequent revisions. All subjects signed written informed consent form before the enrollment.

Inclusion criteria

Patients of both sexes, older than 18 y.o., willing to participate in the study were eligible.

In the group of pancreatic cancer, we enrolled patients with histologically or cytologically confirmed pancreatic ductal adenocarcinoma.

In the control group we enrolled generally healthy subjects who applied for routine check-up or treatment of other non-malignant gastrointestinal conditions, and whose data of abdominal ultrasound and/or computed tomography revealed no signs of focal lesions in the pancreas.

Exclusion criteria

Other/indeterminate types of pancreatic cancer, or non-malignant lesions beside PDAC (for the main group); positive blood test for HBsAg, HCV or HIV antibodies; past surgery for pancreatic cancer; current or previous treatment with interferons, nucleos(t)ide analogues for HBV infection or other reasons; clinically significant diseases or health disorders, making impossible to perform procedures required by the study protocol.

Confirmation of the conditions of interest

Previous HBV infection was defined as the presence of anti-HBc with or without anti-HBs or HBV DNA in serum[19].

Control subjects were matched for age (within 2 years), sex, and race/ethnicity with the PDAC patients. Study design is shown in Figure 1.

Study procedures

To exclude health conditions able to affect results of the study, all patients underwent routine diagnostic procedures (including, but not limited to: blood tests, ECG, abdominal ultrasound, chest X-ray) within standards of care.

All the participants were tested for HBV biomarkers in blood (HBsAg, anti-HBc, anti-HBs). Those HBsAg-negative with positive anti-HBc result were tested for HBV DNA in blood. Anti-HBc-positive patients with PDAC were examined for HBV DNA in the pancreatic tumor tissue.

Tumor tissues of anti-HBc-positive patients with PDAC underwent examination for HBV biomarkers (HBV pregenomic RNA and cccDNA) and immunohistochemistry staining for HBxAg and Ki-67 protein in case of the signed informed consent for these tests and sufficient quantity and good quality of the samples.

All anti-HBc-positive participants were tested for the presence of HBV DNA in the blood. In addition, all 18 anti-HBc-positive patients with PDAC were tested for the presence of HBV DNA in pancreatic tumor tissue. In 8 of them, the quality and quantity of samples were suitable for additional testing for HBV pregenomic RNA and cccDNA. Five patients had eligible samples according to these criteria and gave additional consent for immunohistochemical staining for HBxAg and Ki-67 protein.

Collection of samples. Blood samples were taken after overnight fasting, coded and processed immediately at the local laboratory according to the standard instructions.

Pancreatic tumor tissue samples were obtained during surgery or diagnostic biopsy, coded and processed locally, stained with Hematoxylin-Eosin and assessed by qualified morphologist.

Immunology. Serum samples were tested for HBsAg, anti-HBc IgG and anti-HBs, HCV and HIV antibodies. These tests were performed with the use of Sunrise analyzer (Tecan GmbH, Austria) and specific immunoassays kits (Vector-Best Co., Russia).

Analysis of HBV nucleic acids.

Plasma HBV DNA was isolated using commercial AmpliSens Riboprep kit (AmpliSens Biotechnologies, Russia) according to manufacturer's instructions and quantified using polymerase chain reaction (PCR) assay AmpliSens HBV-FL (AmpliSens Biotechnologies, Russia) kit (lower limit of detection of 10 IU/mL).

To isolate nucleic acids from biopsies, samples were first homogenized in the MagNA Lyser (Roche Diagnostics, Switzerland). HBV DNA was isolated by AmpliSens Riboprep kit (AmpliSens Biotechnologies, Russia) and quantified by AmpliSens HBV-FL (AmpliSens Biotechnologies, Russia) kit.

To quantify covalently closed circular DNA HBV (cccDNA), nucleic acids were first treated with T5 exonuclease (New England Biolabs, UK) at 37°C for 60 min and inactivation at 70°C for 20 min[20]. HBV cccDNA was quantified with specific sets of primers and probes and normalized to genomic β-globin.

Specific sets of primers (tab. 1) and TaqMan fluorescent probes were used for PCR analysis to detect HBV DNA in pancreatic tissue samples.

To analyze pregenomic HBV RNA (pgRNA HBV) analysis, nucleic acids were treated with RNase-free DNase I (NEB) for 30 min at 37 °Ç, purified by using AmpliSens Riboprep kit (AmpliSens Biotechnologies, Russia), reverse transcribed by AmpliSens Reverta-FL (AmpliSens Biotechnologies, Russia), and quantified by AmpliSens HBV-FL

(AmpliSens Biotechnologies, Russia) kit. CFX96 Real-Time System (Bio-Rad, USA) PCR machine was used for the analysis of plasma and pancreatic tissue samples.

Immunohistochemistry of pancreatic tissues was performed after deparaffinization. Slides were fixed in 4% paraformaldehyde, washed 3 times in Tris-HCl (50 mM, pH 8.0) followed by incubation with a blocking buffer (0.02% of Triton X-100, 10% horse serum, and 150 mmol/L NaCl in Tris-HCl, 50 mmol/L, pH 8.0) for 30 *min* and 1 *h* staining with primary rabbit anti-HBx (ab39716) (Abcam, UK). Then, slides were washed 3 times for 5 minutes in a washing buffer (0.02% of Triton X-100 and 200 mmol/L NaCl in Tris-HCl, 50 mmol/L, pH 8.0), incubated for 1 *h* with secondary Alexa Fluor 594 goat anti-rabbit antibodies (ab150080) (Abcam, UK). After that, the slides were treated with primary tagged Alexa Fluor® 488 rabbit anti-Ki-67 (ab197234) and Hoechst 33342 (ab228551) for 1 *h*, washed 3 times for 5 *min* in washing buffer and finally mounted with a Fluoroshield reagent (Abcam, UK). Images were captured using Thunder imaging systems (Leica Microsystems, Germany) with 10× objectives. Ki-67 and HBxAg staining was analyzed using LAS X (Leica Microsystems, Germany). Ki-67 index was counted as the percentage of Ki-67-positive cells[21].

Statistical analysis. Statistica 12.0 software (StatSoft Inc., USA) was used for analysis of the data. Statistical processing of the obtained data was carried out using nonparametric statistics. Quantitative indicators were preliminarily assessed for compliance with the normal distribution using the Kolmogorov – Smirnov and Lilliefors tests. When quantitative indicators' distribution differed from normal, we used medians (Me) and the interquartile ranges (IQR, [25%-75%]) for the description, and processed the data using Mann-Whitney U-test. Nominal data were compared using Pearson $\chi 2$ test with Yates's correction. *P*- values <0.05 were considered significant. Odds ratio (OR) and 95% confidence interval (95%CI) calculations were performed to assess the association between PDAC and previous HBV infection markers detection.

RESULTS

Data of 60 patients with PDAC and 70 participants of the control group were available for the final analysis. Demographic and viral characteristics of the participants are shown in tab. 2.

In patients with PDAC anti-HBc antibodies were found more often than in the control group (P = 0.029). Accordingly, the odds of PDAC in anti-HBc-positive subjects were significantly higher compared to those who had no PBI, Odds ratio (OR) [95% confidence interval (CI) limits]: 2.905 [1.191-7.084], Standard Error 0.455.

Overall, HBV DNA was found in eight anti-HBc-positive patients with PDAC: in two subjects it was detected in both, the blood and pancreatic tumor samples, whereas in the other six participants the testing gave positive results only in the pancreatic tumor tissues. No positive results on HBV DNA were obtained in the control group.

The data of special examination of blood and pancreatic tissue samples are shown in table 3. In five patients with PDAC markers confirming replicative competence of hepatitis B virus (cccDNA with or without pgRNA) were found in the pancreatic tumor tissue samples. In one subject with positive test on HBV DNA in the pancreatic tissue examination on cccDNA and pgRNA was not performed.

In those with detectable HBV DNA, viral load in the pancreatic tissue was (Me [25%-75%]) 632 [390-851] IU/mL.

HBxAg expression and active cells proliferation was revealed by immunohistochemistry in four participants with PDAC in the pancreatic tumor tissue samples (table 3, figure 2). The number of HBx-expressing cells in them did not exceed 10%.

Ki-67 proliferative index in subjects with PDAC in cohort of special examination was (Me [25%-75%]) 79.1 [45.2–86.4]. All HBx-expressing cells were also Ki-67 positive.

DISCUSSION

The results of the present study demonstrate association of previous HBV infection with PDAC and provide direct molecular evidence for the presence of HBV biomarkers in the pancreatic tumor tissue. In eight of our patients with PDAC HBV

DNA was detected in the pancreatic tumor tissue. In five of them, replicative competence of HBV DNA in the pancreas was supported by detection of cccDNA (with or without pgRNA). Identification of cccDNA and pgRNA (transcribed only from cccDNA) additionally suggests that these patients saved a silent replication of the virus in the pancreatic tissue. Detection of the virus nucleic acids in pancreatic tissue only (with no HBV DNA present in blood) in most of subjects excludes the possibility of artificial contamination of the tumor tissue samples.

Viral infections, including those caused by hepatitis B virus were recognized among the modifiable risk factors of pancreatic cancer development[22, 23]. The data of meta-analysis of case-control and observational studies (number of subjects with PC 5883) showed that odds of pancreatic cancer are significantly higher in chronic or inactive HBsAg carries (OR 1.60 (95%CI: 1.26-2.05)) and anti-HBc-positive but anti-HBs-negative individuals (OR 1.76 (95%CI: 1.05-2.93)) compared to those who were never exposed to HBV infection[7, 24]. In our study, the odds of PDAC were even higher, and anti-HBc-positive subjects had almost 3-fold greater chances of pancreatic ductal adenocarcinoma compared to the controls.

Only few studies have demonstrated molecular evidence of possible HBV involvement in pancreatic tumor development by identifying HBV DNA (cccDNA) and / or its antigens in pancreatic tumor tissue, and only limited number of subjects (especially HBsAg-negative, but anti-HBc-positive) were involved[25, 26]. Although certain pathogenetic mechanisms of PC associated with hepatitis B infection need to be explained in specially planned studies, some assumptions could be made.

Hepatitis B virus is a known carcinogen and is one of the main causes of hepatocellular carcinoma in endemic regions[8]. However, in HBV endemic areas, such as Africa and East Asia, show relatively low PC related death. Probably due to high mortality from other causes (including HBV-associated HCC), there is not enough time for the development of PC in people with previous HBV infection.

It integrates into genome of infected cells, causes genomic aberrations, enhances expression of oncogenes or inhibits tumor suppressors leading to the cancer

development [14]. Similar mechanisms are possible in non-liver cancerogenesis, including pancreas[25, 27]. Pancreatic beta cells and hepatocytes develop from the ventral foregut endoderm during ontogenesis and thus may share characteristics that are favorable for HBV replication and virus-induced tumor development[28]. It seems that malignant transformation in the pancreas is not provoked by direct cellular damage and is caused by the integration of HBV DNA into the genome of pancreatic cells and subsequent disruption of the functions of anti-oncogenes, or by stimulation of pro-oncogenes' activity [29]. The replication of HBV in pancreatic tissue may decrease with time. However, DNA fragments of the virus integrated into the genome of host cells continue to express viral proteins (especially regulatory protein X) of HBV responsible for carcinogenesis. Expression capability of HBx from integrated fragments of the viral genome in tumor tissues when replication is absent confirmed for HCC[30-32].

In our study, immunohistochemistry revealed expression of HBx in the pancreatic tumor tissue in four out of five HBsAg-negative and anti-HBc-positive patients with PDAC. Replicative competence of HBV (detected cccDNA) was found in three of them. This may mean that in one patient, expression of HBx was caused only by the integration of the virus into the genome of pancreatic cells. These fragments of viral DNA, which preserve the open reading frame and express HBx, may serve a basis for cancerogenesis in subjects with pancreatic ductal adenocarcinoma. Although this mechanism may play role in primary cancer development, its role in pancreatic cancer recurrence is not clear and further studies are necessary. The low-grade replication may also play role in HBV reactivation, especially in cases when immunodepressants are used. However, this question is insufficiently studied yet.

It is not clear whether the number of HBxAg expressing cells is important for cancer development. In hepatocellular carcinoma of HBsAg-negative HBV DNA-positive subjects, the relative number of HBxAg expressing cells is about 30% within the tumor tissue and 20% in the rest of the liver tissue[33]. Similar data for pancreatic cancer are lacking. According to our results, the number of cells producing HBxAg in PDAC is about 4%. It seems, that the number of cells producing HBx protein is less important

than their presence, at least for pancreatic cancer development. This may be indirectly confirmed by the fact that in all HBxAg-positive subjects in our study proliferative index Ki-67 was significantly higher than 50%, whereas similarly high values of this marker are found only in about 12% of subjects with PDAC[21, 34].

Detection of cccDNA in pancreatic tissue in HBsAg-negative subject supports the need for revision of the statements of the Taormina Workshop (2018), which defines occult HBV infection as the presence of replication-competent HBV DNA (*i.e.*, cccDNA in the liver and/or HBV DNA in the blood of people who test negative for HBsAg by currently available assays[16]. As extrahepatic HBV replication may occur in HBsAg-negative subjects (which was confirmed in the course of our study), it is reasonable not to indicate in the statement specific organ for HBV DNA (cccDNA) detection.

Involvement of previous HBV infection in pancreatic cancer development requires revision of the ultimate targets of antiviral treatment. "Sterilizing cure" (undetectable HBsAg in blood in combination with the absence of DNA HBV in any tissues, including cccDNA and integrated viral DNA) was recognized unachievable in the nearest future[35]. However, "functional cure" (defined as sustained clearance of HBs with or without HBs-seroconversion, and non-detectable HBV DNA in blood after the course of treatment) evidently cannot affect the expression of oncogenic proteins of HBV (especially HBx) and thus diminish the chances of the cancer development. Although it is impossible to achieve eradication of cccDNA and integrated fragments of the viral genome with currently existing means, this should be stated as the ultimate goal for future therapy options.

The limitation of the study is a relatively small number of the patients. Moreover, examination of the tumor tissues on cccDNA, pgRNA and HBxAg was possible for only part of 18 antiHBc-positive subjects with PDAC due, mostly due to the quality of the obtained specimens. Further randomized multicenter studies are necessary to confirm the obtained results, prove the role of HBV infection in the etiology of PC and clarify carcinogenic mechanisms in them.

CONCLUSION

Almost threefold risks of PDAC are found HBsAg-negative, but anti-HBc-positive subjects. Detection of silent viral replication and pro-oncogenic HBx protein expression in the tumor tissue prove involvement of HBV infection in pancreatic cancer development. Previous HBV infection seems to be an underestimated cause of pancreatic ductal adenocarcinoma to the moment.

ARTICLE HIGHLIGHTS

Research background

Etiology of pancreatic cancer (PC) is unclear. This limits possibilities for its prevention and effective treatment. Hepatitis B virus (HBV) is responsible for the development of hepatocellular carcinoma and different types of extrahepatic cancer, but its role in etiology of PC is still being discussed.

Research motivation

The epidemiological relationship of previous HBV infection with PC and identification of viral biomarkers within the tumor tissue may provide support for this. However, there is still a lack of such reports, especially from non-endemic regions for HBV infection.

Research objectives

In our study, we aimed to assess the prevalence of previous HBV infection and to identify viral biomarkers in patients with pancreatic ductal adenocarcinoma (PDAC) to support the role of the virus in etiology of this cancer.

Research methods

The data of 130 HBsAg-negative subjects were included in the final analysis (60 patients with PDAC confirmed by cytology or histology, and 70 sex and age matched controls). All the participants were tested for HBV biomarkers in blood (anti-HBc, anti-HBs, HBV

DNA), and those with PDAC — the biomarkers in resected pancreatic tissues (HBV DNA, HBV pregenomic RNA and cccDNA). Additionally performed immunohistochemistry staining of pancreatic tissues for HBxAg and Ki-67 protein. Non-parametric statistics was used for the analysis.

Research results

We have established that 18\60 (30%) patients with PDAC and in 9\70 (13%) participants in the control group (p=0.029) were anti-HBc-positive. HBV DNA was detected in eight anti-HBc-positive patients of PDAC (in six of them — in the pancreatic tumor tissue samples only), but none of the control group subjects. In five patients with PDAC we revealed signs of replicative competence of the virus (cccDNA with or without pgRNA) in the pancreatic tumor tissue samples. HBxAg expression and active cells proliferation was revealed by immunohistochemistry in four participants with PDAC in the pancreatic tumor tissue samples.

Research conclusions

Previous HBV infection seems to be an underestimated cause of PDAC to the moment.

Research perspectives

Larger studies are necessary to assess risks of PDAC in subjects with previous HBV infection and define HBV-associated mechanisms of cancerogenesis in them.

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