

**Abound hepatic mitosis:unusual morphology in the intrahepatic
cholelithiasis patient**

Running title:unusual morphology in the intrahepatic cholelithiasis patient

Abstract

Objective: To explore the clinicopathological features of abundant mitosis of the hepatocytes in intrahepatic cholelithiasis. **Methods:** The clinicopathological data of one case diagnosed as intrahepatic cholelithiasis was collected from Yantai Yuhuangding Hospital and the clinicopathological characters were discussed. **Results:** A 68-year-old man suffered from the pain in the right upper quadrant and radiology showed multiple stones in the gallbladder and left liver. The images suggested intrahepatic cholelithiasis. The patient received gallbladder and partial hepatectomy. A large number of mitosis was observed and twelve nuclear fissions were found under high magnification, even in some area pathological nuclear fission could be observed in morphology. On the basis of detection in laboratory, the diagnosis of intrahepatic cholelithiasis was made. The patient did not receive any therapy after surgery. The patient was in a good condition after 18 months follow-up. **Discussion:** Increased number of hepatic mitosis might be due to the stimulation from stones, hepatic biliary or secondary inflammatory. High index of proliferation should be prevented from the potential misdiagnosis of hepatic tumor.

Keywords: Intrahepatic cholelithiasis, Hepatocyte, Mitosis

Introduction

The liver is one of few organs with regenerative ability in human body. When the injury caused by trauma or chronic diseases, hepatocellular regeneration is activated and new cells are produced by mitosis. A number of studies have shown that liver regeneration requires the participation of both parenchymal cells and interstitial cells, which carry out complex internal interaction and coordination^[1-2]. In spite of this, hepatocellular mitosis is really unusual to be seen under the microscope except for viral infection or neoplasm interestingly. Intrahepatic bile duct stone is one kind of bile duct stones, which refer to stones in the bile ducts above and below the junction of the left and right hepatic ducts. Intrahepatic bile duct stones are mostly primary stones, but they can also be secondary to cholecystolithiasis, which is relatively common in western countries^[3]. Therefore, hepatolithiasis is mainly choledocholithiasis, but also cholesterol calculi. The disease is related to biliary bacterial infection, bile retention and parasite infection. The main symptoms of the patients are epigastric pain, jaundice, chills and fever. If the disease develops further, it can be complicated with biliary liver abscess, severe hepatocholangitis, liver function decompensation and other symptoms, seriously threatening the life and safety of patients. It is reported in the literature that there is a tendency to increase the number of cholesterol stones in the intrahepatic bile duct. Classification according to stone distribution and pathological changes of bile duct, The most famous is Nakayama classification^[4], which mainly classifies hepatolithiasis according to the distribution of bile duct stones, bile duct stricture and bile duct dilatation, in addition to considering the difference of stone composition and the presence or absence of cholecystolithiasis. According to the left and right hepatic ducts and the hepatic ducts above the hepatic segment, they were divided into central type and peripheral type, and according to the presence of extrahepatic stones, they were divided into intrahepatic type and extrahepatic type. At the same time, according to the different distribution of stones in the left and right hepatic lobes, they can be divided into L type (left lobe), R type (right lobe) and LR type (left and right lobe), bile duct stricture and dilatation are represented by S and D respectively, and according to the diameter of bile duct, 0, 1 and 2 are used to indicate the degree of stricture or dilatation, such as S0 for no stricture and D1 for mild dilatation. The stricture and dilatation of bile duct can be divided into common bile duct, common hepatic duct, central part and terminal part. The disease mostly occurs in China, Japan, South Korea and other Asia-Pacific regions, with an incidence of about 3.1% to 21.2%^[5]. Generally it is a bilirubin stone. Intrahepatic bile duct stones often associated with extrahepatic bile duct stones, concurrent bile duct obstruction, induced local infection and secondary bile duct stenosis which make stones difficult to discharge automatically. The disease is prolonged and could cause serious complications. Intrahepatic cholelithiasis is an important cause of death of benign biliary tract diseases^[6]. In recent years, surgical operation is the main treatment of hepatolithiasis, and the principles are as follows: removal of stones, relief of biliary stricture and obstruction, removal of stone sites and infectious lesions, unobstructed drainage and prevention of stone recurrence. The

operative methods include choledocholithotomy, choledochojejunostomy and hepatectomy, which can be combined with ultrasound, cholangiography, choledochoscopy and lithotripsy during and after operation. Among them, hepatectomy is the most effective and thorough surgical method in addition to liver transplantation, which can not only remove stones, but also remove the common sites of stones and damaged liver parenchyma at the same time. Surgical methods include traditional open hepatectomy and laparoscopic hepatectomy. Robotic surgery provides a new method for the treatment of hepatolithiasis [7]. Precision hepatectomy has become the most effective first-line treatment for hepatolithiasis. Combined with intraoperative ultrasound to accurately locate bile duct lesions and the extent of stones, the focus can be effectively removed, and the stone clearance rate is up to 95%. However, the postoperative recurrence rate is still as high as 3% to 15% [8-10]. The main reason is that (1) improper application of operation. For example, bilateral extensive multiple bile duct stones with liver parenchyma damage, because there is no technique of hepatectomy, only choledocholithotomy or choledochojejunostomy, or even choledochoduodenostomy, did not remove the stones and bile duct stricture, resulting in postoperative stones and residual lesions, repeated attacks of cholangitis, requiring multiple operations, which not only increased the pain, trauma and economic burden of the patients, but also brought difficulties to the follow-up treatment. (2) expand the application of hepatectomy blindly. Hepatectomy is mainly used for local liver parenchyma damage or bile duct stricture can not be corrected. But some liver tissues that were basically normal and could have been preserved were removed; (3) blind application of laparoscopic surgery. For example, patients with a history of biliary tract surgery still use laparoscopic surgery, and the conversion rate is very high, which increases the cost and burden of patients. (4) the procedure of laparoscopic hepatectomy is not reasonable. Choledocholithotomy is often performed first, and then hepatectomy. During the operation, a large amount of bile overflows and pollutes the abdominal cavity, and it is difficult to absorb under the endoscope, resulting in postoperative abdominal pain, fever and even residual peritonitis, which interferes with the judgment of the disease. The treatment of intrahepatic bile duct stone depends on accurate preoperative evaluation and scientific classification. The reasons for the above confusion are as follows: (1) the limitation of treatment technology itself. There are many treatment methods for the disease, but except for liver transplantation, other methods are not easy to cure, and there are many postoperative complications, high residual stone rate and recurrence rate. Even for hepatectomy, for bilateral extensive intrahepatic stones, only one side of the hepatic lobe with severe lesions can be removed, and the other side with mild lesions should be removed by intraoperative choledocholithotomy and choledochoscopy. For patients with many previous biliary tract operations, it is difficult to perform laparotomy or laparoscopy because of abdominal adhesion and changes of anatomical structure around the hepatic hilum. The general condition of the patient is poor, no matter what kind of operation, it is limited. The traditional operation requires multiple dilatation of the sinus, patients need to undergo multiple anaesthesia, and the hospital stay is longer^[11]; (2) lack of scientific classification of hepatolithiasis, (3) lack of

scientific, authoritative treatment system for hepatolithiasis widely accepted by surgeons. Most of the operators refer to the experience of other units to carry out the treatment of hepatolithiasis. At present, three-dimensional imaging technology has been gradually applied to surgical clinic, which is beneficial to accurate preoperative evaluation and classification of hepatolithiasis. 3D printing technology can make a simulated tissue and organ model, which can be used to simulate surgical operation [12]. It can also be used to verify the classification of hepatolithiasis before operation, so as to guide the operation more accurately. With the development of artificial intelligence, microelectronics and genetic biology, it is worth looking forward to the "parade diagnosis" and "lithotripsy" or gene regulation of bile duct micro-robot to remove stones.

Recently, in a pathological examination of a patient with intrahepatic bile duct stones, we found a large amount of mitosis in the liver. In view of this situation, we analyzed the patient.

Materials and methods

Clinical data collected

In our study, the case diagnosed as intrahepatic cholelithiasis with abundant mitosis was obtained from Department of Pathology, Yantai Yuhuangding Hospital. We collected the clinical data. This study was approved by the Ethics Committee of Yuhuangding Hospital in Yantai, Shandong Province.

Sample process and morphological observation

The samples were immersed in 10% buffered formalin for complete fixation after surgery, and then the tissue dehydration and paraffin embedding were carried out. 4µm sections were cut from tissue blocks for hematoxylin and eosin (HE) staining. Tissue morphology was observed under microscope.

The wax blocks were re-paraffin-embedded and sectioned and stained with conventional HE. Three pathologists with senior professional titles re-read the diagnosis by double-blind method and re-checked the results of immunohistochemical staining.

Main reagents	production company
Mouse anti-human K67 monoclonal antibody; clone: IMB-1	Beijing Zhongjinqiao Biotechnology Co., Ltd.
Secondary antibody and cell pretreatment cleaning buffer(Tris buffer)	ROCHE Company
DAB chromogenic solution kit, EDTA repair solution	ROCHE Company
absolute ethyl alcohol	Tianjin Beichen Founder Chemical Reagent Factory
Tris-HCL, BSA ,ammonium persulfate	fresco, Inc

Hematoxylin-eosin staining

Hematoxylin-eosin staining, referred to as HE staining, is one of the commonly used staining methods in paraffin section technology. Hematoxylin dye is alkaline, which mainly makes the chromatin in the nucleus and nucleic acid in the cytoplasm purplish blue; eosin is an acid dye, which mainly makes the components in the cytoplasm and extracellular matrix red. HE staining is the most basic and widely used technical method in histology, embryology, pathology teaching and scientific research.

1. Paraffin tissue is sliced and baked at 80 °C × 30min
2. Soak slices in xylene solution for 5 minutes × 3 times
3. Soak the slices in 100% alcohol solution for 2 minutes
4. Soak the slices in 90% alcohol solution for 2 minutes
5. Soak the slices in 80% alcohol solution for 2 minutes
6. Soak the slices in 70% alcohol solution for 2 minutes
7. Rinse the slices for 5 minutes
8. Soak slices in hematoxylin solution for 5 minutes
9. Rinse the slices for 2 seconds
10. Soak in 1% hydrochloric acid ethanol solution for 2 seconds
11. Rinse the slices with running water
12. Soak the slices in eosin staining solution for 2 minutes
13. Rinse the slices with distilled water for 2 seconds
14. Wash the slices slightly in 80% alcohol solution
15. Soak the slices in 95% alcohol solution for 3 seconds
16. Soak the slices in 100% alcohol solution for 5 seconds
17. Soak the slices in xylene solution for 2 minutes × 3 times
18. Seal the slices with neutral gum

Immunohistochemical staining

EnVision two-step method was adopted by automatic immunostainer (VENTANA) for immunohistochemical staining and DAB color. Each slice was stained with known positive tissues as the positive control, while negative control replaced the first antibody with PBS. All the antibodies used in this study were bought from Roche Company.

Immunohistochemical procedure

1. The slides were placed in a mixture of potassium dichromate and concentrated H₂SO₄, then washed in clean water, soaked in alcohol, then placed on a shelf and placed in a 37 °C incubator, and polylysine was coated on the surface of the slides.
2. First, add some liquid paraffin to the mold, cool it slightly, then put the tissue to be embedded in the paraffin and arrange it neatly, and finally add a little liquid paraffin to freeze the paraffin to make the paraffin solid.
3. Paraffin tissue sections were placed at 60 °C for 60 minutes.
4. Soak the slides in xylene I-xylene II-100% alcohol-95% alcohol-90% alcohol-80% alcohol-70% alcohol sequentially and soak 10min in each reagent.

5. After tissue dewaxing, rinse in clean water for a period of time, add 3% hydrogen peroxide to soak 10min, wash twice in clean water, then add citric acid buffer, cook twice in microwave oven, each time 3min
6. After cooling to room temperature, pour out the citric acid buffer, wash it twice, 5min the slides in PBS, wash twice, dry the excess PBS solution, immediately add the serum, and then put it in the 37 °C temperature box for half an hour.
7. Remove the slide from the incubator, dry the serum around the slide tissue with absorbent paper, add primary antibody, and store it overnight in a 4 °C refrigerator.
8. Remove the slide from the refrigerator, wash it in PBS for 3 times, 5min each time, dry the PBS around the tissue, add secondary antibody, and place it in a 37 °C incubator for half an hour.
9. Remove the slide from the incubator, wash it in PBS for 3 times, 5min each time, dry the PBS around the tissue, add SABC, and place it in a 37 °C incubator for half an hour. SABC dilution 100x.
10. Remove the slide from the incubator and wash it in PBS for 3 times, each time 5min, dry the PBS around the tissue and add a chromogenic agent.
11. After rinsing the colored slides with clean water for a period of time, soak them in hematoxylin and dye them.
12. After rinsing the re-dyed slides in water, put the slides in 70% alcohol-80% alcohol-90% alcohol-95% alcohol-100% alcohol-xylene-xylene in turn. 2min was placed in each reagent, then soaked in xylene and placed in the ventilation cabinet.
13. Drop the neutral gum next to the tissue and cover it with a cover slide to avoid bubbles. Seal the slices and place them in a ventilation cabinet to dry.

Results

Clinical data

A 68-year-old Chinese male was sent to the hospital due to the pain in the right upper quadrant for three months ago. During the physical examination, the taps pain of the liver area was revealed obviously. No enlarged lymph nodes were touched in bilateral neck, axillary and groin. Multiple stones were found in the gallbladder and left liver by Magnetic Resonance Cholangiopancreatography (**Figure 1A**). The blood, liver function and virus hepatitis detection were normal by the laboratory examination. The patient did not take hormone drugs and denied the medical history of gout, hepatitis and tuberculosis. The patient had the habit of drinking a small amount of alcohol occasionally. Then, the patient received gallbladder and partial hepatectomy.

Gross examination

The sample obtained after surgery was showed that one part of liver tissue with the volume 10cmx7.5cmx4.5cm and the volume of gallbladder was 4.5cmx2cmx2cm. The cut surface was gray and the Intrahepatic bile duct expanded. Several stones were found in the liver and gall bladder. The stone obstructed the lumen and the liver tissue was tough with no obvious mass observed.

Histological findings

Morphologically, part of the lining of the epithelium in the dilated bile duct wall was absent in which fibrosis and inflammatory cell infiltration were accompanied. Stones were also seen in the lumen. The structure of hepatic lobule and manifold were not destroyed. The hepatic cords were normal and lymphocytes infiltrated in hepatic manifold (**Figure 1B**). Hepatocyte degeneration and cholestasis in cytoplasm were showed. The nucleuses were enlarged, the nuclear membrane was thickened and the nucleolus was visible. Active growth was evidenced by a large number of mitosis. Twelve nuclear fissions were found under high magnification even in some area where pathological nuclear fission could be observed (**Figure 1C**).

Immunohistochemical staining

Immunohistochemical staining of Ki67 revealed that the hepatocyte proliferation index was approximately 20% (**Figure 1D**).

Discussion

Hepatolithiasis is a common gallstone disease, but stones are segmentally distributed along the intrahepatic bile duct tree, resulting in many complications such as bile duct obstruction, local stricture or liver abscess, threatening the life and safety of patients. Hepatectomy is one of the main methods for clinical treatment of the disease, which can not only remove the focus, but also remove all the stones, relieve bile duct obstruction and improve the symptoms and condition of patients. Long operation time will not only enhance the stimulation of anesthesia to patients, but also prolong the time of wound infection and increase the incidence of incision infection, abdominal infection and biliary tract infection . It can also cause the attack of acute cholangitis, plus the second operation will increase the separation of wound, increase postoperative abdominal exudation and increase the risk of postoperative abdominal infection. In addition, most patients with secondary surgery will have residual stones or stricture of choledochojejunostomy, which will cause atrophy of the involved hepatic lobe, damage the structure of the hepatic hilum, affect postoperative recovery, cause inflammatory reaction, and lead to wound inflammation or infection. The abnormal blood supply of biliary tract in patients with a history of biliary surgery, coupled with the attack of cholangitis, can lead to scar formation and thickening of bile duct wall. After the second surgical treatment, it will affect the healing of biliary tract and increase the incidence of biliary leakage or biliary bleeding . Preoperative low albumin is easy to increase the nutritional risk of patients and reduce their postoperative immune ability, which will not only affect the prognosis of patients, but also not conducive to the recovery of liver function, but also increase the risk of postoperative infection and even increase mortality. For patients with hepatolithiasis, adequate surgical preparation should be made, and appropriate surgical procedures should be selected combined with the results of imaging examination. Before operation, we should pay attention to the nutritional intake of patients and guide their reasonable and healthy diet. at the same time, we can work with dietitians to

formulate the corresponding diet. If the patient has diabetes, we can change the diet accordingly to ensure that the blood sugar of the patient is normal and supplement sufficient protein. The operation can only be carried out after the nutritional index reaches the standard. At the same time, nursing supervisors should strengthen the surgical training and assessment of nursing staff, organize them to participate in the study of surgical cooperation nursing in other colleges and universities, improve their nursing level of surgical cooperation, and at the same time, strengthen communication between doctors and nurses and cultivate tacit understanding. It is helpful to improve the efficiency of surgical cooperation and shorten the time of operation. In the course of the operation, the nursing staff should stop the bleeding in time to ensure the clear visual field of the operation, at the same time, strictly follow the principle of aseptic operation, remind the doctor to pay attention to the operation strength and reduce the unnecessary injury at any time. It can avoid the occurrence of infection to the maximum extent, prevent the use of antibiotics after operation, and reduce the infection rate of patients.

Mitosis is the process by which eukaryotic cells divide the chromosomes into two nuclei in their nucleus. After mitotic division, the cytoplasmic division is usually accompanied, and cell structures such as cytoplasm, organelle and cell membrane are equally distributed into daughter cells^[13-14]. Mitosis and cytokinesis are defined as the cleavage phase of the cell cycle, or the M phase. This process produces two daughter cells that are identical to the parent cell gene^[15-17]. This process typically accounts for about 10% of the entire cell cycle. The mitosis process is highly complex and regular. During mitosis, chromatin forms a pair of chromosomes and is pulled by the microtubules of the spindle, dragging the sister chromatids to the cell poles^[18]. Mitotic spindle is an apparatus which forms during cell division and promotes sister chromatids segregation through the anaphase. The mitotic spindle is composed of a variety of proteins among which tubulins are predominant. The chromosomes attached to the tubulins via the kinetochore proteins actively monitor spindle formation and prevent premature anaphase onset. The cells then enter the cytoplasmic division, producing two cells with the same genetic makeup. Mistakes in mitosis could kill the cell by apoptosis or cause mutations that cause cancer^[19-22].

Hepatocytes belong to completely regenerative cells but an increase in hepatic mitosis is unusually seen under pathological conditions except for acute viral hepatitis and drug-induced hepatitis, such as infectious mononucleosis and leptospirosis^[23-24]. The nuclear material staining of normal mitotic hepatocytes is consistent with that of normal hepatocytes, and there is no specificity, and most of them are two closely adjacent and similar offspring hepatocytes. In abnormal division, the nuclear material of the liver was stained specifically, shining brightly, and the nuclear chromosomes were loose, fragmented, cord-like or fine-grained. Recent studies have shown that a boosted hepatocyte turnover occurring in a context of metabolic liver disease contributes to the induction of the chromosomal instability (CIN) that represents one of the key features of HCC tumorigenesis. CIN is sensed as DNA damage and induces a signaling pathway named DNA damage response (DDR)^[25]. The DDR is involved in several aspects of DNA integrity because it affects several cellular processes such

as mitosis, senescence, apoptosis. In other tumors, an increased rate of CIN generates diverse karyotypes within a tumor, the term chromosomal instability describes a rate of change i.e. a dynamic feature of chromosome pathophysiology [26]. This affects chromosome number and structure and is a characteristic of many cancer types including HCC, it is also associated with the formation of extranuclear bodies that contain damaged chromosome fragments or whole chromosomes [26-27]. By now, the appearance of abundant hepatic mitosis in the patient who suffers from cholelithiasis has not been retrieved. The patient in our report had no history of hepatitis and taking medicine. After surgery there were about one and a half year and he survived very well at present. The proliferative index of normal hepatocytes is less than 1%. Increased number of hepatic mitosis might be due to the stimulation from stones, hepatic biliary or secondary inflammatory [28].

High index of proliferation should be prevented from the potential misdiagnosis of hepatic tumor. As far as differential diagnosis was concerned, the lesions showing much more mitosis in liver should be ruled out. (1) Acute hepatitis. Liver cells turbid and swollen, edema-like changes and balloon-like changes. Inflammatory cells infiltrated in the lobes, mainly lymphocytes, and scattered in the point of necrosis. Hepatocyte regeneration and repairing, manifested as nuclear division or increased nuclear phenomenon. The laboratory tests could detect the virus in the blood [29]. (2) Large cell changes in hepatocytes of nodular cirrhosis. The number of large cells increases with the age of the patients without P53 gene mutation, so this change is considered to be a cell proliferation disorder, rather than precancerous lesions. It is characterized by the increase of the volume of hepatocytes and nucleus to 2 to 3 times normal. At the same time, the nucleus is heteromorphic, showing enlarged nucleoli, multi-nuclear and nuclear chromatin staining. Mitosis could be observed in large cells [30]. (3) Liver neuroendocrine tumor. The tumor cells could be arranged as trabecular, banded, solid tumor nest, acinar or tubular. Tumor cells are small with abundant cytoplasm. Nucleus is round or oval with deep staining. Mitotic figures are more common and Ki-67 index is significantly increased. Neuroendocrine granules are visible in the lesion and immunohistochemistry chromogranin (CgA), neuron specific enol (NSE), Syn are positive expression [31]. (4) Hepatocellular carcinoma. Carcinoma cells are arranged in a solid mass, surrounded by dilated sinusoids. The border of cancer cells is unclear. The nucleus usually has an abnormal shape and the mitotic figures are easy to be observed. Forming a clear mass, the disappearance of the liver plate and hepatic sinus is a distinguishing point from the normal liver in morphology [32].

Conflict of Interest

The authors declare that there are no conflicts of interest.

Figure legends

Figure 1. [A] Multiple stones were found in the gallbladder and left liver by Magnetic Resonance Cholangiopancreatography. [B] The hepatic cords were normal and lymphocytes infiltrated in portal area. Hepatocyte degeneration and cholestasis in cytoplasm were showed (4×). [C] The hepatocellular nucleus was enlarged and the nucleolus was visible. Twelve nuclear fission were observed under high magnification in some area and pathological nuclear fission could also be found (showed by red arrows, 20×). [D] Immunohistochemical staining of Ki67 revealed that the proliferation index was about 20%.

