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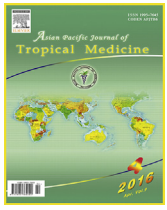

Original research http://dx.doi.org/10.1016/j.apjtm.2016.03.001

Pregnane receptor gene polymorphisms, pathogenic bacteria distribution and drug sensitivity, and TCM syndrome differentiation in patients with cholelithiasis

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ARTICLE INFO

Article history:
Received 15 Jan 2016
Received in revised form 20 Feb 2016
Accepted 1 Mar 2016
Available online 8 Mar 2016

Keywords:
Cholelithiasis
Cholesterol gallstone disease
Drug resistance test
Pregnane X receptor
Polymorphism
Traditional Chinese medicine syndromes

ABSTRACT

Objective: To investigate the distribution of pathogens and drug resistance in bile and the association between the pregnane X receptor (*PXR*) gene polymorphisms, traditional Chinese medicine (TCM) syndromes and the risk of cholesterol gallstone disease (CGD).
Methods: A total of 392 samples were enrolled in this study from January 2014 to February 2015, among which 192 patients were with CGD, and 200 samples were healthy. Strains were isolated and susceptibility testing was the disk diffusion method susceptibility testing. The patients were divided into hepatochlic hygropyrexia, stagnation of liver-qi, and the accumulation of damp. The *PXR* gene polymorphisms were analyzed by polymerase chain reaction-restriction fragment length polymorphism. The association between the *PXR* gene polymorphisms and the risk of CGD was examined by logistic regression analysis.
Results: A total of 192 cases were detected in 230 of bile culture pathogens, including Gram-negative bacteria 133 (57.83%), Gram-positive bacteria 76 (33.04%), and fungi 21 (9.13%). The top five pathogens were *Escherichia coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Candida albicans*, and *Enterococcus feces*, of which 110 cases was of single infection, 48 cases of mixed infection of two strains, eight cases of mixed infection of three bacteria. Among 59 *Escherichia coli*, the yield extended-spectrum beta-lactamases had 40 (67.80%). The hepatochlic hygropyrexia was the most TCM syndrome, followed by stagnation of liver-qi, and the accumulation of damp was least. Different pathogens and the *rs6785049* genotypes distributed differently in cholelithiasis patients with different TCM syndromes ($P < 0.05$). In hepatochlic hygropyrexia patients the Gram-negative bacteria was most. There was significant differences between CGD group and control group in *rs6785049* ($P < 0.001$). Comparison with wild-type portable GG, GA genotype increased the risk of the occurrence of gallstones ($OR = 0.40$, 95%*CI*: 0.16–0.79); likewise, carrying the GA+AA genotype also increased the risk ($OR = 0.38$, 95%*CI*: 0.19–0.81). There was no significant differences in *rs2276707*, *rs3814055* site polymorphic loci alleles in CGD group and control group.

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Peer review under responsibility of Hainan Medical College.

Foundation Project: This study was supported by the National Science Foundation of China (30672698).

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Conclusions: In the treatment of cholelithiasis, bile samples should be collected for bacterial culture and sensitivity test, and drugs should be strictly chosen based on the results. The *rs6785049* polymorphisms in *PXR* gene may increase the risk of gallstones ontogeny, and gallstones can be early detected and prevented by detecting genotypes. *rs6785049* polymorphisms in *PXR* gene may has relationship with TCM syndromes.

1. Introduction

Cholelithiasis, also known as cholesterol gallstone disease, involves the formation of gallstones that disrupt the proper draining of bile from the gallbladder. Symptoms include a swollen, painful abdomen, radiating pain, yellowing skin, and sometimes fever. Improved living conditions and nutritional status among the Chinese has contributed to an increase in cholelithiasis in recent years [1]. The formation of gallstones has been linked to a variety of environmental and dietary factors, and some studies suggest that genetics also play a role [2]. Specifically, the pregnane X receptor (PXR) has been found to be closely associated with cholelithiasis, as *PXR* gene polymorphisms have been shown to reduce the risk of gallbladder and bile duct stone formation [3–6]. PXR is a member of the nuclear receptor subfamily and numerous studies have found that alterations to the *PXR* gene can affect cholesterol metabolism, reduce gallbladder motility, influence the excretion of bile and cholesterol, and regulate the cholesterol saturation index so as to affect the formation of gallstones [3,7–11].

According to traditional Chinese medicine (TCM), chronic gallstone formation is the result of qi stagnation, blood stasis, and accumulation of fluid in the body, or dampness [12]. TCM suggests that the pathogenesis of chronic cholelithiasis involves the invasion of exogenous factors via skin, hair, or interstices of the flesh into the interpleuro-diaphragmatic space, where they then accumulate and interfere with bile drainage [13]. The accumulated and stagnated bile then leads to the formation of stones. Emotional disorders may also contribute to the stagnation of the liver qi and qi depression in the gallbladder, further resulting in stagnated bile. Additional causes may be related to excess phlegm, blood extravasation, or roundworm stagnation in the liver and gallbladder, causing poor bile excretion and coagulation into stones. These factors may act alone, or in combination.

The relationship between bile duct bacterial strains and TCM syndrome differentiation related to cholelithiasis was assessed in this study, and the role of *PXR* gene polymorphisms in the development of cholelithiasis was also determined. Bile bacterial cultures and blood samples collected from cholelithiasis patients were compared to samples from healthy individuals and the bacteria were subjected to antibiotic screening.

2. Materials and methods

2.1. Study participants

Samples from 192 patients with cholelithiasis treated in our hospital between January 2014 and February 2015 were selected for this study. The patient group was made up of 110 males (57.29%) and 82 females (42.71%), with an average age of (61.18 ± 9.72) years. The diagnosis of all 192 patients was clinically confirmed, and 200 healthy individuals receiving physical examination during the same period in our hospital were selected as a control group. The control group was made up of 118 males (59.00%) and 82 females (41.00%), with an average age of (60.35 ± 8.16) years. There were no statistically significant differences in gender or age between the case group and the control group. All patients and healthy participants were Han Chinese. This study was approved by the Ethics Committee of Affiliated Hospital of Hainan Medical University and informed consent was obtained from all participants.

2.2. Identification of bacterial strains

A total of 4–6 mL bile were collected from patients and placed in sterile culture tubes. Each sample was divided into two portions; one to be used for bacterial culture and drug sensitivity testing and another for anaerobic culture. Strain identification was performed according to the National Clinical Laboratory Procedures manual. A VITEK-2 Compact Automated Bacteria Analyzer (bioMerieux, France) was used to perform drug sensitivity testing using a paper strip. Drug sensitivity determination was performed in accordance with the Executive Standard for Sensitivity Testing for Antimicrobial Drugs published by the Clinical and Laboratory Standards Institute.

2.3. Antibiotic resistance test

Antibiotic resistance was determined among *Escherichia coli* (*E. coli*) and *Klebsiella pneumoniae* samples by testing for the presence of extended-spectrum beta-lactamases (ESBLs) using the disk diffusion test recommended by the Clinical and Laboratory Standards Institute.

2.4. PXR gene polymorphism determination

Genomic DNA was extracted using phenol/chloroform and polymerase chain reaction (PCR) restriction fragment length polymorphism was used to determine the *PXR* gene polymorphism. Genotyping was performed as follows: *rs2276707*: upstream PCR primer 5′CTGAGAAGCTGCCCCTCGAT3′, downstream PCR primer: 5′CTCATGCAGCTGCA GCTTCTTC3′; annealing temperature: 57 °C; restriction endonuclease: *Mbo* I. *rs6785049*: upstream primer: 5′CCCA TAATCCAGAAGTTGGGCG3′, downstream primer: 5′GCA GAGCTGTCTGCTGGGTTGT3′; annealing temperature: 51 °C; restriction endonuclease: *Hin*6 I. *rs3814055*: upstream primer 5′TCATTTTTTGGCAATCCCAGGAT3′, downstream primer: 5′TCTGGCAACAGTAAAGCAGGGG3′; annealing temperature: 50 °C; restriction endonuclease: *Mbo* I.

Each PCR reaction totaled 20 μL, and contained 10 μmol/L of primers (0.3 μL), 0.8 μmol/L dNTPs, 1.5 μmol/L $MgCl_2$, 80 μg/μL genomic DNA, 0.5 unit of REDTaq DNA polymerase, and 15.7 μL H_2O. A PTC 200 Therma Cycler (Promega Corporation, USA) was used to perform the reactions. Each PCR reaction involved denaturing at 95 °C for 5 min, followed by 35 cycles at 95 °C, each for 30 s, annealing at 70 °C, and extension at 70 °C for 45 s. For restriction fragment length polymorphism analysis, the PCR products of *rs2276707*, *rs6785049*, and *rs3814055* were digested with *Mbo* I, *Hin*6 I, and *Mbo* I endonuclease, respectively. PCR products were electrophoresed in 2% sepharose gel and stained with ethidium bromide for band visualization.

2.5. TCM syndrome differentiation

Cholelithiasis patients were divided into three types according to the Guiding Principles for Clinical Research on New Drugs of TCM and Specifications for TCM Syndrome, published by the Chinese Ministry of Health. Patients were determined to have dampness-heat syndrome of the liver and gallbladder based on the presence of yellow urine, bitter taste in the mouth, fullness and distention or paroxysmal pain of the lateral thorax, occasional unbearable angina extending to back and shoulder, loss of appetite, yellowness of body and eye, constipation, yellow and greasy tongue coating, and wiry pulse. Hepatic qi stagnation was determined based on the presence of significant angina at the right lateral thorax or under the xiphoid process, lateral thorax pain or angina radiating to the back, nausea, vomiting, loss of appetite, bitter taste, dry mouth, aversion to greasy foods. Some patients also suffered from fever, fiery temperament, irritability, hiccups, belching, dark red tongue, thin yellow or thin white tongue coating, and thread, slippery pulses. Noxious dampness accumulation was determined by the presence of fever and aversion to cold or alternating episodes of fever and chills, unbearable pain below the right lateral thorax, abdominal tenderness, tension of abdominal muscles, bitter taste, dry throat, yellow body and eyes, cold limbs, in severe cases, coma and delirium, red or crimson tongue, yellow dry tongue coating, and slippery, rapid pulses.

2.6. Statistical analysis

Epidata 3.1 (Epidata Software, Denmark) was used to create a database for data entry using the double entry method, followed by logic checks. SAS 9.2 (SAS Institute, USA) was used for data processing. The statistical methods used to analyze data were the χ^2 test and non-conditional logistic regression analysis. A $P < 0.05$ was considered to be statistically significant.

3. Results

3.1. Pathogenic bacteria distribution

A total of 230 strains of pathogenic bacteria were detected among the bile cultures, including 133 strains of Gram-negative bacteria (57.83%) and 76 strains of Gram-positive bacteria (33.04%), as well as 21 strains of fungus (9.13%). The five most common organisms were *E. coli*, *Klebsiella pneumoniae*, *Enterococcus faecalis*, *Candida albicans*, and *Enterococcus faecium*. There were 110 cases of single-fungus infection, 48 cases of mixed infection of two bacteria, and eight cases of mixed infection involving at least three types of bacteria and/or fungus (Table 1).

Table 1
Bile pathogen distribution in cholelithiasis disease.

Pathogens	*n*	% Of total cases
Gram-negative bacteria	133	57.83
Escherichia coli	59	25.65
Klebsiella pneumoniae	17	7.39
Enterobacter cloacae	12	5.22
Bacteroides fragilis (anaerobic)	12	5.22
Enterobacter birth	9	3.91
Acinetobacter baumannii	8	3.48
Pang proteus	7	3.04
Other gram-negative bacteria	9	3.91
Gram-positive bacteria	76	33.04
Enterococcus faecalis	16	6.96
Feces Enterococcus	13	5.65
Streptococcus viridans	9	3.91
Lead yellow *Enterococcus*	8	3.48
Quail chicken *Enterococcus*	7	3.04
Clostridium perfringens (anaerobic)	6	2.61
Streptococcus anaerobic digestion (anaerobic)	6	2.61
Hard acid bacteria (anaerobic)	6	2.61
Other Gram-positive bacteria	5	2.17
Fungus	21	9.13
Candida albicans	16	6.96
Candida tropicalis	5	2.17
Total	230	100.00

3.2. Antibiotic resistance among bile bacteria cultures

Fifty-nine strains of *E. coli* were identified among the cholelithiasis patients and 40 tested positive for ESBLs (67.8%). Gram-negative bacteria were resistant to ampicillin, nitrofurantoin, erythromycin, ciprofloxacin, and ceftriaxone, while remaining

Table 2
Antibiotic resistance of Gram-negative bacilli.

Antibacterials	*Escherichia coli*		*Klebsiella pneumoniae*	
	Investigated number (*n*)	Number and resistance rates [(*n*)%]	Investigated number (*n*)	Number and resistance rates [(*n*)%]
Ampicillin	59	50 (84.75)	17	17 (100.00)
Sulbactam	42	23 (54.76)	13	8 (61.54)
Amikacin	59	3 (5.08)	17	3 (17.65)
Aztreonam	59	32 (54.24)	17	7 (41.18)
Nitrofurantoin	59	10 (16.95)	17	5 (29.41)
Ertapenem	55	0 (0.00)	17	0 (0.00)
Amoxicillin	59	10 (16.95)	17	11 (64.71)
Trimethoprim	59	38 (64.41)	17	15 (88.24)
Ciprofloxacin	59	49 (83.05)	17	11 (64.71)
Meropenem	53	0 (0.00)	16	0 (0.00)
Tazobactam	59	2 (3.39)	17	3 (17.65)
Cefepime waning	59	25 (42.37)	17	5 (29.41)
Gentamicin	59	22 (37.29)	17	5 (29.41)
Cefoperazone	56	1 (1.79)	17	4 (23.53)
Ceftriaxone	59	44 (74.58)	17	14 (82.35)
Ceftazidime	57	24 (42.11)	17	8 (47.06)
Cefoxitin	59	12 (20.34)	17	4 (23.53)
Cefazolin	59	48 (81.36)	17	15 (88.24)
Tobramycin	59	13 (22.03)	17	5 (29.41)
Imipenem	51	0 (0.00)	17	0 (0.00)
Tigecycline	50	0 (0.00)	15	0 (0.00)
Levofloxacin	59	47 (79.66)	17	15 (88.24)

Table 3
Antibiotic resistance of enterococci.

Antibacterials	*Enterococcus faecalis*		Feces *Enterococcus*	
	Investigated number (*n*)	Number and resistance rates [(*n*)%]	Investigated number (*n*)	Number and resistance rates [(*n*)%]
Ampicillin	16	6 (37.50)	13	9 (69.23)
Nitrofurantoin	15	0 (0.00)	13	3 (23.08)
Erythromycin	16	4 (25.00)	13	13 (100.00)
Ciprofloxacin	16	2 (12.50)	13	9 (69.23)
Clindamycin	14	14 (100.00)	13	13 (100.00)
Quinupristin	16	16 (100.00)	13	0 (0.00)
Linezolid	16	0 (0.00)	13	1 (7.69)
Streptomycin (high units)	14	2 (14.29)	13	9 (69.23)
Moxifloxacin	16	2 (12.50)	13	8 (61.54)
Penicillin G	15	5 (33.33)	13	9 (69.23)
Gentamicin	15	4 (26.67)	13	6 (46.15)
Tetracycline	16	8 (50.00)	13	5 (38.46)
Teicoplanin	16	0 (0.00)	12	0 (0.00)
Tigecycline	16	0 (0.00)	13	1 (7.69)
Levofloxacin	16	3 (18.75)	13	7 (53.85)
Vancomycin	15	1 (6.67)	13	2 (15.38)

sensitive to ertapenem, tigecycline, and imipenem. *Enterococcus* were highly sensitive to teicoplanin, linezolid, nitrofurantoin, and tigecycline. *Enterococcus faecalis* was highly resistant to clindamycin and quinupristin, while *Enterococcus faecium* was highly sensitive to erythromycin, clindamycin, levofloxacin, and ciprofloxacin. Complete drug resistance profiles among Gram-negative and *Enterococcus* bacteria were shown in Tables 2 and 3.

3.3. Correlation of bacterial strain and pregnane receptor gene polymorphism with TCM syndrome identification

TCM diagnostic factors identified dampness and heat in the liver and gallbladder as the prevailing syndrome of cholelithiasis patients, followed by hepatic qi stagnation, with the smallest number of patients showing signs of noxious dampness accumulation. Different bacterial strains from cholelithiasis patients were significantly associated with the syndromes identified by TCM ($P < 0.05$), and Gram-negative bacteria were detected in the largest number of patients with dampness and heat of the liver and gallbladder.

The *rs6785049* genotype of the *PXR* gene was found to be significantly associated with TCM syndromes ($P < 0.05$), while there was no statistically significant difference observed in the distribution of the *rs2276707* and *rs3814055* genotype. The results were shown in Table 4.

3.4. PXR gene polymorphism linked to cholelithiasis

The only genotype to show significant association with cholelithiasis diagnosis was the *rs6785049* polymorphism ($P < 0.001$), and after combining GA and AA, there was still statistically significant difference between the combined genotype and GG ($P < 0.001$). Logistic regression analysis indicated that the GA genotype within the *rs6785049* polymorphism carried an increased risk of gallstones compared to those carrying the GG wild type genotype (*OR* = 0.40, 95%*CI*: 0.16–0.79). Individuals carrying the GA+AA genotype were also at increased risk of gallstones (*OR* = 0.38, 95%*CI*: 0.19–0.81).

Table 4
TCM syndromes, pathogens, and *PXR* gene polymorphism [(*n*)%].

TCM	Pathogenic bacteria			*PXR* gene polymorphism								
	G (+)	G (−)	Fungus	*rs2276707*			*rs3814055*			*rs6785049*		
				CC	CT	TT	CC	CT	TT	GG	GA	AA
Hepatochlic hygropyrexia (n = 81)	31 (37.80)	40 (43.96)	10 (52.63)	19 (54.29)	38 (35.85)	24 (47.06)	36 (42.86)	20 (29.85)	25 (60.98)	13 (41.94)	50 (40.00)	1 (50.00)
Stagnation of liver-qi (n = 62)	27 (32.93)	30 (32.97)	5 (26.32)	10 (28.57)	35 (33.02)	17 (33.33)	25 (29.76)	22 (32.84)	15 (36.59)	10 (32.26)	41 (32.80)	11 (30.56)
Accumulation of damp (n = 49)	24 (29.27)	21 (23.07)	4 (21.05)	6 (17.14)	33 (31.13)	10 (19.61)	23 (27.38)	25 (37.31)	1 (2.43)	8 (25.80)	34 (27.20)	7 (19.44)
P	<0.05			1.73			0.81				0.04	

Table 5
Genotype and allele frequencies among cases and controls and their association with cholelithiasis risk [(*n*)%].

Genotype	Cholelithiasis (*n* = 192)	Control (*n* = 200)	*P*	*OR* (95%*CI*)
rs2276707				
CC	35 (18.23)	32 (16.00)	0.3870	1
CT	106 (55.21)	124 (62.00)		0.13 (0.58.2.0)
TT	51 (26.56)	44 (22.00)		0.89 (0.61.1.8)
CT+TT	157 (81.77)	168 (84.00)	0.5580	1.25 (0.71.2.1)
rs3814055				
CC	84 (43.75)	90 (45.00)	0.9550	1
CT	67 (34.90)	67 (33.50)		0.99 (0.43.1.5)
TT	41 (21.35)	43 (21.50)		1.02 (0.48.1.8)
CT+TT	108 (56.25)	110 (55.00)	0.8030	0.98 (0.55.1.67)
rs6785049				
GG	31 (16.15)	68 (34.00)	0.0002	1
GA	125 (65.10)	106 (53.00)		0.40 (0.16.0.7)
AA	36 (18.75)	26 (13.00)		0.31 (0.14.0.7)
GA+AA	161 (83.85)	132 (66.00)	<0.0001	0.38 (0.19.0.8)

There was no evidence that the *rs2276707* or *rs3814055* gene polymorphism was correlated with risk of cholelithiasis. The results were shown in Table 5.

4. Discussion

Culturing of the bile bacteria collected in this study revealed a high rate of mixed infections (56 cases), higher than that previously reported in Chinese patients [14], and a low rate of fungal infection. Our discovery of *Enterococcus faecalis* and *Enterococcus faecium* in the bile of cholelithiasis patients confirm previous findings [15]. Our analysis revealed a number of ESBLs-producing bacteria and the Gram-negative bacteria displayed resistance to a variety of antimicrobial agents while remaining sensitive to ertapenem, tigecycline, and imipenem, suggesting that these may be the most useful in treating biliary tract infections caused by *Enterobacteriaceae*. Our results also support the practice of collecting and culturing bile samples for drug sensitivity testing as part of routine cholelithiasis treatment. Antibiotics should be selected carefully, so as to avoid double infection and reduce the emergence of resistant strains of bacteria.

It was found that the largest proportion of cholelithiasis patients had dampness-heat of the liver and gallbladder according to TCM, and that pathogenic bacteria were differently distributed in cholelithiasis patients with different TCM syndromes. Gram-negative bacteria were detected in the largest proportion of patients with dampness-heat of the liver and gallbladder, suggesting that the presence of dampness and heat in the liver and gallbladder may be a result of Gram-negative bacteria infection. Different genotypes of the *PXR* gene were differently distributed in cholelithiasis patients with different TCM syndromes, suggesting that the *PXR rs6785049* genotype could play a role in determining how cholelithiasis manifests in different individuals.

Analysis of the *rs2276707*, *rs3814055*, and *rs6785049* polymorphisms revealed a correlation between the *rs6785049* polymorphism and cholelithiasis diagnosis, along with statistically significant differences in GG, GA, and AA genotype distribution within the *rs6785049* polymorphism. After combining GA and AA, there was still a significant difference between the combined genotype and GG, and logistic regression analysis showed that compared to those carrying the GG wild type allele, individuals with the GA genotype were at increased risk for developing gallstones (*OR* = 0.40); similarly, those carrying with GA+AA genotype were also at increased risk of gallstones (*OR* = 0.38). Findings have indicated that bile acids can activate PXR, and the activated PXR can reduce cholestasis, bile acid, and maintain homeostasis of bile acids [16–19]. The mechanism for this may involve PXR induction of fibroblast growth −19 gene expression, thereby strengthening the recovery of bile acids, so as to prevent the formation of gallstones.

Bacterial cultures of bile specimens and blood samples collected from cholelithiasis patients were analyzed and bacteria types, antibiotic sensitivity of the bacteria, and *PXR* gene polymorphisms to samples from healthy patients were compared. The pathogenic bacteria distribution in patients' bile and antibiotic resistance characteristics in relation to TCM syndrome differentiation were also evaluated. The results of our study support the timely collection of bile samples for bacterial culture, drug sensitivity testing and the careful selection of antibiotics to prevent double infection and reduce the emergence of resistant strains. Our results also support a role for *PXR* gene *rs6785049* polymorphisms in influencing the expression of the *PXR* gene and the development of gallstones, providing an opportunity for early detection and prevention at an early stage through genotype detection. It is also found that gallstone development is associated with TCM syndrome differentiation, providing a theoretical basis for the prevention, diagnosis, and treatment of cholelithiasis.

Conflict of interest statement

We declare that we have no conflict of interest.

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