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Expression of MUC5B Protein and mRNA in Gallbladder Membrane Directly Correlated with Gallstone Nucleation

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Abstract

The presence of gallstones in the gallbladder (Cholecystolithiasis) is a common disease in Northern part of India and some other countries like japan, China and Indo-American population. Nucleation is first event for the formation of gallstone in gallbladder sac environment. It was suspected that mucin family gene play role in nucleation process. Out of 20 mucin family members it is still unclear which one playing crucial role in gallstone nucleation process. Our objective was to investigate secretary/jell forming mucins in gallstone formation. Mucin family genes [MUC1, MUC5AC, MUC5B, MUC6, MUC7, MUC9 and MUC11] were evaluated through western blotting and semiquantitative PCR. MUC5B mRNA level was most prominent in expression followed by MUC9 and MUC5AC. MUC5B expression was much significantly higher in cancerous gallbladder than non-cancerous gallbladder ($p<0.001$, CI95%). MUC5B expression was directly correlated with number of stone. Whereas size of stone was not having any impact on MUC5B upregulation. Above result indicate that MUC5B protein may crucially involve in nucleation process during gallstone formation.

Keywords: Gallstone pathogenesis; Mucin; Gallbladder cancer; Cholelithiasis

Introduction

Gall bladder cancer (GBC) is a principal reason of cancer-related mortality in the northern parts of the Indian subcontinent [1]. Late or advanced diagnoses of cancer in most of the patients are suitable for palliative care only. There is a strong association between long-standing gallstone disease and the development of GBC [2]. While proper trials are lacking, potential population-based studies from low-high-risk regions expose that cholecystectomy reduces the GBC mortality [3]. Cholelithiasis is a well-established risk factor for gallbladder cancer and the risk seems to associate with stone size and number [2]. Anomalous junction of pancreaticobiliary ducts (AJPBD), especially without choledochal cyst, and porcelain gallbladder are supplementary factors that influence to gallbladder cancer [4,5]. A total of 20 different mucin genes have been identified and subdivided into two groups, membrane-bound and secreted mucins, according to Human Genome Mapping conventions. Normal mucosal cells secrete a variety of different mucins (high molecular weight glycoproteins, designated as MUC1, MUC2, MUC3, MUC4, MUC5A, MUC5B, MUC10, MUC11…. MUC16); these mucins serve protective and lubricating roles in the normal epithelium of gastrointestinal organs and markedly altered during neoplastic and paraneoplastic condition [6]. The pace of nucleation depends upon a crucial balance between pronucleating and antinucleating factors in bile [7]. Mucin, a high molecular weight glycoprotein secreted by the gallbladder and biliary duct epithelium, is a pronucleating agent in experimental and human gallstone disease. Some ambiguity exists with regard to the type of mucin expression in normal biliary tract and its alteration in gallstone formation. Gallbladder mucin shares with other epithelial mucins the ability to bind lipids and bile pigment. The hydrophobic binding sites in the polypeptide core of mucin may provide a favorable environment for nucleation of cholesterol monohydrate from supersaturated bile. Human gallbladder mucin at concentrations of 2 and 4 mg/ml accelerated the nucleation time of cholesterol crystals in model bile. A critical step in the formation of cholesterol gallstones is nucleation [8-10].

In the gallbladder and/or its associated diseases, at least, expression of nine mucin genes (MUC1, MUC2, MUC3A, MUC3B, MUC4, MUC5AC, MUC5B, MUC6 and MUPCDH) has been confirmed in the literature [11-13]. Many polymorphisms in mucin family also reported as a risk factor for gallstone disease [14-16].

The exact evidence of mucin involvement in gallstone nucleation is still not clear. In this study we have investigated the role of mucin in gallstone nucleation process.

Material and Methods

Ethics statement

This work was approved by institutional human ethical committee from Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. A predesigned query proforma and written informed consent was taken from all subjects.

Subject and sample collection

Total 34 with stone bearing, 15 without stone Gallbladder tissue (whiples procedure) and 10 well define invasive gallbladder cancer (5 with stone bearing and 5 without stone) patients (clinically and pathologically stage IV patients) operated in the Department of

Surgical Oncology, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Patients suffering from other digestive tract abnormality were omitted from this study. Tissues were snap fridge and stored in -800°C up to use.

S. No.	Sample types	No of Sample	Sample character	
1	Stone bearing normal gallbladder tissue	34 (Female only)	Age group	Maximum – 64 Minimum- 31 Average- 45.7±5
2	Without stone normal gallbladder tissue	15 (Female only)	Age group	Maximum – 56 Minimum- 33 Average- 42.4±3.5
3	Well define invasive GBC (with stone)	5 (all stage IV and female only)	Age group	Maximum – 66 Minimum- 37 Average- 49.8±6
4	Well define invasive GBC (without stone)	5 (all stage IV and female only)	Age group	Maximum – 67 Minimum- 35 Average- 48.6±5

Table 1: Characteristics of sample taken for experiments

Protein isolation

Protein was isolated by method described by Maurya et al. [17] Briefly, tissue were homogenized in lysis buffer (Tris HCL 50 mM, Nacl 150 mM, EDTA 0.5 mM, SDS 1%, Protease inhibitor cocktail, PMSF 1 mM). Homogenate were centrifuged at 10,000g for 10 min at 40°C. Clear homogenate were used for protein estimation and aliquots were made and stored in -800°C.

Immunoblotting

Total 25 µg protein were separated by 10% SDS-polyacrylamide gel electrophoresis followed by electrophoretic transfer to a polyvinylidene difluoride membrane (PVDF, Immobilon; Millipore Corp., Bedford, MA) at 50 Volt for overnight at 4°C. The membrane was then blocked with 5% skim milk in Tris-buffered saline (TBS, 50mM Tris-Cl, pH 7.5, 150 mM NaCl) for 3 h at room temperature followed by overnight incubation with primary antibodies in TBST (0.5% Tween 20 in TBS). The membrane was washed three time with TBST for 10 min and then probed for 2 h with secondary antibodies conjugated to horseradish peroxidase. Again membrane was washed with TBST for 10 min each. The membrane was exposed to high performance autoradiography film and visualized using the ECL system (Santa Cruz, CA). For internal control β-actin was used.

RNA isolation, cDNA preparation and Semiquantitative Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Stored gallbladder 50-100 mg of frozen tissue was transfer in a 15 ml tube with 1 ml TRIzol (GIBCO BRL). Tissue were properly homogenized for 60 sec and kept on room temperature for 5 min. 200 µl chloroform were added and mixed by inverting the tube for 15 sec and kept for 3 min at room temperature followed by centrifugation at 12000 g for 15 min. Aqueous phase was transferred into a fresh tube and added 500 µl isopropanol. Mixture was centrifuged at 12000 g for 10 min in at 40°C. The pellets were washed with 500 µl 70 % ethanol three times. The pellet dried on air for 10 min and dissolved in 50-100 µl DEPC-H2O with incubation for 10 min at 60° C. Quality and quantity were checked for further experiment. Isolated total RNA (1µg) was used to prepare cDNA through cDNA preparation kit from Quagene. To check cDNA preparation, RTPCR with GAPDH reaction were done.

PCR was performed on cDNA (5 µl), using specific pairs of primers (Table 1). The GAPDH was used as the internal control. PCR products (10 µl) were separated on a 1.5% agarose gel containing ethidium bromide run in 1X TBE. The MUC5B/GAPDH gene ratio was calculated after scanning DNA bands with GelAnalyst-GelSmart software (Claravision).

Statistical methods

Quantified RT-PCR signals were normalized to GAPDH levels and presented as the mean ± S.D. to determine the significances of differences in expression data Student's t test was used. Two way ANOVA was used for multiple comparison. Statistical analysis was performed using the program SPSS 11. Differences were considered to be significant at values less than $p>0.05$.

Result

Expression analysis of secretary mucin family genes in normal gallbladder cancer

Our first objective was to investigate most abundant secretary mucin glycoproteins in 34 stone bearing gallbladder. For this, reverse transcriptase PCR reaction was done to evaluate the expression of MUC1, MUC5AC, MUC5B, MUC6, MUC7, MUC9 and MUC11 mRNA. MUC5B mRNA level was most prominent and high expression followed by MUC9 and MUC5AC (Figure 1). This information was necessary to further investigate for the role of mucin family genes in gallstone formation.

Expression of MUC5B in with stone and without stone gallbladder tissue

It was interesting to see whether presence of stone in gallbladder tissue having any effect on MUC5B expression or not. So that we chose with and without stone gallbladder tissue to compare MUC5B expression. MUC5B mRNA expression was significantly higher in stone bearing normal gallbladder compare to stone bearing gallbladder ($p<0.036$, CI 95%) (Figure2). Parallely, we have also checked effect of stone in gallbladder cancer tissue with stone and without stone. In cancerous tissue, MUC5B expression was non-significantly higher in stone bearing gallbladder when compared with non-stone bearing gallbladder ($p<0.089$, CI 95%). MUC5B expression was much significantly higher in cancerous gallbladder than non-cancerous gallbladder ($p<0.001$, CI95%). Same result was also noticed in protein expression profile in above samples.

Effect of number of stone in gallbladder on MUC5B expression

From above result it was hypothesized that either stone may upregulate MUC5B expression or high MUC5B initiate stone formation. So we segregated tissue on the basis of number of stone in gallbladder tissue and divided in to groups (Figure 3). There was direct

correlation between number of stones and MUC5B expression. Parallely, we did not find any correlation between size of gallstone and MUC5B expression (Figure 4).

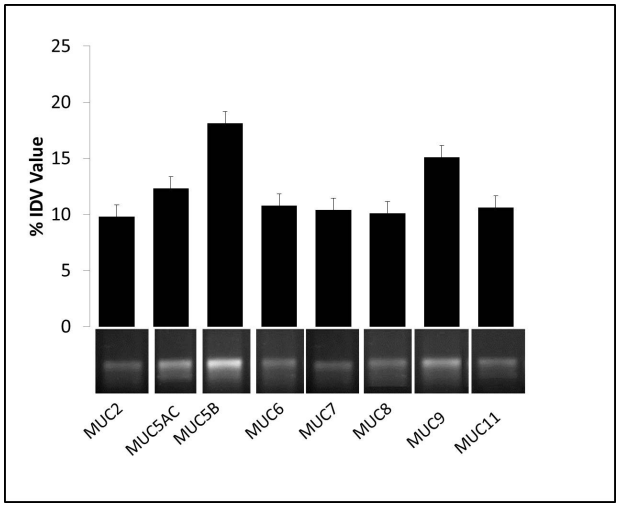

Figure 1: Expression of mucin family genes in gallstone bearing normal gallbladder epithelia: Secretary and gel forming mucin family gene expression at mRNA level in cholelethiasis gallbladder epithelial tissue. For loading control GAPDH were used (not shown in figure). Error bar showing the value of standard error of mean.

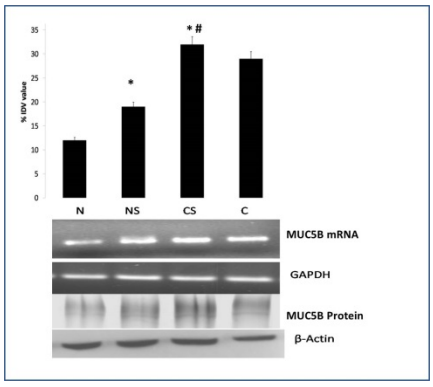

Figure 2: MUC5B mRNA and Protein expression in gallstone baring and non gallstone bearing cholelithiasis and cancer gallbladder epithelial tissue: Lane 1 show non stoned and lane 2 show stone bearing gallbladder epithelial tissue. Lane 3 show stoned and lane 4 show non stoned cancerous lesion of gallbladder tissue.

Discussion

Glycoproteins are diverse functions and appear in nearly every biological process studied. Mucins are high molecular weight O-glycosylated proteins present at the surface of most epithelial cells. Out of two families, secreted or gel-forming/polymerizing mucins are particularly important to investigate the role of mucins in nucleation of gallstone [18]. Gallbladder mucin synthesis and secretion is initiated by lysolecithin and polyunsaturated free fatty acids via the prostanoid pathway [19]. Mucin known as a storng pronucleating agent in experimental [20,21] and human gallstone disease [22].

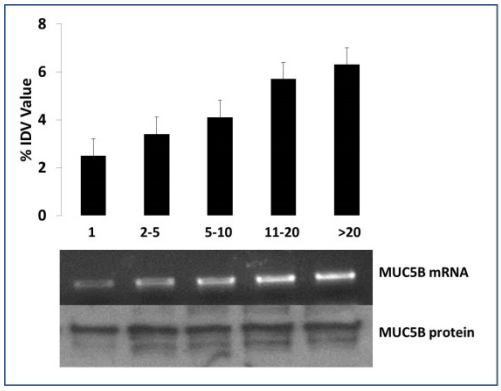

Figure 3: Effect of stone and its number in stone bearing gallbladder epithelial tissue: Normal gallbladder tissue with increasing number of stones and MUC5B mRNA and protein expression.

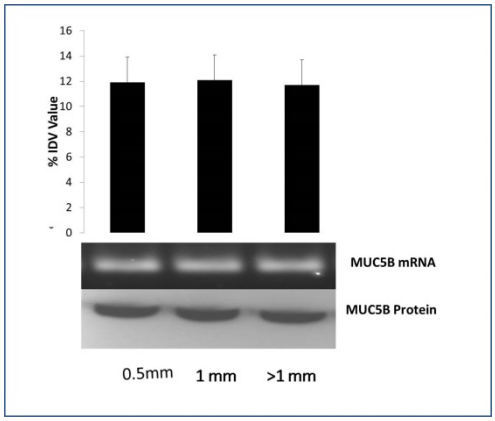

Figure 4: Effect of stone size in stone bearing gallbladder epithelial tissue on expression of MUC5B mRNA and protein

Firstly, it is crucial to investigate mucin/s which is more prominently present in stone bearing gallbladder tissue than without stone. There are many studies which shows presence of different mucins in gallbladder tissue like MUC1 [23], MUC2 [24], MUC4 [25], MUC5AC [26], MUC5B [27], MUC6 [28] etc. So we decided to see all secretary/ gellforming mucins in stone bearing gallbladder tissue. Semi quantitative RTPCR were done to know level of all eight secretary mucins in stone containing gallbladder tissue (Figure 1). MUC5B was the most prominently upregulated mucin protein in stone baring gallbladder tissue followed by MUC9 and MUC5AC respectively.

This result shows that prominent presence of MUC5B glycoprotein, compare to other mucin protein, may have some role in nucleation of gallstone. To validate this hypothesis we used variable number of stone bearing gallbladder epithelia to test MUC5B mRNA and protein expression study. Result shows significant positive correlation with number of gallstones in gallbladder and MUC5B expression (mRNA as well as protein). It is appears that MUC5B expression may determine number of gallstone (it is our hypothesis/suspect only). In

other experiment, expression of MUC5B was determined in according to gallstone size. No any significant correlation was observed among the different size gallstone containing gallbladder. This result shows that size of gallstone don't have any influence in MUC5B expression. This result further strengthening our hypothesis that MUC5B expression determines number of gallstone. Finally MUC5B expression was also checked in gallbladder carcinoma tissue with and without gallstone. As expected, stone bearing gallbladder carcinoma tissue shows maximum MUC5B expression compared to non-stone bearing gallbladder carcinoma or other normal stone and without stone gallbladder tissue.

Above results indicate that MUC5B is one of the major mucin family gene which influence gallstone nucleation.

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