RESEARCH ARTICLE

Elevated Expression of Maspin mRNA as a Predictor of Survival in Stage II and III Gallbladder Cancer Cases

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Abstract

Background: Maspin expression is a potential prognostic factor for various malignancies but its relation with gallbladder cancer is unknown and needs to be investigated needs to be investigated. We therefore here focused on maspin mRNA expression in normal, gall stone disease and gallbladder cancer subjects, with particular attention to prognostic importance in individuals with malignancies. Materials and Methods: This study was carried out at the Department of Surgical Gastroenterology, King George's Medical University, Lucknow, India. Gallbladder samples from normal (n=25), gall stone disease (n=25) and cancer patients (n=38) were analysed for maspin mRNA expression by semi-quantitative reverse transcriptase PCR and quantitative real time PCR. Statistical analysis was carried out using the Students t test or ANOVA. Survival analysis was conducted according to the Kaplan-Meier method and correlations were assessed using the Pearson correlation method. $p<0.05$ was considered statistically significant. Results: Significant increase ($p=0.028$) in expression of maspin mRNA was observed in gallbladder cancer as compared to gall stone disease, whereas no expression was found in normal tissues. Significant correlation (Pearson's coefficient(r)=-0.798, $p<0.0001$) was observed between relative quantification of maspin mRNA and survival of cancer patients after surgery, with significantly shorter ($p=0.002$) survival in patients having relative quantification >1.5 as compared to those having relative quantification <1.5. Similarly, significant differences in patient survival for maspin mRNA expression was observed for stage II ($p=0.025$) and III ($p=0.011$) cancer. Conclusions: Higher expression of maspin mRNA in gallbladder cancer has prognostic significance for stage II and III cancer, which needs to be investigated further.

Keywords: Gallbladder cancer - maspin expression - overall survival - prognosis

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Introduction

Gallbladder cancer (GBC) is the most common malignancy of biliary tract with marked ethnic and geographical variations including Northern Indian gangetic belt (Pandey et al., 2001; Misra et al., 2003; Gupta et al., 2012; Kazmi et al., 2013). Poor prognosis due to late diagnosis and early dissemination is the common characteristic of this dreaded disease (Dhir and Mohandas, 1999).

Tumor-suppressive activity of maspin is attributed to its potential to inhibit metastasis in breast cancer (Zou et al., 1994). Increased maspin expression predicts a better prognosis for breast (Mohsin et al., 2003), non-small-cell lung cancer (Takanami et al., 2008) and oral squamous cancers (Xia et al., 2000) but its relation with GBC is unknown and needs to be investigated.

Gall stone disease is said to be most important etiological factor for GBC development and the risk seems to increase with stone size >3cm (Diehl, 1983; Lowenfels et al., 1989; Zou and Zhang, 2000). However, the same need to be established at molecular level also. Since maspin over expression is observed in early GBC (Kim et al., 2010), it might play a role in its development from gallstone disease. In light of the potential applications of maspin as a molecular marker and differential expression of maspin gene in various cancers, we studied maspin gene expression in normal, gall stone disease and GBC patients and investigated its prognostic significance for patients with gallbladder malignancy.

Materials and Methods

The study protocol was approved by the Institutional ethics committee and all patients provided written informed consents. Resected gall bladder tissues specimens of histopathologically proven GBC (n=38), gallstones disease (n=25) and normal subjects (n=25) who underwent surgery between May 2010 and October 2012 at the Department of Surgical Gastroenterology, King George's Medical University, Lucknow were used as material for the study. To assess the presence of gallstones

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or GBC, abdominal imaging (ultrasonography and computerized tomography) was done. Histopathological examination was used for diagnosis of GBC and staging was done according to the American Joint Committee on Cancer (AJCC) tumor node metastasis (TNM) staging, 2010 (Edge and Compton, 2010). Only resected stage II and III GBC patients (diagnosed preoperatively) were included while incidental cases of GBC (stage I) and patients with advanced malignancy (not amenable for a curative resection) were excluded. For controls, we selected twenty-five normal gallbladder tissues from patients in which gallbladder is removed as a part of other surgeries (Choledochal cyst excision (n=12), Whipples pancreaticoduodenectomy (n=9) or following hepatobiliary trauma (n=4)). Tissue samples were immediately put into TRIzol (Invitrogen) reagent and stored at -80°C till further processing.

RNA isolation and cDNA synthesis

Total RNA was isolated from tissue as per manufacturer's instruction. 2μg of RNA was reverse transcribed into cDNA using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) and 50μl of its reaction mixture contains 10× Reverse transcriptase (RT) buffer, 10× RT Random primers, 25× dNTP mix (100mM), 50 U/μl of Reverse Transcriptase enzyme. RT reaction was carried out by incubating the reaction mixture at 25°C for 10 minutes followed by 37°C for two hour. Reaction was terminated by incubating mixture at 85°C for 30 seconds.

Semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis

Prior to amplification of Maspin gene, normalization was carried out with beta actin, the housekeeping gene. The PCR reaction mixture of 20 μl for β actin contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2mM dNTP mix, 0.5 μM of each ß-actin primers, 2 μl of cDNA and 1 μl Taq enzyme (MBI Fermentas, USA). PCR for β actin was carried out using 35 cycles of denaturation at 94°C for 5 min, annealing of primers at 58°C for 30s, extension at 72°C for 45s and final extension at 72°C for 10 min. The 20 μl reaction mixture of maspin gene contained 1× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 0.5 μM of each respective primers (Forward-5'-CTGACAACAGTGTGAACGAC-3' and Reverse-5'-CAAGCCTTGGGATCAATCATCT-3'), normalized cDNA and 1 μl Taq enzyme (MBI Fermentas, USA). PCR for maspin was carried out in Gene Amp PCR system 9700 of Applied Biosystem using 35 cycles of denaturation at 94°C for 5 min, annealing of primers at 60°C for 1min, extension at 72°C for 1.5 min and final extension at 72°C for 10 min. PCR products (446bp for maspin and 175bp for ß-actin) were analysed in agarose gel stained with ethidium bromide in VERSA DOC Imaging system, Model 1000 (Biorad, USA). Densitometric analysis was carried out using Quantity One Quantitation Software version 4.3.1 (Biorad, U.S.A).

Real time qPCR analysis

Real time quantitative PCR reaction was performed using Power SYBR Green PCR master mix (Applied Biosystems, USA) according to manufacturer's instruction. Housekeeping gene, beta-actin was used as an internal standard and experiments were carried out in triplicates. The thermal cycling conditions consist of 50°C for 2 min followed by denaturation step of 95°C for 15 min. Further, 40 cycles of denaturation (95°C for 15 seconds) were performed. Annealing (Forward primer-5'-CTACTTTGTTGGCAAGTGGATGAA-3', Reverse primer- 5'-ACTGGTTTGGTGTCTGTCTTGTTG 3') and extension was carried out at 60°C for 60 seconds. Gene expression changes were calculated using relative quantification ($2^{-\Delta\Delta Ct}$) method. Analysis was done using 7900HT Sequence Detector System software version 2.2.1 (Applied Biosystems, USA).

Statistical analysis

Statistical analysis was carried out using one-way ANOVA for multiple groups or by student t-test for two groups. Correlation analysis was performed using Pearson correlation method. Kaplan-Meier method was used for survival analysis and the difference between survival curves was analyzed by log-rank test. Cox proportional hazard analysis was used for estimating the hazard ratio. Two-tailed P value less than 0.05 was considered to be statistically significant.

Results

Study population consists of 88 patients including 25 cases each of resected normal, Gall stone disease and 38 cases of GBC group with 34 male and 54 females. Gall stone disease group included all the cases of chronic calculus cholecystitis with no case of polyp or adenoma. Table 1 shows the characteristic profiles of normal, gallstones disease and GBC patients.

Expression of 446bp amplified product of maspin mRNA was detectable in all gallstones disease and GBC tissue sample but undetectable in normal resected gall bladder tissues. Maspin mRNA expression was detected in a significantly (p=0.028) greater proportion in GBC (21.34%) as compared to gallstones disease by densitometric analysis of band intensity (Figure 1).

Table 1. Characteristics of Normal (controls), Gallstones and GBC Patients

Parameters	Normal (controls), n (%)	Gallstone, n (%)	GBC, n (%)
Male	10 (40)	12 (48)	12 (31.57)
Female	15 (60)	13 (52)	26 (68.42)
Age (years)±S.D.	41.16±13.19	41.32±13.81	48.08±11.35
Range	19-65	15-65	21-65
Gallstone present	0	All	27 (71.05)
Gallstone absent	All	0	11 (28.94)
Jaundice	0	7 (28)	14 (36.84)
Stage	N/A	N/A	
II			20 (73.68)
III			18 (47.36)
Cellular Differentiation	N/A	N/A	
Poor & Moderate			21 (55.26)
Well			17 (44.73)
Total	25	25	38

*N/A: Not Applicable, GBC: Gallbladder cancer, S.D: Standard Deviation

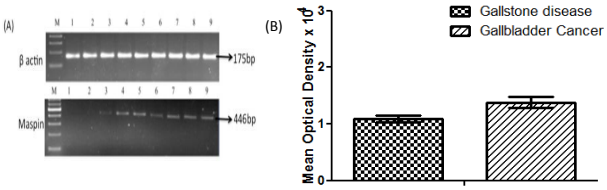

Figure 1. Expression of Maspin mRNA in Human Gallbladder Tissues Detected by RT-PCR. (A) Electrophoresis of agarose gel (2%) in response to RT-PCR assay in which upper lane is of 175bp beta- actin while lower one is of 446bp amplified maspin mRNA. Lane L: 100bp ladder, Lane 1, 2 and 3: normal tissues, Lane 4, 5 and 6: Gall stone disease and Lane 7, 8 and 9: GBC tissues. (B) Graphical representation in which values are represented as mean±standard error. Statistically significant difference (p=0.028) in expression was observed between gall stone disease and GBC. (Statistical test: unpaired student t test, RT-PCR: Reverse Transcriptase PCR, GBC: Gallbladder cancer)

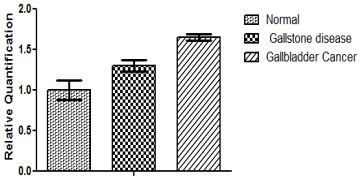

Figure 2. Graphical Representation of Expression of Maspin mRNA in Tissue Samples by Real Time PCR. All the values are mean±standard error. Statistically significant (p<0.0001) difference in expression was observed between all the groups (Statistical test: One way ANOVA, GBC: Gallbladder cancer)

Similarly, 29.5% and 64.37% increase (p<0.0001) in expression of maspin mRNA was observed in gallstones disease and GBC tissue samples as compared to normal by quantitative real time PCR (Figure 2). Further within cancer patients, statistically significant (p<0.0001) increase in maspin mRNA expression was observed in stage III as compared to stage II (Table 2). For cellular differentiation, higher expression of maspin mRNA was observed in poor and moderate tumors as compare to well differentiated (p=0.001). No significant difference in expression of maspin mRNA was observed for age (p=0.255), sex (p=0.858), jaundice (p=0.209) and presence or absence of gallstones (p=0.148) for GBC patients.

Correlation scatter diagram (Figure 3) between relative quantification (RQ) of maspin mRNA and survival of patients after surgery indicate significant correlation at 0.01 level (2-tailed) with Pearson correlation coefficient, r=-0.798, p<0.0001. As, there is markedly decrease in survival of GBC patients with RQ>1.5, we classified patient pool in two subgroup, one with RQ>1.5 and other with RQ<1.5.

The Kaplan-Meier survival curves demonstrated that the overall survival was significantly shorter (p=0.002) in patients of GBC with RQ>1.5 (median survival; 11 months) as compared to those patients in which RQ<1.5 (median survival; 19 months) (Figure 4A). Patients with RQ>1.5 had a hazard ratio (HR) of 3.263 for death as compared to patients with RQ<1.5 (95%Confidence interval [CI]=1.536-6.932). Statistically significant difference (p=0.025) in overall survival was observed for stage II patients with RQ>1.5 (median survival; 17 months) in comparison to patients with RQ<1.5 (median survival; 20.5 months) (Figure 4B). Similarly, for stage III, there was significant difference (p=0.011) in overall survival between patients with RQ>1.5 (median survival; 9 months) and RQ<1.5 (median survival; 13 months) (Figure 4C). The HR for stage II and III disease was 4.043 (95%CI =1.191-13.72) and 5.003 (95%CI=1.442-17.36) respectively.

Table 2. Correlation of Maspin mRNA Expression with various Clinicopathological Factors for Gallbladder Cancer Patients

Parameters		RQ±S.E.	p value
Age (years)	>45(n=23)	1.68±0.05	0.21
	<45 (n=15)	1.58±0.06	
Sex	Male (n=12)	1.63±0.08	0.85
	Female (n=26)	1.64±0.05	
Gall stones	Present (n=27)	1.68±0.05	0.14
	Absent (n=11)	1.54±0.06	
Jaundice	Present (n=14)	1.71±0.09	0.2
	Absent (n=24)	1.60±0.04	
Stage	II (n=20)	1.46±0.03	<0.0001*
	III (n=18)	1.83±0.05	
Cellular	Poor and Moderate (n=21)	1.76±0.06	0.001*
Differentiation	Well (n=17)	1.48±0.04	

* Indicates statistically significant difference. p value <0.05 is considered as statistically significant, Data is represented as relative quantification + standard error (RQ + S.E.) obtained through real time PCR. Differences were tested by unpaired Student's t test

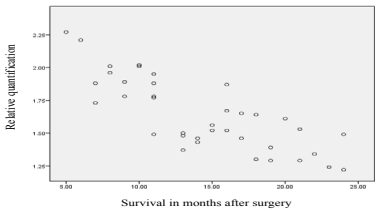

Figure 3. Scatter Graph Showing Correlation between Relative Quantification of Maspin mRNA and Survival of Gall Bladder Cancer Patients (n=38). Correlation is significant at 0.01 level (2-tailed) with Pearson correlation coefficient, r=-0.798, p<0.0001

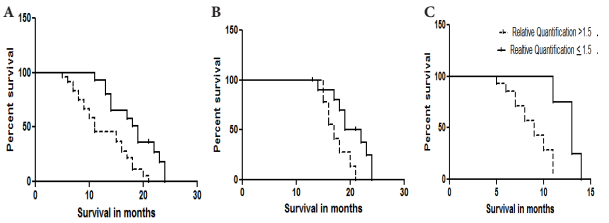

Figure 4. Kaplan-Meier Survival Curves for Maspin mRNA Expression. Statistical analysis was carried out using log rank test between RQ value >1.5 and RQ <1.5 (A) Significant difference (p=0.0021) in survival curve was observed for all resected GBC patients (n=38), (B) Significant difference (p=0.025) in survival curve was observed for resected stage II GBC patients (n=20), (C) Significant difference (p=0.0112) in survival curve was observed for all resected stage III GBC patients (n=18). (RQ: Relative Quantification, GBC: Gallbladder cancer)

Discussion

Multiple genetic changes are involved in the development of human cancers and role of such genes (tumor suppressor genes, oncogenes and DNA repair

gene) are being studied (Singh et al., 2004). Cancer of the gall bladder is the fifth most common gastrointestinal tumor and emerging as the major killer in northern India in particular the gangetic belt (Unisa et al., 2011; Chhabra et al., 2012; Gupta et al., 2012). However, literature review has shown only limited information regarding molecular events involved in the pathogenesis of GBC. Detailed understanding of molecular abnormalities may provide methods for risk assessment and early detection of GBC.

The mean age of GBC patients at presentation is around 55 years with female preponderance in various studies (Kumar et al., 2006; Gupta et al., 2012). The reported mean age of gallstones disease at presentation in German population is around 61 years (Volzke et al., 2005) whereas Shukla et al. (2008) reported 39.7 years in Indian population. In agreement with this, observed mean age in our study for gallstones patients is 41.32 years and for GBC patients is 48.08 years with male to female ratio of 1:2.17 for cancer patients. The early presentation of GBC in our population could be explained by the fact that people in endemic areas like Northern India have early exposure to the gallstone disease, an important risk factor of GBC.

Poor prognosis, one of the distressing features of GBC, is mainly due to its anatomical position and lack of specific signs, symptoms and biomarkers for early identification (Misra et al., 2003). Newer diagnostic and prognostic markers are needed for early diagnosis and management of this lethal disease. The gene expression profiling of GBC through cDNA microarray revealed maspin as one of the emerging gene for gallbladder carcinogenesis (Kim et al., 2008). There is accumulated functional evidence that demonstrates that maspin blocks tumor metastasis, tumor cell motility and invasion, and apoptosis in vitro (Zou et al., 1994). However, some in vivo analyses have shown that gain of maspin expression is associated with malignant behavior (Maass et al., 2001; Sood et al., 2002). Thus, the role of maspin in tumor biology remains controversial. The high incidence of aberrant maspin expression in different types of cancer cells encouraged us to examine its expression in normal, gall stone disease and GBC patients and its prognostic significance in cancer patients.

Kim et al. (2010) demonstrated absence of maspin protein in normal gall bladder epithelium. Similarly, Maass et al. (2001) also failed to find maspin expression in normal human pancreas by Northern blott and immunohistochemistry. In agreement with these studies, we also found absence of maspin mRNA expression in normal gall bladder tissue, which suggest transcriptional inactivation of maspin gene in normal population.

The presence of gallstones is believed to be most important risk factor for GBC (Kumar et al., 2006; Randi et al., 2006). Presence of gallstones over a period of time may lead to chronic inflammation, bile retention within the gallbladder and infection. Genetic abnormalities in the gall bladder epithelium may be triggered by repetitive cycles of damage and regeneration of gall bladder mucosa (Singh et al., 2004). Maesawa et al. (2006) found expression of maspin protein in gallstones containing gallbladder tissues. In our study, we also observed maspin mRNA expression in gall stone disease patients indicating transcriptional activation of maspin gene due to presence of long standing gallstones. The promoter hypo-methylation is one of the most probable cause for such activation, which needs to be investigated further.

Elevated maspin expression in relation with better prognosis for breast (Mohsin et al., 2003) prostrate, colon, oral, squamous cancers (Takanami et al., 2008) and esophageal squamous cell carcinoma (Wang et al., 2013) is observed in which it shows tumor suppressor activity. However, it is not always true and in some in vivo studies this activity has been questioned as contrary results were found. Kim et al. (2010) observed overexpression of maspin in surgically resected GBC tissue as compared to normal tissue. Maspin overexpression was also observed in pancreatic (Maass et al., 2001) and ovarian (Sood et al., 2002) cancer, whereas normal tissue was maspin negative. In these tumors, maspin seems to behave as an oncogene rather than a tumor suppressor gene. In present study RT-PCR and real time analysis revealed overexpression of maspin mRNA in resected tissue samples, revealing its oncogenic property in GBC also.

Prognostic significance of maspin gene in GBC has hardly been studied. Further no siginificant difference in overall survival and disease free survival in intermediate stages was observed in the only one study done so far (Kim et al., 2010). Further to analyze association of maspin mRNA expression with survival after surgical resection of gallbladder, we categorised maspin expression data of GBC patients on the basis of RQ value obtained through real time PCR. We observed that in stage II and III disease, expression with RQ more than 1.5 is associated with poor outcomes, suggesting that overexpression of maspin may contribute to tumor progression and carcinogenesis. To best of our knowlede this is the first study which evualated association of maspin mRNA with survival of resected GBC patients in our endemic belt. Maspin mRNA over expression is found in stage III as compared to stage II tumors. Poor and moderate differentiated tumors show higher expression as compared with well differentiated ones. This probably indicates its potential role in gallbladder carcinogenesis which needs to be explored. Further maspin mRNA expression is not found to be associated with any of the clinical parameter studied.

This study has limitation of stage I and IV GBC tissues. We generally do not experience patients of stage I GBC as most of such cases are detected incidentally during cholecystectomy performed for benign diseases at others centers and present to us with histopathology report only. Similarly, Stage IV GBC is inoperable and retrieving tissue samples from such cases remains difficult. Maspin protein expression in these cases is also advocated and is area of further research. Longitudinal studies with larger sample size may be required to validate the present obtained results.

Recently, the potential role of maspin as a therapeutic agent using different approaches is matter of investigation (Berardi et al., 2013). A similar approach of maspin-targeted treatment may be helpful in management of patients with GBC. Also, its mRNA expression might be useful as a prognostic and possibly predictive factor that can guide physicians in selecting therapy (Berardi

et al., 2013).

In conclusion, increased maspin mRNA expression from gallstone disease to GBC indicates presence of gallstones may lead to malignant transformation and poor survival. Maspin mRNA can therefore be used as a prognostic marker for resected stage II and III tumor.

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