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Full Length Research Paper

Stage dependent expression of MUC1 glycoprotein in gallbladder carcinoma

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Gallbladder carcinoma is a relatively aggressive and frequently lethal cancer. It is defined as a clinicopathological presentation including a wide spectrum of lethal disease from simple gallstones to dysplasia, to carcinoma *in situ* and invasive carcinoma. MUC1, a cell surface antigen expressed in glandular epithelia, is overexpressed and aberrantly glycosylated in adenocarcinoma. In this report, the immunohistochemical and immunofuorescence expression of MUC1 was studied in formalin-fixed, paraffin-embedded surgical specimens from patients with gallbladder carcinomas. In T2 tumor, 48.3% cases showed weak expression and 51.7% showed moderate expression. In T3 tumors, expression was weak in 23.1 %, moderate in 46.2% and strong in 30.8% of cases. In T4 tumors, 9.1% cases showed weak, 40.9% moderate and 50% strong expression of MUC1. We have shown that early gallbladder cancer pathology may involve MUC1 in the disease progression. We have defined the localization of expression of the MUC1 genes during GBC development and correlated them with expression and its stage dependent nature.

Key words: Gallbladder carcinoma, metastasis, membrane protein, cancer diagnosis.

INTRODUCTION

Gallbladder carcinoma (GBC) is commonly associated with gallstones formation and chronic cholecystitis (Shukla et al., 1981). A relatively well-defined sequence of changes in GB epithelium begins as pre-malignant dyspalsia and progression to carcinoma *in situ* stage and ultimately develops as invasive carcinoma (Hirasawa et al., 2000). The histological changes are a result of progressive molecular upheavals that commence in apparently histologically normal looking epithelium of chronic cholecystitis. From a clinical standpoint, the development of methods that are sensitive and specific enough to permit an early diagnosis of gallbladder carcinoma may greatly facilitate the detection and subsequent treatment of this disease.

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Abbreviation: GBC; Gallbladder carcinoma, **GSD;** gall stone diseases, **WD;** well differentiated, **MD;** moderately differentiated, **PD;** poorly differentiated, **T;** tumor stage.

A variety of biochemical, genetic and imaging techniques have been developed for diagnostic purposes and/or for monitoring the outcome of GBC treatment. In consideration of the potentials and limitations inherent in each of these methods, the need for efficient differential diagnostic markers that specifically discriminate GBC cannot be overemphasized. In that regard, MUC1 may represent potential candidates for such a purpose, with respect to their biochemical properties relating to malignant conditions.

Normal mucosal cells secrete a variety of different mucins (high molecular weight glycoproteins, designated as MUC1, MUC2, MUC3, MUC4, MUC5A and MUC6) a heterogeneous family of O-linked glycoproteins, which are divided into two classes: epithelial and gel-forming mucins. MUC1 is believed to play a key role in immune protection and anti-adhesion (Carraway et al., 2007; Carraway, 2002; Gendler et al., 2001; Gendler et al., 1990). Transformation of epithelia to carcinomas is associated with marked over-expression of MUC1 throughout the entire cell membrane (Kufe et al., 1984). MUC1, a cell surface antigen expressed in glandular epithelia, is over-

expressed and aberrantly glycosylated in adenocarcinoma. The peptides and glycans of MUC1 are being studied as substrates for cancer vaccines. A natural humoral immune response to MUC1 has been associated with a favorable disease outcome in patients with breast, lung and pancreatic cancer (Hamanaka et al., 2003; Taylor-Papadimitriou et al., 2002, Mensdorff-Pouilly et al., 2000). The majority of human adenocarcinoma cells express MUC1 and it may strongly relate to tumour progression (Anon, 2008; Ferreira et al., 2008; Sellers et al., 2008; Hinoda et al., 2003; Tamada et al., 2002).

In normal gallbladder, mucins protect the underlying mucosa against potential injuries such as reflux of gastroduodenal contents including bile acids (Corfield et al., 2000). Mucin secretion and accumulation in the gallbladder are determined by multiple mucin genes (Verma et al., 1994; Gendler et al., 1995). The Muc1 gene regulates a membrane-associated mucin that is abundant in the secretary epithelia of the gallbladder (Kim et al., 1995; Buisine et al., 2000). Increased epithelial MUC1 mucin enhances cholelithogenesis by promoting gallbladder cholesterol absorption and impairing gallbladder motility can be used as marker of malignant transformation of gallbladder (Wang et al., 2006; Ghosh et al., 2005; Kawamoto et al., 2004).

The expression levels of MUC1 in invasion/metastasis-related substances in gallbladder carcinoma may provide valuable information for understanding the mechanisms responsible for the malignant behavior of carcinoma cells. In this report, the immunohistochemical and immunofuorescence expression of MUC1 was studied in formalin-fixed, paraffin-embedded surgical specimens from patients with gallbladder carcinomas. Our prime objective is to extend the investigation of MUC1 protein expression and localization in GBC tissue by immunohistochemisty and Immunofluorescence.

MATERIAL AND METHODS

The subject consisted of 30 - 75 years (mean 50.25 ± 13.44) old Indian male (39/175) and female (136/175) GBC patients and male (05/25) and female (20/25) GSD (mean 51.37 ± 10.04) as control patients who had been admitted from December 2006 to January 2008 for treatment at SS Hospital, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. GBC cases were included on the basis of histologically confirmed adenocarcinoma of the gallbladder without any previous cancer. The study was approved by the human ethical committee of IMS, BHU.

Tumor invasion (T) and lymph node (N) classification fallowed the 5th edition of the UICC criteria. All of the resected tumors were histologicaly evaluated by one of co-author of this paper by H&E staining. Resected tumors were stored in 10% formalin at room temperature for immunohistochemistry and immunofluorescence.

Immunohistochemistory (IHC)

IHC has been done on 4 µm thick paraffin embedded gallbladder tumor tissue. Immunostaining was performing using a biotine-streptovidine alkaline phosphate method. Two adjacent section were used, one for immunostaining and one as a negative control. In brief after deparaffinization in xylene the slides were washed with water and rehydrated with graded alcohol. Antigen retrieval was done in boiling cooker for 10 min in a citrate buffer. Endogenous peroxidase activity was quenched by 15 min incubation in methanol with 3% hydrogen peroxides. Non specific bonding was blocked by the application of normal goat serum in a humidity chamber at a 1:7.5 dilution for 30 min. Primary anti-MUC1 antibody (Santacruze biotech, USA) were applied to sections at a dilution of 1:200 and were incubated for 2 h at room temperature, fallowed by incubation for 20 minutes with biotinylated antimouse secondary antibody and 20 min with HRP conjugated streptovidine. Peroxidase chromogen substrate was used for color development for 10 min. The sections were than counter-stained with hematoxylin and mounted in DPX by coverslip.

Immunofluroscence

FITC conjugated immunofluoresence staining of the formalin fixed tumor tissue section was performed. Briefly after deparafinization, rehydration, blocking as describe above in method of immunohistochemistry. Section were incubated for 12 h at 4°C with a mixture of blocking serum and anti human MUC1 anti sera with 1:200 dilution. After 5 rings in TBST, they were exposed to 10% rabbit serum for 1 h fallowing secondary antibody conjugated with FITC (1:700) thereafter, section were washed with TBST mount in glycerol and viewed under a fluorescence microscope (Zeiss) with appropriate filters. Negative controls were performed by replacing primary antibody.

Image analysis

Staining was analyzed using an automated imaging system with an Olympus IX50 microscope and Olympus imaging-analysis software (Olympus, Hamburg, Germany). Immunostaining intensity of MUC1 in gallbladder tissue was graded as strong (+++), moderate (++), weak (+) or nil (0).

Statistical analysis

Statistical analyses were performed using the SPSS 11.0 software (SPSS, Chicago, IL, USA). Independent sample t-test was used to compare patients' ages and tumor sizes between the patient groups with or without elevated MUC1 expression. Chi-square or Fisher's exact test was used to analyze the relationship between MUC1 expressions in different stages of tumor. The difference was considered significant if the p value was less than 0.05 (two-tailed test).

RESULTS

Anti-MUC1monoclonal antibodies were used to investigate MUC1 protein expression in gallbladder carcinoma and GSD, by immunohistochemistry. The apical, cytoplasmic and stromal MUC1 localization was observed in GBC. Whereas in GSD only apical MUC1 staining were observed. Other noncancerous lesions like hyperplasia, metaplasia and dysplasia, MUC1 also showed only apical staining (data not shown) (Figure 1). In cancerous lesion of GB, MUC1 expression showed diverse pattern with depth of invasion from T1 carcinoma in to T3 and T4 carcinoma. There were only two T1 cases and in both MUC1 expression was weak. In T2 tumor, 48.3% (14/29) cases showed weak expression and 51.7% (15/29) showed moderate expression. In T3 tumors, expression

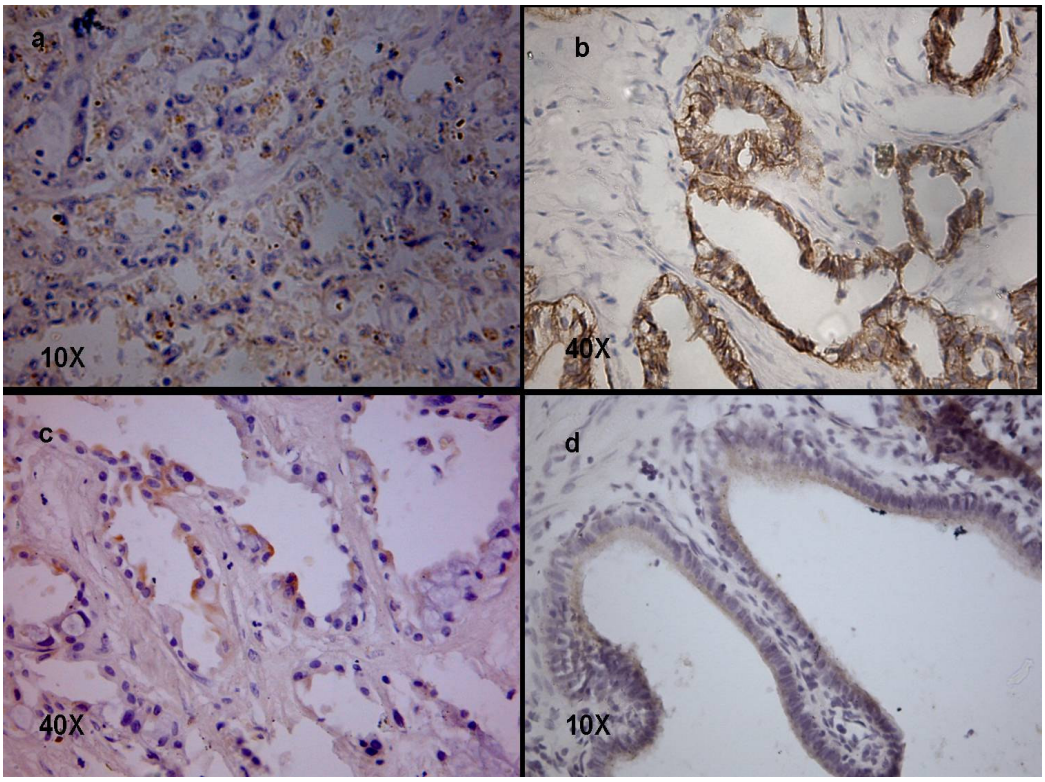

Figure 1. Immunohistochemical localization of MUC1 protein in GBC and GSD tissue section. a. MUC1 distribution in GBC tissue at 10X magnification. b. MUC1 is present in secretary glandular cells (40X) high magnification. c,. MUC1 polarization in high (40X) magnification. d. apical staining of MUC1.

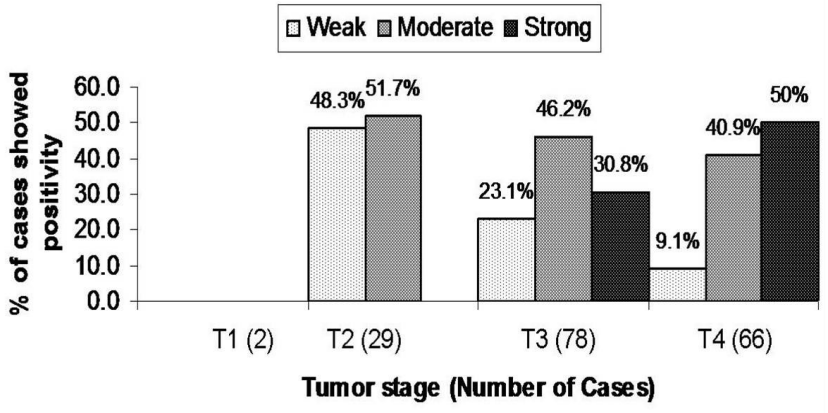

Figure 2. MUC1 expression in different stages (T1-T4) of GBC tumor tissue. Immunohistochemical expression of MUC1 in different tumor stages. On the basis of MUC1 expression each stage is divided in to weak, moderate and strong staining. * represent value that are significantly different from T2 tumor $p < 0.0001$.

was weak in 23.1 % (18/78), moderate in 46.2% (36/78) and strong in 30.8% (24/78) of cases. In T4 tumors, 9.1% (6/66) cases showed weak, 40.9% (27/66) moderate and 50% (33/66) strong expression of MUC1 (Figure 2). In T2 tumor we did not find any strong expression. Where as strong MUC1 staining found in only T3 and T4 tumor.

In respect to histological grades in WD tumor, 20% (12/60) cases showed weak, 43.3% (26/60) moderate and 36.7% (22/60) showed strong expression. In MD tumors, expression was weak in 26.2 % (16/61), moderate in 52.5% (32/61) and strong in 21.3% (13/61) of cases. In PD tumors, 20.4% (11/54) cases showed weak, 37.1%

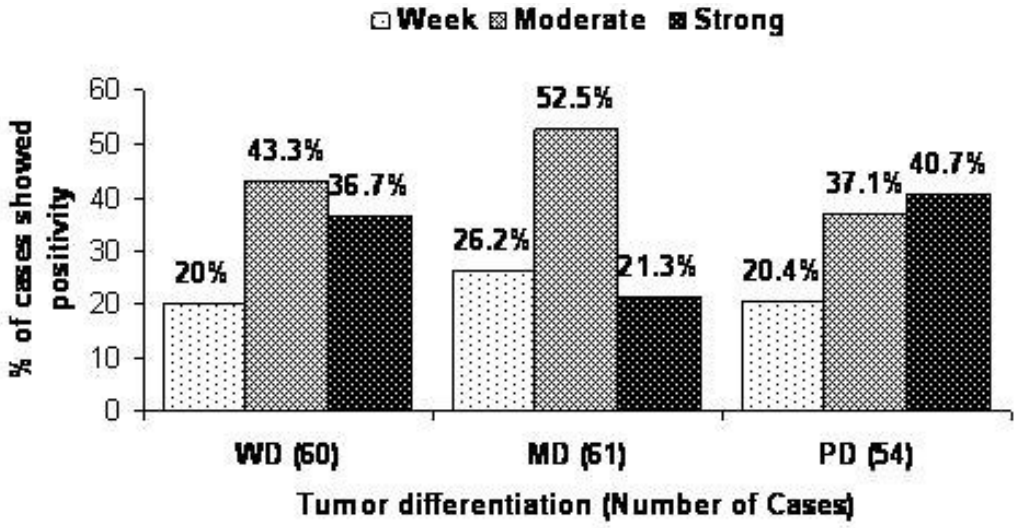

Figure 3. MUC1 expression in different grades of GBC tumor tissue. Immunohistochemical expression of MUC1 in tumor grades. All three grades well, moderate and poorly differentiated adenocarcinomas are divided, on the basis of MUC1 expression, in to weak, moderate and strong staining. Details are mentioned in material and method section. Results are shown in percentage value.

(20/54) moderate and 40.7% (22/54) strong expression of MUC1 (Figure 3).

Staining was most frequently seen in the form of scattered single or clustered cells in zones of the tumour equivalent to high suprabasal layers (Figures 1 and 2). Where as the in some cases staining was uniformly distributed within the cytoplasm (Figure 3) but in other positive cases the cells showed asymmetrical staining (Figure 4). Expression of MUC1 significantly increased in close correlation with the neoplastic process and reached its highest values in GBC ($p < 0.001$).

Immunofluorescence staining to see depolarization pattern in GBC and GSD

Immunofluorescence experiments were carried out to ascertain immunohistochemical result with more detail polarization study of MUC1 membrane protein (Figure 4). Expression and depolarization rate of MUC1 in the epithelial layer increases with (only two cases and both was showed no depolarization of MUC1 protein) advancing stages; that is, 55.2% (16/29) in T2, 89.7% in T3 (70/78) and 93.9% in T4 tumors (62/64), respectively. All of the cells in the MUC1-positive T1 cancers displayed apical staining (Figure 5).

On the other hand, 85% (51/60) of WD, 82% (50/61) of MD and 87% (45/54) of PD tumor showed depolarized pattern (Figure 6). This information confirmed the presence of MUC1 at the apical surface of both glandular and luminal epithelial cells in GBC and GSD tissue, but appears further profuse in tubal tissue where the distribution is again exclusively up-regulated and depolarized. These results clearly indicated that the MUC1 expression is up-regulated in GBC, independent from tumor differentiation.

DISCUSSION AND CONCLUSION

We examined immunohistochemically the expression patterns and quantity of MUC1 protein expression in GBC and GSD tissue. For many years researchers had observed a link between the aberrant expression of MUC1 and cancer progression. Earlier studies revealed that MUC1over expression is related with poor prognosis in numerous cancers (Uen et al., 2006; Egea et al., 1995; Reis et al., 1989; Lundy et al., 1985). For metastasis along with the members of cell adhesion family, MUC1 show unique properties and functions in cell to cell attachment and metastasis during cancer progression (Kawamoto et al., 2001; Guddo et al., 1998). It is thought that MUC1 strongly affects the survival of GBC patients. MUC1 is also involved in many cellular properties including cell adhesion, motility, proteolysis and angiogenesis (Ghosh et al., 2003; Ghosh et al., 2005). Based on our immunohistochemical result there was a significant correlation between MUC1 positivity with GBC tumor stages.

MUC1 expression was weak in GSD and T1 stage but reached maximum at T4 stage. In the patients who had undergone surgery with curative intent for less-advanced pT2 gallbladder carcinoma, these with a high expression rate of stromal localization of MUC1 at the deepest invading sites in the subserosal layer attributable to peritoneal dissemination or metastasis at distant organs, than those with a low expression rate. The results indicate that gallbladder carcinoma cells in the deepest invading sites

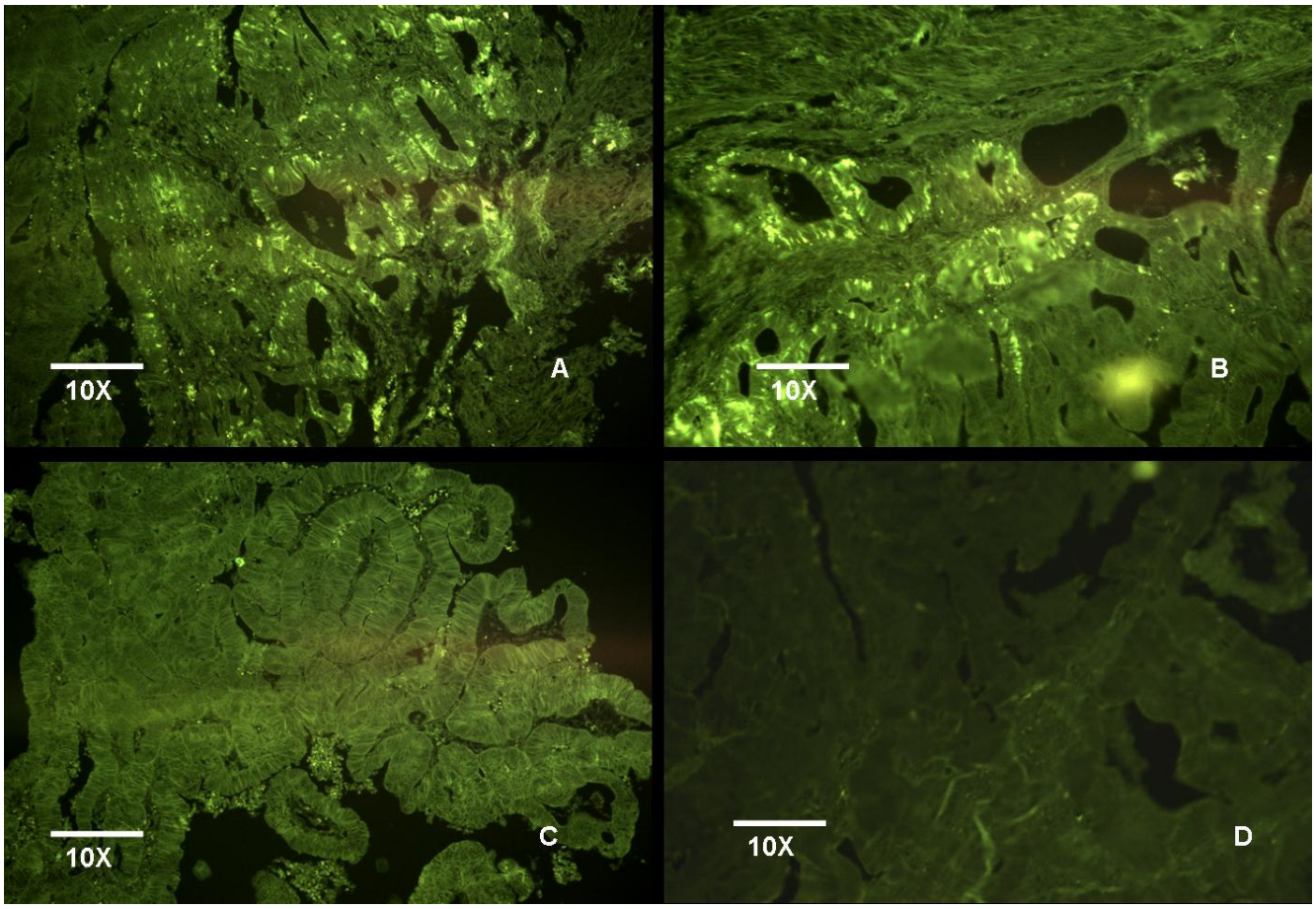

Figure 4. Immunofluorescence localization of MUC1 protein in GBC tissue. a and b. MUC1 staining is localized in glandular cells only. c. represent negative without primary antibody. d. auto fluorescence of GBC tissue.

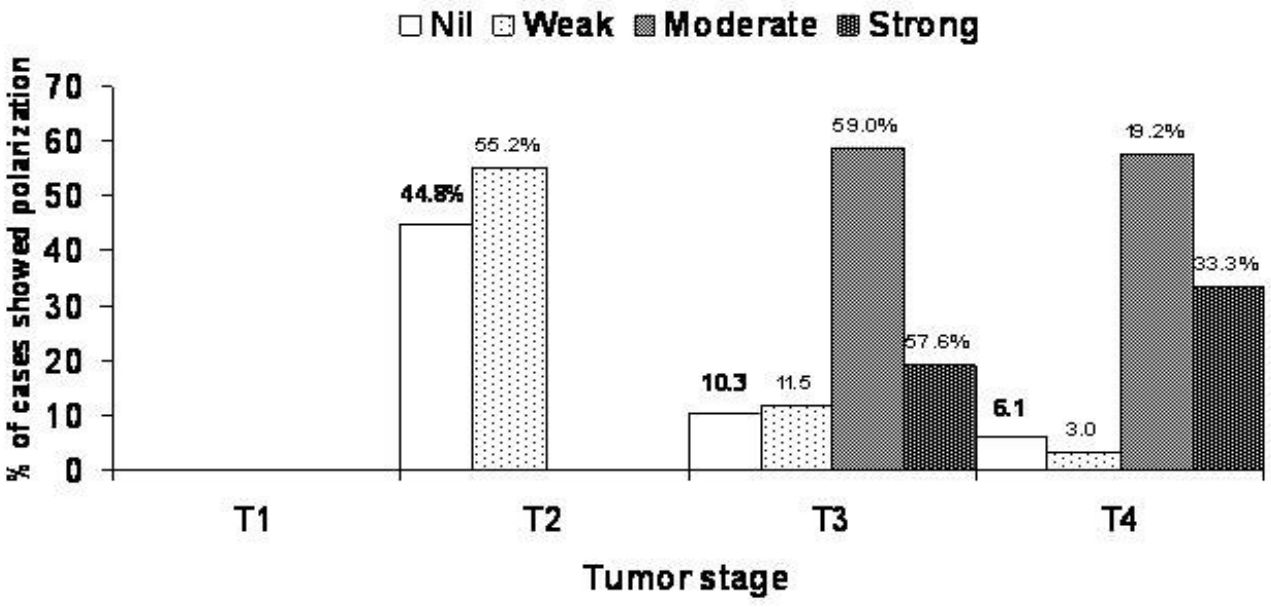

Figure 5. Percent of Polarization of MUC1 protein in GBC tissue (T1-T2). Polarization pattern of MUC1 protein in cell smear obtained from different stages of GBC.

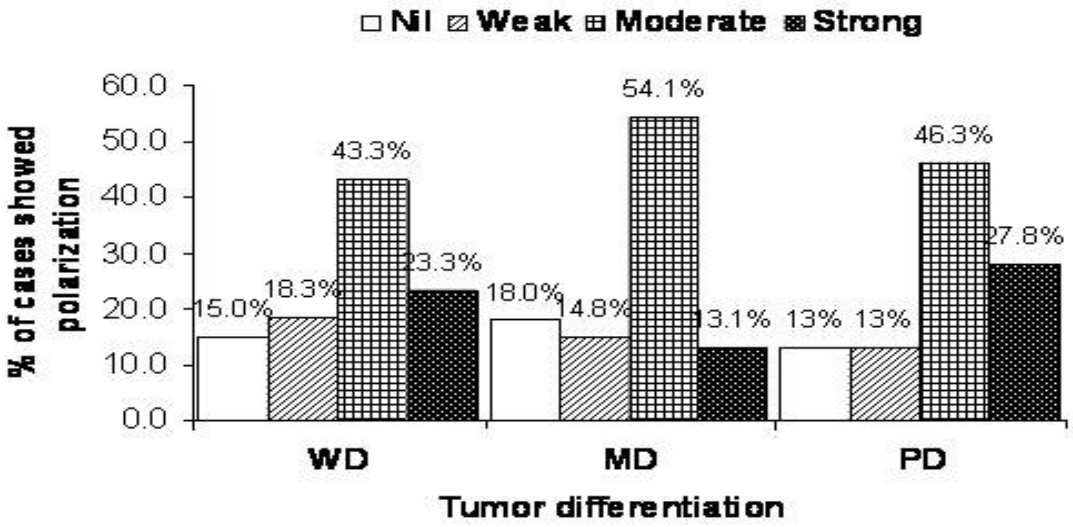

Figure 6. Percent of Polarization of MUC1 protein in GBC tissue (WD, MD and PD). Polarization pattern of MUC1 protein in cell smear obtained from different grades of GBC.

showing a high expression rate of stromal localization of the MUC1 mucin have a strong potential for metastasis and that micrometastasis may already have occurred at the time of surgery if the expression rate was high at the deepest invading sites.

Membrane-bound mucins MUC1 was less detectable in the cholilithiasis and the cholicystitis. Hinoda et al. (2003) also demonstrated MUC1 expression may be related to progression of pancreatic cancer.

Histologic type of GBC may represent separate disease entities with different invasion and metastasis behaviors. Because tumor invasion is associated with changes in cell adhesion molecules (Uen et al., 2006), we have studied the involvement of MUC1 in different grades of GBC tumor. MUC1 expression is independent of tumor differentiation because we did not find significant differences WD, MD and PD GBC tumor.

The mechanism of elevated expression of MUC1 is not known in GBC. But in colon cancer it has been observed that the activity of GalNAc transferase is increased in cancer, predominantly inside the rough endoplasmic reticulum (RER). Generally O-glycosylation occurs in golgi bodies of typical cell, but studies suggested that it occurred in the inflamed RER in spite of Golgi bodies (Egea et al., 1993). High levels of MUC1 protein at the cell membrane reduce cell-cell and cell-matrix interactions, possibly through steric hindrance (Wesseling et al., 1995; Hilkens et al., 1992). We have seen that the depolarized staining pattern is dominant in cancer cells and correlated with tumor stages. Our result shows strong MUC1 immunostaining in GBC tumor tissue and there was significant correlation between MUC1 expession and membrane localization. Strong MUC1 immunoreactivity in gallbladder tumor has been reported (Wang et al., 2006; Takagawa et al., 2005; Ghosh et al., 2005; Lee et al., 2002; Yamato et al., 1999) and this suggests that MUC1 might participate in cell migration and metastasis. During the development of gallbladder carcinoma MUC1 transcripts/ protein are seen, though their patterns of distribution are different. MUC1 expression in GBC indicated that MUC1 protein/transcript positivity was stage dependant.

Our results have also shown a close association among the depolarization of MUC1 in GBC and its depolarization by the tumor cells enhance the migration and metastasis. This study may provide scientific rationale for exploring therapeutic approached that can target cell surface adhesion molecule MUC1 in GBC.

In conclusion we have shown that early gallbladder cancer pathology may involve MUC1 in the disease progression. We have defined the localization of expression of the MUC1 genes during GBC development and correlated them with expression and its stage dependent nature. The identification of cells that show both overexpression and depolarization provides a logical starting point for further molecular analysis of mucin expression and glycosylation in specific epithelial cell types in gallbladder carcinoma. We believe that MUC1 expression may relate with tumour progression but also metastatic potentials.

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