Biomarkers of Gallbladder Cancer

R. Dixit*, V. K. Shukla

Maximillian de Stoll firstly identified Gallbladder cancer in two autopsy cases in 1777, and Keen was the first one to perform cancer resection in 1891.[1]Gallbladder cancer is a most common biliary tree cancer in the world but it is only 0.5% of all gastrointestinal cancer though lethal malignancy with marked ethnic and geographical variations. The representing signs and symptoms are commonly imprecise,so patient presents at a later stage. The overall mean survival rate for patients having advanced gallbladder cancer is 6 months, with a 5-year survival rate of 5%.[2,3] Cholecystectomy is the potential cure for early gallbladder cancer (restricted to the mucosa), though rare. The majority (>80%) of gallbladder cancers are adenocarcinomas which originate from fundus (60%), body (30%), as well as neck (10%). The premise likely is actually genetic susceptibility, perhaps elicited simply by chronic gallbladder inflammation, normally a result of cholelithiasis.[4] One particular reasonable hypothesis targets continual tenderness of the mucosa (e.g., through the actual physical vicinity of the rocks and/or superimposed chronic infection such as through *Salmonella typhi*) which leads to dysplasia (may be abetted by mutagenic secondary bile acids) and ending in cancerous change.

Ethnicity, genetic susceptibility, lifestyle factors and infections are the different risk factors for developing gallbladder cancer. If we see worldwide, gallbladder malignancy carries a low occurrence of <2 for every 100,000, but carries a large difference similar to U.S., it represents 0.5% of all gastrointestinal malignancies, representing short of 5,000 cases for every year (1 to 2.5 for every 100,000). High yearly frequency rates happen in North and South American Indians, creating an exorbitant mortality, especially among females: 15.5 for every 100,000 vs. 7.5/100,000 in men from La Paz, Bolivia, and 11.3 for every 100,000 in females vs. 4/100,000 in males from New Mexico.[5,6] Different high- risk places tend to be dispersed even though Eastern Europe (14/100, 000 in Poland), northern Indian (as higher as 21.5/100, 000 for women in Delhi) as well as South Pakistan (11.3/100,000).[7,8] Moderate frequencies (3.7 to 9.1 for each 100,000) occur somewhere else in South Americans of Indian origin, as well as in Israel (5/100,000) and in Japan (7/100,000).[7] The rate of occurrence is rising in Shanghai, China and now represents the most repeated gastrointestinal malignancy and is also a considerable reason for death.[9] Although the majority of the world possesses decreasing fatality developments in gallbladder cancer, Iceland, Costa Rica, and Korea have an elevation in mortality for men.[10] This gives off an impression of being an unassuming decrease in predominance in the course of recent decades [National Cancer Institute, Surveillance, Epidemiology and End Results (SEER) Program]. Around the world, GBC affects on females two to three times more than males. To some degree, this unique sexual orientation predisposition has been ascribed to the pervasiveness of cholelithiasis, which is more professed in females.[11] In spite of the fact that the specific etiology of GBC stays vague, a few elements are known to enhance the possibility such as cholelithiasis, chronic cholecystitis, calcified "porcelain" gallbladder, choledochal cysts, abnormal pancreatobiliary duct junction, along with gallbladder polyps. Various dietary components, chronic gallbladder diseases and environmental exposure to particular chemicals have also been related with the progression of GBC.[12] Growth of gallbladder cancer increases with age. Size of gallstones and length of time of gallstone illness additionally raise danger to developing cancer. Enhanced morbid obesity and repetitive salmonella contamination excessively creates risk for GBC.[13-16]

There are definitely genetic and environmental components which coincide leading to gallbladder cancer. The only liable gene discovered until now is the apolipo health proteins B (the APOB gene), which impacts cholesterol handling, however, is not associated with gallstones. Indeed, the connection between cholesterol gallstones and gallbladder tumor may possibly correspond toan interdependent disposal path that will boost the export of both cholesterol and ecological harmful toxins into bile.[17] A family history of gallbladder malignancy is unmistakably a risk factor.[18,19] GBC has familial predisposition. The nationwide Swedish Cancer Registry reported high risk for familial GBC and also found maternal

*Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi – 221 005, India.
Correspondence to: Dr V.K. Shukla, Professor, Department of General Surgery, Institute of Medical Sciences, Banaras Hindu University, Varanasi – 221 005, India. ***E- mail:*** vkshuklabhu@gmail.com

transmission favoring over paternal.[19] By far the most repeated molecular modifications in GBC are classified as the mutations of TP53 as well as KRAS gene, loss of cell-cycle regulation, micro satellite instability and loss of heterozygosity. Nevertheless, most of these mutations have a small percentage of GBC. Epidemiological studies have identified the role of various low penetrance variants in GBC susceptibility[12]; however, a lot more genes together with modest effects and their associations with one another and with environment are yet to be found. However, a recent genome-wide association study (GWAS) analysis on Japanese populace[20] has recognized a single nucleotide polymorphism (SNP) in the deleted in colorectal malignancy gene (DCC) for being implicated in GBC.

Ultrasound, CT scans, MRI, MRCP, MR angiography, Endoscopic retrograde cholangiopancreatography (ERCP), Percutaneous transhepatic cholangiography, Endoscopic and Laparoscopic Ultrasound, Diagnostic Laparoscopy and Guided Biopsies are common diagnostic imaging techniques of GBC.

Carcinoma of the gallbladder advances from dysplasia to carcinoma insitu progressing onto invasive carcinoma in about 15 years.[21] In spite of recent advances as well as passionate progress to recognizing potential biomarkers with regard to early on diagnosis as well as proper management, GBC continues to be a demanding tumor with a very poor prognosis. Distinctive types of gallbladder cancer are known as: adenocarcinoma (well differentiated, moderately differentiated as well as poorly differentiated adenocarcinoma), squamous cell carcinoma, adenosquamous, carcinoma insitu, small cell carcinoma, sarcomas and papillary cancer. Therefore, a few biomarkers are needed pertaining to earlier diagnosis of GBC. Detection of a protein which indicates the specific disease state is known as a biomarker. It particularly shows an expression change which associates with the development of a disease. Biomarkers can be of predictive or prognostic marker. The predictive marker predicts reaction to treatment like estrogen receptors in breast tumor while prognostic marker decides prognosis like lymph node status in breast malignancy. Tumor itself produces a tumor marker; if it is present in an enormous amount, it shows the presence of a cancer. It may be available in tissues or may be appearing in serum.[22-25]Alongwith the serum, other body liquids, for example, urine, effusions, saliva, cerebrospinal fluid (CSF), and nipple release can likewise be utilized for assessment of tumor markers.

Different biomarkers have been developed which have beenfound to be helpful in the diagnosis of gallbladder cancer. Some important ones are: CA 242, CA 19-9, CA 15-3, CA 125, CA 50, CA 72-4, CEA, Mucins, PCNA, and RCAS1. The two most usually used markers are Carbohydrate antigen 19-9 (CA19-9), and carcinoembryonic antigen (CEA), which are frequently used in the determination of GBC. They cannot be used for early diagnosis of gallbladder cancer because of low specificity and delayed increase in concentration levels usually found in late stages of gallbladder cancer. Significant concentration of CA19-9 can also be seen in mucin rich saliva, seminal fluid, gastric juice, amniotic fluid, urine, ovarian cyst fluid, pancreatic, gallbladder as well as duodenal aspirate.[26] Shukla et al. examined, CA242, CA19-9, CA15-3 as well as CA125 assay preoperatively within serum involving 39 affected individuals of carcinoma in the gallbladder, 4 affected individuals with cholelithiasis as well as 8 healthy controls by using ELISA technique.[27] CA 19-9 level was 86.06 u/ml in patients of cholelithiasis in comparison to 211.27 u/ml in patients of gallbladder cancer, the difference being statistically significant.[27] Combination of CA 19-9 and CA 125 gave precision of 80.65% in determination of gallbladder cancer.[27] The cellular presentation of carcinoembryonic antigen and its role in gallbladder cancer detection has been well described.[28-30] Serum levels of CEA are raised in those tissues which develop from normal to invasive adenocarcinoma.[31] Various studies found the elevated level of CEA in the GBC.[32-35] RCAS1 (Receptor Binding Cancer Antigen Expressed on Siso cells) is a membrane protein of type II which is associated with various human cancer cells. RCAS1 expression hinders the development and incites apoptosis in immune and natural killer cells.[36] Oshikiri et al. examined RCAS1 interpretation by immunohistochemistry in 60 patients with EBDC (Extra Hepatic Bile Duct Cancer).[37] RCAS1 expression was higher in 76.7% of cases and sensitivity and specificity was found to be around 74.4% and 91% respectively.[37] CA125 is a tumor associated glycoprotein of more than 200 KDa. Their concentration levels were significantly higher in gallbladder cancer in comparison to cholelithiasis.[38-42] CA 15-3 is a heterogeneous 300 KD glycoprotein antigen and is elevated in serum breast cancer.[43] CA242 antigen is a monoclonal antibody and has been obtained by immunizing mice with cell lines of human colorectal cancer (COLO 205).[44] It is associated, however not indistinguishable, to the epitope of CA 19-9 and CA 50.[45] Shukla et al. discovered altogether high, serum level of CA 242 (42.19u/ml)

in carcinoma of the gallbladder in comparison to12.10 u/ml in cholelithiasis.[27] A high positive relationship between values of CA242 and serum bilirubin or alkaline phosphatase levels has previouslybeenreported.[46] But more research is needed to establish its usefulness in gallbladder cancer. CA 50 can be utilized to distinguish betweenbenign and malignant gallbladder lesions. Sera from pancreatic diseases, gallbladder diseases and bile duct diseases were examined for CA-50 tumor marker and were observed to be important in discriminating cancerous from non-cancerous gallbladder diseases. Despite the fact that the effectiveness of this marker is entrenched in pancreatic malignancy, very little work has been carried out in gallbladder cancer.[47] CA 72-4 antigen in gallbladder bile was discovered to be better than any serum and pancreatic juice examination as for affectability and specificity.[48] The clinical significance of CA 72-4 has been established as itwas found to be elevated in carcinoma in situ and first stage of gallbladder cancer.[49] We have also looked at the enzyme activity of arginase in gallbladder cancer. Tissue arginase activity was found to be significantly higher from stage I to stage II and similarly from stage I to stage III.[50]Very few studies have been carried out on arginase. Mucins are usually a group of large molecular weight glycoproteins composed of a mucin core proteins as well as O-linked carbohydrate. To date, nine apomucins (MUC l to 8, and MUC5B) have been recognized. Latest studies have proven that MUC1 will be expressed in various types of tumors, and could work as an anti-adhesion molecule which inhibits cell-to-cell adhesion, causing metastasis.[51] Kashiwagi et al. studied the expression of MUC1 as well as MUC2 in gallbladder adenocarcinoma.[51] MUC1 immunoreactivity has been diagnosed in the malignant cells and in the stroma of cancer; and has been significantly associated with lymphatic attack, lymph node metastasis ($p<0.001$) and poor outcome. Then again, MUC2 was infrequently associated and had no prognostic importance with its immunoreactivity located just in the cancer goblet cells.[51]

Positivity rate of PCNA (Proliferating Cell Nuclear Antigen)indicates the cell proliferation activity, and subsequently, indirect measure of cell kinetics. Isozaki et al. analyzed PCNA expression in GBC patients with PBM and observed a 14.2% positivity rate in malignant lesions having maljunction in comparison to 3.9% rate among cancer patients with normal pancreaticobiliary junction.[32] They further found a 11.6% positivity rate among non-cancerous cases with maljunction as well as 1.5% positivity amongst normal patients.[52] Various studies have also found PCNA expression to be significantly higher in malignant lesion than benign.[53,54] Expression of E-Cadherin has been shown in 17/49 patients of gallbladder malignancy[55] and it was not found to be associated with age, sex, tumor measurement, grading and lymph node status.[56] Vimentin, somatostatin, gastrin, human chorionic gonadotropin, serotonin and pancreatic polypeptide have also been studied but their accurate role in gallbladder cancer has not been established.

Molecular studies in several neoplasms have established the activation of predominant proto-oncogenes along with inactivation of recessive tumor suppressor genes. Tumor suppressor genes are frequently inactivated by a mutation at a single allele along with removal of one more allele.[57] p53, a tumor suppressor gene, assumes a critical part in the genesis of numerous malignancies. The incidence rate of p53 overexpression in GBC is changeable (20-92%).[58-62] Roa et al., in an investigation of 157 primary GBC, found its over-expression in about 45% tumors.[61] In a study, they observed a high occurrence of p53 over-expression in GBC and in dysplasia associated with GBC and chronic cholecystitis, proposing that dysplasia is a critical and early event in the pathogenesis and progression of GBC.[63] Over-expression of p53 in GBC and in malignancy associated with chronic cholecystitis recommends that p53 mutations are the early events in GBC progression.[63] Allelic loss of short arm of chromosome number 9 has been recognized in many human cancers. A new tumor suppressor gene has been distinguished close to the interferon gene on chromosome number 9p21-22. This gene CDKN2, also known as MTS1 or even p16ink4, suppresses a formerly identified inhibitor (p16) associated with cyclin dependent kinase 4. A study reported that decreased expression of p16 is associated with degree of malignancy and metastasis of carcinoma of the gallbladder.[64] Other study also found LOH at 9p which is more regularly seen in intramucosal portion of invasive gallbladder cancer than insitu lesions.[65] It has been described that promoter hypermethylation of the p16 gene could have transpired as an early event, followed by homozygous deletion considered as a late event. They suggest two main pathways as LOH and homozygous deletion for inactivating the p16 gene in gallbladder cancers. This mechanism can be useful in the gallbladder cancer detection and treatment.[66] Theras gene family is a set of three closely related proto-oncogenes which are involved in the pathogenesis of many cancers.

H-ras, K-ras and N-ras are the three family members of the ras gene family.[67] The K-ras oncogene could be the other important genetic factor which is related with the pathway of pathogenesis of gallbladder cancer.[68] Mutations of K-ras are frequent in GBC associated with the anomalous anomalous union of the pancreaticobiliary duct (AUPBD).[69] Lee et al. examined the expression of ras oncogene by immunohistochemistry in gallbladder adenocarcinoma (n=13), dysplasia (n=3) and chronic cholecystitis (n=11); and found that its expression is important for GBC development but not for progression.[70] Another study of Hanada et al. concluded that k-ras mutation may be related with early stage of carcinogenesis of gallbladder mucosa with PBM when compared without PBM.[71] Fragile Histidine Triad (FHIT), CD antigen, Her-2-neu, heat shock protein, cyclo-oygenase enzyme, APL gene, HLA antigen and vascular endothelial growth factor (VEGF) are the other genes which have also been found to be associated with gallbladder cancer.[72-86]

Jayalakshmi et al. recently did an analysis of solid state 13C nuclear magnetic resonance (NMR) of gallbladder stones in gallbladder cancer and benign patients.[87] Differences were identified in the 13C chemical shift between stones from cholecystitis patients and from GBC patients. The study additionally performed qualitative and quantitative 1H NMR investigation of lipid extracts of gallbladder tissue in chronic cholecystitis, xanthogranulomatous cholecystitis (XGC), and GBC patients.[88] They have observed various changes in gallbladder tissue lipid components in chronic cholecystitis, XGC and GBC patients. It has been mentioned that chemical structure of bile displays metabolic functions which correlate with hepatobiliary organs like the gallbladder.[89] Components of bile have also been studied in several studies to find out the potential biomarkers in hepatobiliary diseases including GBC.[90-93] Koopmann et al. assessed a malignancy-related protein, Mac-2-binding protein (Mac-2BP), which had already been expressed in various tumors.[94] They concluded in their study that Mac-2BP in combination with CA19-9 levels can be used as a novel diagnostic tool for GBC.[94] Total protein was examined in GBC and benign gallbladder tissue by two-dimensional gel electrophoresis (2-DE) to identify potential biomarker. They recognized seventeen differentially expressed proteins and distinguished them by relative proteomic analysis. Some up-regulated proteins, like splicing factor 3B subunit 5, cystatin-B, S100A10 protein, histone H2B type 2-E, profilin-1, eukaryotic translation initiation factor 1A, isoform 1 of eukaryotic translation initiation factor 5A-1, FERM domain containing 3, glycer-aldehyde-3-phosphate dehydrogenase, serum amyloid P-component precursor, and harmonin isoform b3 were firstly identified in gallbladder malignancy. Out of all, two up-regulated proteins S100A10 and haptoglobin were further confirmed in patients of gallbladder cancer. They found raised protein levels by using western blotting. In this study, the majority of the patients with gallbladder disease had a tiny, surgically resectable tumor, recommending that the differentially expressed proteins can be conceivably utilized as biomarkers for the early detection of gallbladder malignancy. Further in this study, authors came to a conclusion that these differentially expressed proteins can be used as biomarkers [Tan Y et al. 2010].[95] Mou Y et al. likewise employed MALDI-TOF-MS in order to profile and assess the serum proteins of GBC and healthy controls.[96] An optimum proteomic pattern had been identified which was composed of three statistically different protein peaks, which differentiated GBC patients from control group.[96] Multiple gene expression analysis have been performed to look at the differential gene expression patterns between normal and tumor cells for the identification of potential biomarkers for GBC. Serial analysis of gene expression (SAGE) libraries have been created from GBC and non-neoplastic gallbladder mucosa and found an over-expression of connective tissue growth factor (CTGF) which was statistically significantly related with overall survival.[97]

Alternative splice variants have been identified in numerous tumors, in addition to an enormous number of cancer related genes.[98] These splice variants could either alter the cancer responsiveness or behave as surrogate markers. A thorough investigation of proteins which are coded by mRNA transcript variants of well-known and less known genes may propel the finding of novel diagnostic and prognostic biomarkers, and also new targets for therapy in GBC.

Micro RNAs (miRNAs) are found to be responsible for about 30% of the global gene expression. Various studies have reported the association of aberrant expression of miRNAs with many human cancers.[99] Another group of researchers observed a decreased expression of miR-148a and miR-152 in Mz-ChA-1 cells in relation with H69 non malignant human cholangiocytes invitro as well as tumor xenografts in vivo.[100] Therefore, miRNA may represent as a class of biomarkers in diagnostic approach.

In addition to previously mentioned probable options, latest research have discovered new types of low-molecular-weight metabolites, referred to as pteridines to be considerably increased in a variety of cancers establishing them as a promising toolin cancer diagnosis.[101] In addition to the open-ended genome-wide expression microarray analyses, specific gene families can also be used to identify and interrogate prognostic markers in GBC.[102] The transgenic mouse model (BK5.ErbB-2A mice) for GBC also represents a promising tool for the development of new treatment and/or prevention strategies [Kiguchi K et al. 2008].[103]

Because the underlying molecular processes often go on for decades until the initial clinical symptoms surface, the prognosis of GBC is generally poor and it is difficult to cure by surgery alone. Identification of new prognostic biomarkers would help identify patients who might benefit from additional treatment. The present article gives a brief idea of published studies in GBC on potential gene expression biomarkers. In order to conclude, not one of the biomarkers distinguished in GBC are particular or even efficient as being a routine screening test in a medical practice. More endeavors are needed for better understanding of the prognostic importance of gene expression biomarkers in gallbladder cancer.

References

1. Sheinfeld W. Cholecystectomy and partial hepatectomy for carcinoma of the gallbladder with local liver extension. *Surgery* 1947; 22(1): 48-58.
2. Levy AD, Murakata LA, Rohrmann CA Jr. Gallbladder carcinoma: radiologic-pathologic correlation. *Radiographics* 2001; 21: 295-314.
3. Pandey M, Pathak AK, GautamA et al. Carcinoma of the gallbladder: a retrospective review of 99 cases. *Dig Dis Sci* Jun 2001; 46(6): 1145-51.
4. Pandey M. Risk factors for gallbladder cancer: a reappraisal. *Eur J Cancer Prev* Feb 2003; 12(1): 15-24.
5. Andia ME, Hsing AW, Andreotti G et al. Geographic variation of gallbladder cancer mortality and risk factors in Chile: a population-based ecologic study. *Int J Cancer* Sep2008; 123(6): 1411-16.
6. Serra I, Calvo A, Csendes A et al. Gastric and gallbladder carcinoma in Chile: epidemiological changes and control programs. *Rev Med Chil* 1989; 117: 834-836. 186,187.
7. Lazcano-Ponce EC, Miquel JF, Muñoz N et al. Epidemiology and molecular pathology of gallbladder cancer. *CA Cancer J Clin* 2001; 51: 349-64.
8. Randi G, Franceschi S, La Vecchia C. Gallbladder cancer worldwide: geographical distribution and risk factors. *Int J Cancer* 2006; 118: 1591-602.
9. Hsing AW, Bai Y, Andreotti G et al. Family history of gallstones and the risk of biliary tract cancer and gallstones: a populationbased study in Shanghai, China. *Int J Cancer* 2007; 121: 832-38.
10. Hariharan D, Saied A, Kocher HM. Analysis of mortality rates for gallbladder cancer across the world. *HPB (Oxford)* 2008; 10: 327-31.
11. Gabbi C, Kim HJ, Barros R et al. Estrogen-dependent gallbladder carcinogenesis in LXRbeta-/- female mice. *Proc Natl Acad Sci USA* 2010; 107: 14763–68.
12. Srivastava K, Srivastava A, Sharma KL et al. Candidate gene studies in gallbladder cancer: a systematic review and meta-analysis. *Mutat Res* 2011; 728: 67-79.
13. Caygill CP, Hill MJ, Braddick M et al. Cancer mortality in chronic typhoid and paratyphoid carriers. *Lancet* 1994; 343: 83-84.
14. Chow WH, Johansen C, Gridley G et al. Gallstones, cholecystectomy and risk of cancers of the liver, biliary tract and pancreas. *Br J Cancer* 1999; 79: 640-44.
15. Pandey M, Shukla VK. Diet and gallbladder cancer: a case-control study. *Eur J Cancer Prev* Aug 2002; 11(4): 365-68.
16. Zatonski WA, Lowenfels AB, Boyle P et al. Epidemiologic aspects of gallbladder cancer: a case-control study of the SEARCH Program of the International Agency for Research on Cancer. *J Natl Cancer Inst* 1997; 89: 1132-38.
17. Venniyoor A. Cholesterol gallstones and cancer of gallbladder (CAGB): molecular links. *Med Hypotheses* 2008; 70(3): 646-53.
18. Goldgar DE, Easton DF, Cannon-Albright LA et al. Systematic population-based assessment of cancer risk in first-degree relatives of cancer probands. *J Natl Cancer Inst* 1994; 86: 1600-08.
19. Hemminki K, Li X. Familial liver and gall bladder cancer: a nationwide epidemiological study from Sweden. *Gut* 2003; 52: 592-96.
20. Cha PC, Zembutsu H, Takahashi A et al. A genome-wide association study identifies SNP in DCC is associated with gallbladder

cancer in the Japanese population. *J Hum Genet* Apr 2012; 57(4): 235-37.
21. Roa I, Araya JC, Villaseca M et al. Preneoplastic lesions and gallbladder cancer: an estimate of the period required for progression. *Gastroenterology* 1996; 111: 232–36.
22. Chu TM. Biochemical markers for human cancer. In: Morphological Tumors Markers, general aspects and diagnostic relevance. Seifert G (ed.) *Springer Verlag* 1987: 19-42.
23. Harnden DG. Human Tumor Markers: Biological Basis and Clinical Relevance. *J Roy Soc Med* 1985; 78: 1071–72.
24. Virji MA, Mercer DW, Herberman RB. Tumor Markers and their measurements. *Pathol Res Pract* 1988; 183: 95-99.
25. Bates SE, Longo DL. Use of serum tumor markers in cancer diagnosis and management. *Semin Oncol* 1987; 14: 102-38.
26. Malati T. Tumor markers: An overview. *Indian Journal of Clinical Biochemistry* 2007; 22(2): 17-31.
27. Shukla VK, Gurubachan, Sharma D et al. Diagnostic value of serum CA242, CA 19-9, CA 15-3 and CA 125 in patients with carcinoma of the gallbladder. *Trop Gastroenterol* 2006; 27(4): 160-65.
28. Bordes M, Michiels R, Martin F. Detection by immunofluorescence of carcinoembryonic antigen in colonic carcinoma, other malignant and benign tumor and non-cancerous tissues. *Digestion* 1973; 9: 106-15.
29. Isaacson P. Tissue demonstration of carcinoembryonic antigen (CEA) in ulcerative colitis. *Gut* 1976; 17: 561-67.
30. O'Brien MJ, Zamcheck N, Burke B et al. Immunocytochemical localization of carcinoembryonic antigen in benign and malignant colorectal tissues: Assessment of diagnostic value. *Am J ClinPathol* 1981; 75: 283-90.
31. Strom BL, Iliopoulos D, Atkinson B et al. Pathophysiology of tumor progression in human gallbladder: Flow cytometry CEA and CA 19-9 levels in bile and serum in different stages of gallbladder diseases. *J Natl Cancer Inst* 1989; 81: 1575-80.
32. Okami J, Dohno K, Sakon M et al. Genetic detection for micrometastasis in lymph node of biliary tract carcinoma. *Clin Cancer Res* Jun 2000; 6(6): 2326-32.
33. Kanthan R, Radhi JM, Kanthan SC. Gallbladder carcinomas: an immune prognostic evaluation of P53, Bcl-2, CEA and alpha-fetoprotein. *Can J Gastroenterol* 2000; 14(3): 181-84.
34. Dowaki S, Kijima H, Kashiwagi H et al. CEA immunohistochemical localization is correlated with growth and metastasis of human gallbladder carcinoma. *Int J Oncol* 2000; 16(1): 49-53.
35. Wang YF, Feng FL, Zhao XH et al. Combined detection tumor markers for diagnosis and prognosis of gallbladder cancer. *World J Gastroenterol* Apr 2014; 20(14): 4085-92.
36. Doval DC, Azam S, Sinha R et al. Expression of epidermal growth factor receptor, p53, Bcl2, vascular endothelial growth factor, cyclooxygenase-2, cyclin D1, human epidermal receptor-2 and Ki-67: Association with clinicopathological profiles and outcomes in gallbladder carcinoma. *J Carcinog* Aug 2014; 13: 10.
37. Oshikiri T, Hida Y, Miyamoto M et al. RCAS1 expression as a prognostic factor after curative surgery for extrahepatic bile duct cancer. *Br J Cancer* 2001; 85: 1922-27.
38. Bast RC Jr, Freeney M, Lazarus H et al. Reactivity of a monoclonal antibody with human ovarian carcinoma. *J Clin Invest* 1981; 68: 1331-37.
39. O'Brien TJ, Raymond LM, Bannon GA et al. New monoclonal antibodies identify the glycoprotein carrying the CA 125 epitope. *Am J ObstetGynecol* 1991; 165: 1857-64.
40. Davis HM, Zurawski VR, Bast RC et al. Characterization of the CA 125 antigen associated with human epithelial ovarian carcinomas. *Cancer Research* 1986; 46: 6143-48.
41. Magnani J, Nilsson B, Brockhous M et al. The Antigen of a Tumor Specific Monoclonal Antibody is a Ganglioside containing sialylated Lacto-N-Fucopentaose II: Federation Proceedings 1982; 41: 898.
42. Chaube A, Tewari M, Singh U et al. CA 125. A potential tumor marker for gallbladder cancer. *J Surg Oncol* 2006; 93(8): 665-69.
43. Atkinson BF, Ernst CS, Herlyn M et al. Gastrointestinal Cancer-associated Antigen in Immunoperoxidase Assay. *Cancer Research* 1982; 42: 4820-23.
44. Lindholm L, Holmgren J, Svennerholm L et al. Monoclonal antibodies against gastrointestinal tumour-associated antigens isolated as monosialogangliosides. *Int Arch Allergy Appl Immun* 1983; 71: 178–81.
45. Johansson C, Nilsson O, Lindholm L. Comparison of serological expression of

different epitopes on the CA50 carrying antigen CanAg. *Int J Cancer* 1991; 48: 757–63.
46. Pasanen PA, Eskelinen M, Partanen K et al. Multivariate analysis of six serum tumor markers (CEA, CA 50, CA 242, TPA, TPS, TATI) and conventional laboratory tests in the diagnosis of hepatopancreatobiliary malignancy. *Anticancer Res* 1995 Nov-Dec; 15(6B): 2731-37.
47. Johansson C, Nilsson O, Baeckstrom D et al. Novel epitopes on the CA50-carrying antigen: chemical and immunochemical studies. *Tumour Biol* 1991; 12: 159–70.
48. Brockmann J, Emparan C, Hernandez CA et al. Gallbladder bile tumor marker quantification for detection of pancreaticobiliary malignancies. *Anticancer Res* 2000; 20: 4941-47.
49. Stefanovic D, Novakovic R, Perisic-Savic M et al. The evaluation of tumor markers levels in determination of surgical procedure in patients with gallbladder carcinoma. *Med Pregl* 1993; 46(Suppl 1): 58-59.
50. Shukla VK**,** Tandon A, Ratha BK et al. Arginase activity in carcinoma of the gallbladder. A pilot study. *Eur J Cancer Prev* 2009; 18(3): 199-202.
51. Kashiwagi H, Kijima H, Dowaki S et al. MUC1 and MUC2 expression in human gallbladder carcinoma: a clinicopathological study and relationship with prognosis. *Oncol Rep* 2001; 8(3): 485-89.
52. Isozaki H, Okajima K, Hara H et al. Proliferating cell nuclear antigen expression in the gallbladder with pancreaticobiliary maljunction. *J SurgOncol* 1997; 65(1): 46-49.
53. Lee CS. Differences in cell proliferation and prognostic significance of proliferating cell nuclear antigen and Ki-67 antigen immunoreactivity in in situ and invasive carcinomas of the extra hepatic biliary tract. *Cancer* 1996; 78(9): 1881-87.
54. Qing D, Xia L, Zhang X. [The expressions of proliferating cell nuclear antigen (PCNA) in primary gallbladder carcinoma and its significance]. *ZhonghuaWaiKeZaZhi* 1995; 33(2): 102-104.
55. Wistuba II, Sugio K, Hung J et al. Allele-specific mutations involved in the pathogenesis of endemic gallbladder carcinoma in Chile. *Cancer Res* 1995; 55(12): 2511-15.
56. Sasatomi E, Tokunaga O, Miyazaki K. Spontaneous Apoptosis in gallbladder carcinoma. Relationship with clinicopathlogic factors, expression of E-cadherin, bcl2 protooncogene, and p53 oncosuppressor gene. *Cancer* 1996; 78: 2101 -10.
57. Knudson AG Jr. The ninth Gordon Hamilton-Fairley memorial lecture. Hereditary cancers: clues to mechanisms of carcinogenesis. *Br J Cancer* 1989; 59(5): 661-66.
58. Wistuba II, Gazdar AF, Roa I et al. p53 protein overexpression in gallbladder carcinoma and its precursor lesions: An immunohistochemical study. *Hum Pathol* 1996; 27: 360-65.
59. Ajiki T, Onoyama H, Yamamoto M et al. p53 protein expression and prognosis in gallbladder carcinoma and premalignant lesions. *Hepatogastroenterology* 1996; 43: 521-26.
60. Kamel D, Pääkkö P, Nuorva K et al. p53 and c-erbB-2 protein expression in adenocarcinomas and epithelial dysplasias of the gall bladder. *J Pathol* May 1993; 170(1): 67-72.
61. Roa I, Villaseca M, Araya J et al. p53 tumor suppressor gene protein expression in early and advanced gallbladder carcinoma. *Histopathology* 1997; 31: 226-30.
62. Misra S, Chaturvedi A, Goel MM et al. Overexpression of p53 protein in gallbladder carcinoma in North India. *Eur J Surg Oncol* 2000; 26(2): 164-67.
63. Agrawal V, Goel A, Krishnani N et al. p53, carcinoembryonic antigen and carbohydrate antigen 19.9 expression in gall bladder cancer, precursor epithelial lesions and xanthogranulomatous cholecystitis. *J Postgrad Med* Oct-Dec 2010; 56(4): 262-66.
64. Ma HB, Hu HT, Di ZL et al. Association of cyclin D1, p16 and retinoblastoma protein expressions with prognosis and metastasis of gallbladder carcinoma. *World J Gastroenterol* Feb 2005; 11(5): 744-47.
65. Hidaka E, Yanagisawa A, Sakai Y et al. Losses of heterozygosity on chromosomes 17p and 9p/18q may play important roles in early and advanced phases of gallbladder carcinogenesis. *J Cancer Res Clin Oncol* 1999; 125(8-9): 439-43.
66. Tadokoro H, Shigihara T, Ikeda T et al. Two distinct pathways of p16 gene inactivation in gallbladder cancer. *World J Gastroenterol* Dec 2007; 13(47): 6396-403.
67. Barbacid M. Ras genes: *Ann Rev Biochem* 1987; 56: 779 -827.

68. Wistuba II, Gazdar AF. Gallbladder cancer: lessons from a rare tumour. *Nat Rev Cancer* 2004; 4: 695-706.
69. Funabiki T, Matsubara T, Miyakawa S et al. Pancreaticobiliary maljunction and carcinogenesis to biliary and pancreatic malignancy. *Langenbecks Arch Surg* 2009; 394: 159-69.
70. Lee JC, Lin PW, Lin YJ et al. Analysis of K-ras gene mutations in periampullary cancers, gallbladder cancers and cholangiocarcinomas from paraffin-embedded tissue sections. *J Formos Med Assoc* 1995; 94(12): 719-23.
71. Hanada K, Itoh M, Fujii K et al. K-ras and p53 mutations in stage I gallbladder carcinoma with an anomalous junction of the pancreaticobiliary duct. *Cancer* 1996; 77(3): 452-58.
72. Tanaka H, Shimada Y, Harada H et al. Methylation of the 5' CpGisland of the FHIT gene is closely associated with transcriptional inactivation in esophageal squamous cell carcinomas. *Cancer Res* 1998; 58(15): 3429-34.
73. Zöchbauer-Müller S, Fong KM, Maitra A et al. 5' CpG island methylation of the FHIT gene is correlated with loss of gene expression in lung and breast cancer. *Cancer Res* 2001; 61(9): 3581-85.
74. Wistuba II, Ashfaq R, Maitra A et al. Fragile histidine triad gene abnormalities in the pathogenesis of gallbladder carcinoma. *Am J Pathol* 2002; 160(6): 2073-79.
75. Priya TP, Kapoor VK, Krishnani N et al. Fragile histidine triad (FHIT) gene and its association with p53 protein expression in the progression of gall bladder cancer. *Cancer Invest* Aug 2009; 27(7): 764-73.
76. Riquelme E, Tang M, Baez S et al. Frequent epigenetic inactivation of chromosome 3p candidate tumor suppressor genes in gallbladder carcinoma. *Cancer Lett* 2007; 250: 100–06.
77. Yamaguchi A, Zhang M, Goi T et al. Expression of variant CD44 containing variant exon v8-10 in gallbladder cancer. *Oncol Rep* 2000; 7(3): 541-44.
78. Yanagisawa N, Mikami T, Mitomi H et al. CD44 variant overexpression in gallbladder carcinoma associated with tumor dedifferentiation. *Cancer* 2001; 91(2): 408-16.
79. Su WC, Shiesh SC, Liu HS et al. Expression of oncogene products HER2/Neu and Ras and fibrosis-related growth factors bFGF, TGF-beta, and PDGF in bile from biliary malignancies and inflammatory disorders. *Dig Dis Sci* 2001; 46(7): 1387-92.
80. Seitz G, Thelsinger B, Tomasetto G et al. Breast cancer-associated protein pS2 expression in tumors of the biliary tract. *Am J Gastroenterol* Oct 1991; 86(10): 1491-44.
81. Kanoh K, Shimura T, Tsutsumi S et al. Significance of contracted cholecystitis lesions as high risk for gallbladder carcinogenesis. *Cancer Lett* 2001; 169(1): 7-14.
82. Kawamoto T, Shoda J, Asano T et al. Expression of cyclooxygenase-2 in the subserosal layer correlates with postsurgical prognosis of pathological tumor stage 2 carcinoma of the gallbladder. *Int J Cancer* 2002; 98(3): 427-34.
83. Tamiolakis D, Simopoulos C, Kotini A et al. Prognostic significance of HLA-DR antigen in dysplasia, carcinoma in situ and invasive carcinoma of the gallbladder. *East Afr Med J* 2003; 80(11): 554-58.
84. Quan ZW, Wu K, Wang J et al. Association of p53, p16, and vascular endothelial growth factor protein expressions with the prognosis and metastasis of gallbladder cancer. *J Am Coll Surg* 2001; 193: 380–83.
85. Mishra K, Behari A, Kapoor VK et al. Vascular endothelial growth factor single-nucleotide polymorphism in gallbladder cancer. *J Gastroenterol Hepatol* Oct 2013; 28(10): 1678-85.
86. Doval DC, Azam S, Sinha R et al. Expression of epidermal growth factor receptor, p53, Bcl2, vascular endothelial growth factor, cyclooxygenase-2, cyclin D1, human epidermal receptor-2 and Ki-67: Association with clinicopathological profiles and outcomes in gallbladder carcinoma. *J Carcinog* Aug 2014; 13: 10.
87. Jayalakshmi K, Sonkar K, Behari A et al. Solid state (13)C NMR analysis of human gallstones from cancer and benign gall bladder diseases. *Solid State Nucl Magn Reson* 2009; 36(1): 60-65.
88. Jayalakshmi K, Sonkar K, Behari A et al. Lipid profiling of cancerous and benign gallbladder tissues by 1H NMR spectroscopy. *NMR Biomed* 2011; 24(4): 335-42.
89. Kristiansen TZ, Bunkenborg J, Gronborg M et al. A proteomic analysis of human bile. *Mol Cell Proteomics* 2004; 3(7): 715-28.
90. Gowda GA. Human bile as a rich source of biomarkers for hepatopancreatobiliary cancers. *Biomark Med* 2010; 4(2): 299-314.

91. Park JY, Park BK, Ko JS et al. Bile acid analysis in biliary tract cancer. *Yonsei Med J* 2006; 47(6): 817-25.
92. Somashekar BS, Ijare OB, Gowda GA. Analysis of bile and gallbladder tissue by NMR: A route for diagnosis of gallbladder cancer. *Proc Intl Soc Mag Reson Med* 2007; 15: 127.
93. Uchida N, Tsutsui K, Ezaki T et al. Combination of assay of human telomerase reverse transcriptase mRNA and cytology using bile obtained by endoscopic transpapillary catheterization into the gallbladder for diagnosis of gallbladder carcinoma. *Am J Gastroenterol* 2003; 98: 2415–19.
94. Koopmann J, Thuluvath PJ, Zahurak ML et al. Mac-2-binding protein is a diagnostic marker for biliary tract carcinoma. *Cancer* Oct 2004; 101(7): 1609–15.
95. Tan Y, Meng HP, Wang FQ et al. Comparative proteomic analysis of human gallbladder carcinoma. *Zhonghua Zhong Liu ZaZhi* 2010; 32: 29–32.
96. Mou Y, Xing R, Liu C. Diagnosis of gallbladder cancer using matrix-assisted laser desorption/ionization time-of-flight profiling. *Am J Med Sci* 2012; 343(2): 119–23.
97. Alvarez H, Corvalan A, Roa JC et al. Serial analysis of gene expression identifies connective tissue growth factor expression as a prognostic biomarker in gallbladder cancer. *Clin Cancer Res* May 2008; 14(9): 2631-38.
98. Brinkmann D, Ryan A, Ayhan A et al. A molecular genetic and statistical approach for the diagnosis of dual-site cancers. *J Natl Cancer Inst* Oct 2004; 96(19): 1441-46.
99. Pang Y, Young CY, Yuan H. MicroRNAs and prostate cancer. *Acta Biochim Biophys Sin (Shanghai)* 2010; 42: 363–69.
100. Braconi C, Huang N, Patel T. MicroRNA-dependent regulation of DNA methyltransferase-1 and tumor suppressor gene expression by interleukin-6 in human malignant cholangiocytes. *Hepatology* 2010; 51: 881–90.
101. Koslinski P, Bujak R, Daghir E et al. Metabolic profiling of pteridines for determination of potential biomarkers in cancer diseases. *Electrophoresis* 2011; 32: 2044–54.
102. Jeong Y, Xie Y, Xiao G et al. Nuclear receptor expression defines a set of prognostic biomarkers for lung cancer. *PLoS Med* 2010; 7: e1000378.
103. Kiguchi K, DiGiovanni J. Role of growth factor signaling pathways. In: Biliary Tract and Gallbladder Cancer: Diagnosis & Therapy. Thomas CR, Fuller CD, (eds.) *New York: DemoMedical*, 2008: 19–36.