Study of AP Endonuclease (APEX1/REF1), a DNA Repair Enzyme, in Gallbladder Carcinoma

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Abstract. *Aim: This study investigated the levels of Apurinic/Apyrimidinic Endonuclease (APEX1) in gallbladder carcinoma (CaGB) tissue and co-related these levels with various clinicopathological parameters. Patients and Methods: Twenty cases of CaGB and cholelithiasis were included in the study. Western blot analysis of APEX1 protein was performed using actin as the reference point. Densitometric analysis and the integrated density value (IDV) of APEX1 protein samples were determined. The ratio of IDV of APEX1/actin was determined. Results: The mean IDV ratio of APEX1 in CaGB was 0.63±0.33 and 0.45±0.19 in cholelithiasis. The mean IDV ratio of a variant of APEX1 (ΔAPEX1) in CaGB was 0.50±0.09, whereas it was 0.40±0.16 in cholelithiasis. Calculating the mean IDV ratio of total APEX (APEX1+ΔAPEX1) in CaGB was 1.13±0.31 whereas in cholelithiasis, 0.85±0.23. The differences were statistically significant ($p<0.05$). Conclusion: A significant correlation was found between the relative expressions of APEX1 in cancer as compared to that in cholelithiasis patients. There was significant association between APEX1 expression and perineural invasion. A variant of APEX1 correlated with tumor infiltration. Hence APEX1 may be of use as a prognostic marker in patients with CaGB.*

Gallbladder cancer is a highly malignant neoplasm of the biliary tract and is more common in and around the northern region of India than South India (1). The low survival rate makes the development of new techniques for prediction prognosis and for better treatment an urgent need.

AP Endonuclease, also known as (APEX1/REF1), is the main apurinic/apyrimidinic (AP) endonuclease in eukaryotic cells which is responsible for the DNA base excision repair (BER) pathway of all DNA lesions. APEX1/REF1 protein is involved in DNA repair, growth signaling pathways and pathways involved in tumor promotion and progression (2-6). AP sites block DNA replication, lead to DNA breakage, mutagenesis and cytotoxicity (7, 8); information is limited regarding the molecular mechanisms responsible for this. The overexpression of mammalian APEX1 has been observed in tumor cells, although its down-regulation leads to induction of apoptosis of those tumor cells (9). APEX1 has dual function or behavior acting as in AP repair endonuclease, as well as a redox factor (10). Disruption of APEX1 function has been shown to have a detrimental effect on cancer cell viability and its elevated level in certain cancers has been implicated in resistance to chemotherapy/radiotherapy and cell killing (11-14). Therefore, the levels of APE1 may be proven to have a prognostic significance in cancer management.

It has been shown that oxidative DNA damage is higher in the case of solid tumors (15). It has been shown that reactive oxygen species are higher in gallbladder cancer in comparison to gall stones and this oxidative DNA damage is detected and repaired by the BER pathway. APEX1 identifies the damaged sites and allows their repair. The levels of APEX1 have been shown to be increased or elevated in prostate, cervical and lung cancer (16-18). But there is no such study reported for gallbladder cancer. The aim of the present study was to investigate the level of APEX1 in gallbladder cancer tissue and to establish the correlation between the level and the grade and stage of gallbladder cancer.

Patients and Methods

Patients. Tissue samples were obtained from patients admitted to the surgical unit of the SS Hospital, Banaras Hindu University, Varanasi, India. Twenty patients newly diagnosed with gallbladder cancer and twenty patients undergoing cholecystectomy for cholelithiasis constituted the control.

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Table I. *Integrated Density Value (IDV) ratio of APEX1 in CaGB and control groups.*

APEX1	Carcinoma of the Gallbladder (CaGB)	Cholelithiasis (Control)	*t*-Value	*p*-Value
APEX1/actin	0.63±0.33	0.45±0.19	2.039	0.048
Δ APEX1/actin	0.50±0.09	0.40±0.16	2.467	0.018
APEX1/actin + ΔAPEX1/actin	1.13±0.31	0.85±0.23	3.218	0.003

Sample collection, tissue lysate preparation and protein estimation. Gallbladder tissue and gallstones were collected from the operation theatre and immediately snap frozen in liquid nitrogen and then stored at –80˚C.

Tissue was pulverized into powder form by pouring the tissue into liquid nitrogen and using a mortar and pestle. Ice cold protease inhibitor lysis buffer (0.2 ml) was added to the powder (15 mg) and incubated on ice for 20 min. Samples were centrifuged at 12, 000 rpm (16, ×128*g*) at 4˚C for 15 min. The supernatant was collected and used as the total cellular lysate. Total protein was determined by Bradford assay (19). After protein estimation, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out using 12% resolving gel. Ten microliters of protein was loaded and ran at 100 V for 2 h. The power supply was stopped when the dye started to come out from bottom of the gel. After running the gel, the gel was kept in coomassie brilliant blue (CBB) stain and kept on shaker for two hours. The gel was destained in destaining solution (50% methanol, 12% acetic acid) and an APEX1 protein band was identified.

Western blot nalysis. Tissue lysate sample was loaded on 12% resolving gel for western blotting and run at 50 V for 15 min. The gel was transferred onto polyvinylidine difluoride (PVDF) membrane and stained with Ponceau- S stain. After destaining, the primary antibody (for APEX1) (Biogenuix, Delhi, India) was added, the membrane was washed with 0.3% tris-buffered saline and tween 20 (TBST); the secondary antibody (for APEX1) (Biogenuix, Delhi, India) was added after three such washes and kept in rotor for two hours. The blot was developed by keeping the membrane in plastic sheet and diamino benzidine (DAB) mixture was added in polythene sheet in dark place. The bands were visualized using autoradiographic film.

Densitometric analysis of the blot was carried out by Alpha Imager 2000 software (Alpha Innotech Corp, San Leandra, CA, US) and the integrated density value (IDV) of the samples was determined. The ratio of IDV of APEX1 to that of actin was used for relative expression estimation in each sample.

Statistical analysis. The statistical analysis was performed using the statistical software SPSS statistics version 17.0 for windows (US). Chi-square test and Mann-Whitney *U*-test were used for non-parametric variables and one-way ANOVA test was used for multiple group comparisons.

Results

Patient demographics. The mean age of patients in the CaGB group was 57.5 (range 25-75) years, while that in the control group was 45.5 (22-75) years. The female to male ratio was 5.6:1 in the CaGB patients while it was 4:1 in controls.

All patients of the CaGB group had adenocarcinoma by histopathology, whereas 18/20 patients had chronic cholecystitis and 2 patients had acute cholecystitis in the control group. Of the 20 cases of histopathologically proven CaGB, 11 patients had well-differentiated, 6 had moderately and 3 had poorly differentiated cancer.

Two patients had perineural invasion on histopathological examination, 14 had serosal invasion by tumor, 5 patients had involvement of the full thickness of gallbladder wall and 1 had involvement up to the sub-serosa.

APEX1 and APEX1 variant expression. The mean IDV ratio of APEX1 in CaGB was 0.63±0.33 and that in controls was 0.45±0.19. The mean the IDV ratio of the variant of APEX1 (ΔAPEX1) in CaGB detected in our samples was 0.50±0.09, whereas in the controls it was 0.40±0.16. The *p*-values ($p<0.05$) for APEX1, APEX1 variant (ΔAPEX1); total APEX1 (APEX1+ΔAPEX1) were 0.048, 0.018, 0.003 respectively all of which were statistically significant (Table I).

For those samples showing perineural invasion on histopathological examination, IDV ratio was determined for APEX1, APEX1 variant (ΔAPEX1); total APEX1 (APEX1+ ΔAPEX1). On comparing the results using the Mann-Whitney *U*-test the *p*-value was found to be <0.001 for APEX1; 0.031 for ΔAPEX1 variant and 0.002 for total APEX1, which are of statistical significance (Table II).

The IDV ratio of the samples was compared in relation to the wall thickness involved *i.e.* up to muscularis; up to serosa or infiltration beyond serosa. The *p*-values for APEX1 and for the total were 0.175 and 0.438 respectively, which were statistically insignificant. But the *p*-value for APEX1 variant was 0.044 which was statistically significant (Table III).

Discussion

Gallbladder cancer is a highly malignant and lethal disease of the biliary tract. The non specific nature of the early disease leads to difficulty in early diagnosis and treatment. The incidence of CaGB is progressively increasing in China (20) as well as north central India (21). Countries with the highest incidence of CaGB include India, Korea, Japan, Czech Republic, Slovakia, Spain, Columbia, Chile, Peru, Bolivia, and Ecuador (22).

Table II. *Comparison of Integrated Density Value (IDV) ratio of APEX1 according to perineural invasion.*

Perineural invasion	APEX1/actin	ΔAPEX1/actin	APEX1/actin+ΔAPEX1/actin
Present	1.3550±0.89803	0.380±.0707	1.7350±0.82731
Absent	0.5494±0.11254	0.524±0.830	1.0733±0.15335
Mann-Whitney *U*-test	<0.001	0.031	0.002

Table III. *Integrated Density Value (IDV) ratio of APEX1 according to tumor infiltration as evident on histopathology.*

Tumor infiltration	No. of cases	APEX1/actin	Δ APEX1/actin	APEX1/actin+ΔAPEX1/actin
Incomplete (up to serosa)	1	0.6400	0.5200	1.1600
Complete (up to serosa)	5	0.8750±0.62851	0.4240±0.07021	1.2980±0.57686
Complete beyond serosa along with liver infiltration	14	0.5421±0.12522	0.5393±0.08343	1.0814±0.16824
F-Value		1.934	3.758	0.867
p-Value		0.175	0.044	0.438

Kelley *et al*. (16) reported that APEX1 may be a diagnostic marker for early prostate cancer and play a role through its repair and redox in the physiology of the early development of prostate cancer. Bobola *et al*. (23) assayed APEX1 activity in human adult gliomas in order to establish any correlations with tumor characteristics. In their case, increased tumor activity was observed in 93% of tumor/normal pairs, indicating that elevations of APEX1 acivity are characteristic of human gliomagenesis. Yang *et al*. (24) also carried out a study on melanoma cells. They used nuclear and cytoplasmic extracts collected separately from different melanoma cell lines and cultured normal melanomacytes then subjected to them western blot assay. Results of this study showed that both nucleus and cytoplasm of neoplasm exhibited the higher APEX1 expression levels compared with melanocytes. Xu *et al*. (17) investigated the levels of APEX1 protein expression using immunohistochemistry in normal cervix, pre-invasive and invasive squamous lesions of the cervix, as well as in cervical cancer cell lines, and reported that the APEX1 protein is predominantly expressed in the nuclei of cells from both primary tumors and cervical cell lines but the levels of the APEX1 protein are significantly elevated in cervical cancer tissue. These results implicate the use of anti-APEX1 antibodies in the early detection of pre-malignant cancer of the cervix.

The expression of the APEX1 protein was also found to be higher in pancreatic cell lines than HPNE, HPDE and nonneoplastic human pancreatic cell lines (24). Cytoplasmic expression of APEX1 is also higher in cancer cells than in non-malignant liver cells within the tumor (25, 26).

In our study, we compared the IDV of APEX1 in CaGB and chronic cholecystitis. We found increased IDV of APEX1 in CaGB and the difference was statistically significant ($p<0.05$). We also observed a variant of APEX1 (ΔAPEX1) which also showed increased expression in CaGB (mean=0.51±0.09) as compared to cholelithiasis (mean=0.40±0.16). The molecular weight of ΔAPEX1 is 35 kDa and that of APEX1 endonuclease is 37 kDa. Similarly, significant correlation ($p=0.444$) was found between the expression of ΔAPEX1 and the tumor infiltration of the gallbladder wall. This finding provides an insight that ΔAPEX1 can be a biomarker for locoregional spread of the tumor. Significant correlation was also found between the expressions of APEX1, ΔAPE, total APEX1 and the presence of perineural invasion. This is the first study of this nature on CaGB.

Perineural invasion is associated with poor prognosis and a poor survival rate. Chijiiwa *et al*. (27) described the presence of perineural invasion and showed significantly poor survival in the multivariate analysis of the study carried out on 28 patients. From our study, we suggest APEX1 to be a surrogate for perineural invasion and hence for prognosis and survival. In addition, there was no significant correlation between APE expression and stage of tumor; nodal involvement; distal metastasis, or on nuclear differentiation or any other biochemical parameters (27).

A significant correlation was found between the relative expressions of APEX1 in cancer as compared to cholecystitis. In the present study, there was also significant association between APEX1 expression and perineural invasion. In addition, a variant of APEX1 was discovered which correlated with tumor infiltration. Hence APEX1 can act as a molecular biomarker for CaGB in predicting locoregional spread and aggressive behavior of the tumor.

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