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Topic Name: "Role of ABC transporter (ABCB1 and ABCG2) genes in Gallbladder cancer among Indian population"

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Findings

ABC transporters are involved in the transportation of various substrates across cell membrane and are engaged in diverse physiological processes like drug resistance, cell signaling, membrane homeostasis, etc. Emerging evidences suggest the role of ABC transporters in tumor biology and cancer progression. ABCB1 is most well-known ABC transporters which have broad specificity of substrates involved in the mechanism of drug resistance in cancer cells. The expression of ABCB1 has been found to be associated with the tumor phenotype in soft sarcoma and colorectal cancer. Similar to ABCB1, ABCG2 is widely studied in multi-drug resistance. The over-expression of ABCG2 has been reported in breast cancer, lung cancer, ovarian cancer, liver cancer and correlated with their poor prognosis. Here, we analysed the polymorphism, methylation and expression pattern of ABCB1 and ABCG2 in gallbladder cancer in Indian descents.

A total of 74 histo-pathologically proven gallbladder cancer patients were enrolled in this study. Tumor and adjacent normal tissue samples were collected from all the patients willing to give the consent for their participation in the study. Whole blood was collected from 74 healthy volunteers for RFLP study. In this study we analysed SNP, promoter methylation, mRNA expression and protein expression of ABCB1 and ABCG2 genes.

To study the polymorphism of ABCB1 C3435T and ABCG2 C421A we performed PCR-RFLP which was further validated by sanger sequencing. In ABCB1, the frequency of CC, CT and TT genotype at position 3435 was 9/74 (12%), 38/74 (51%) and 27/74 (37%) in GBC cases and 6 (8%), 42/74 (57%) and 26 (35%) in healthy control respectively. The distribution of genotype frequency as well as the allele frequency in patient group was nearly similar to the

healthy control group (p= 0.535 &p=0.810 respectively). The genotype frequency of ABCG2 C421A; CC, CA and AA were 53 (71.6%), 19 (25.6%) and 2 (2.7%) in GBC cases and 61 (82.43%), 11 (14.86%) 02 (2.70%) in healthy control subjects. However, the frequency of 'A' allele was more commonly observed in GBC cases when compared to healthy volunteers (p=0.083). We found no difference in the genotype pattern of tumor vs. normal tissue samples of GBC patients. On clinico-pathological correlation, the frequency of 'T' allele in ABCB1 was frequently observed in advance stage (p= 0.135) and also the genotype frequency of ABCG2 has shown association with TNM staging (p=0.034). The CC and CT genotype of ABCB1 showed increase mRNA expression compared to TT genotype, however it was not statistically significant. The genotype pattern of ABCG2 did not show any correlation with mRNA expression

The MS-PCR was performed to analyse the promoter methylation of ABCB1 and ABCG2 in gallbladder tumor and normal tissue sample. Hypomethylation of ABCB1 and ABCG2 promoter was observed in 73% (54/74) and 81% (60/74) cases respectively. On correlation with the clinico-pathological finding, we observed that the hypomethylation pattern of ABCB1 was observed more in female gender (p=0.089) and in patients with moderately differentiated tumors (p=0.018). Whereas, in ABCG2, the hypomethylation pattern showed significant association with T1-T2 depth of invasion (p=0.025) and inverse association with lymph node metastasis (p=0.045).

The mRNA expression of ABCB1 and ABCG2 in gallbladder tumor with respect to normal was analysed by Real-time PCR. In ABCB1 the gene was found to be up-regulated in 38/74 (51.35%) cases with fold change of 4.6 while in ABCG2 the gene was up-regulated in 49/74 (66.21%) cases with fold increase of 5.7. The mRNA of ABCB1 and ABCG2 was significantly up-regulated in patients with moderately differentiated tumors (Mean ± S.E;3.4±0.7, p=0.048) and (Mean ± S.E; 5.2±0.9, p=0.037) respectively. In ABCB1, the increased expression was inversely associated with perineural invasion (PNI) (p=0.009). While in ABCG2 the up-regulation was more frequently observed in female patients when compared to male patients (6.4±2.2vs3.2±0.4, p=0.036) and patient with early stage (5.1±1.0, p=0.041), T1-T2 depth of invasion (4.9±0.9, p=0.023) and without gallstone association (5.1±1.0, p=0.036).

The protein expression of ABCB1/P-gp and ABCG2/BCRP was assessed in tumor and normal tissue samples of GBC patients by Immunohistochemistry and Western blotting. We found strong positive expression of ABCB1/P-gp and ABCG2/BCRP in 36 (48.65%) and 51(68.92%)

tumor tissue compared to 24(32.43%) and 39 (52.70%) normal tissues respectively (p= 0.045 & p=0.0243). The expression was predominantly observed in the cytoplasm and few showed the membranous pattern along with subcellular localisation. The clinico-pathological correlation of IHC revealed that the high expression of ABCB1/P-gp was significantly associated with moderate differentiation (p=0.013). Similarly, strong expression of ABCG2/BCRP protein was associated with moderately differentiated tumors, although it did not attain statistical significance. In addition, the ABCG2/BCRP expression was found to be increased in patients with age >50 years, (p=0.021), in early stage and T1-T2 tumor depth. The quantitative expression of ABCB1 & ABCG2 protein was analysed by western blotting. Out of 74 cases, only 63 patients were considered for the analysis of protein expression by western blot as eleven cases had low protein concentration. In western blot, the tumor tissue samples showed up-regulation of ABCB1/P-gp in 26/63 (41%) and 44/63 (70%) in ABCG2/BCRP. Similar to IHC, the up-regulated expression of P-gp in western blot was significantly correlated with grade II tumors (p=0.045). Though not significant, the protein of ABCG2 was strongly expressed in patients with T1-T2 tumor depth and grade II differentiation.

We also found strong correlation between mRNA expression and hypomethylation of ABCB1 and ABCG2. The mRNA expression of ABCB1 and ABCG2 was significantly raised in hypomethylated cases {Mean ±S.E; 3.0±0.6 (p=0.037) and 4.4±0.7 (p=0.065) respectively}. Also, the mRNA level was higher in patients with increased IHC expression of ABCB1 and ABCG2 (p<0.05) and (p=0.001) respectively. We found higher expression of ABCG2 in comparison to ABCB1 in GBC. Interestingly, we also observed strong association between the expression of ABCB1 and ABCG2 at mRNA level as well as protein level. Out of 49 patients with up-regulated ABCG2 mRNA, 33 (67.34%) cases have shown increased expression of ABCB1 gene (p<0.001). Similarly, in 51 cases with up-regulated ABCG2 protein, 32 (62.74%) cases also had increased expression of ABCB1 protein (p<0.001).

Kaplan-Meier Log rank test was performed for survival analysis. Fifty-four patients were included for survival analysis. The predictive factors significantly associated with the overall survival of GBC patients was TNM stage (p=0.013), tumor depth (p=0.068), LN metastasis (p=0.003), papillary histology (p=0.046), and history of gallstone (p=0.026). The patients with advanced disease and higher tumor depth had shorter OS (p=0.013) and(p=0.068) respectively. Interestingly, the patients with gallstone association and lymph node positivity also had a shorter 1-year and 3-year survival when compared to patients without gallstone association and node positivity.

On analysing the effect of ABCB1 C3435T and ABCG2 C421A polymorphism on overall, we observed that ABCB1 polymorphism has not shown any significant effect on OS of the patients, while in ABCG2, the patient with heterozygous CA genotype had shorter overall survival (p=0.043). The methylation and expression pattern of bothABCB1 and ABCG2 have not shown any significant correlation with the OS.

The Mean DFS was 19.3 ± 12.2 months. In our study the DFS has shown significant association with female patient (p=0.005), advance stage (p=0.002), T3-T4 depth of invasion(p=0.018), lymph node metastasis (p=0.002), poorly differentiated tumors (p=0.048), absence of papillary histology (p=0.010) and presence of gallstone (p=0.004). The recurrence rate in patients with increased expression of ABCB1was lower as compared to the patient with down-regulated ABCB1 (9/32 vs 9/22) with better DFS (34.5 vs 28.2) months. However, the expression of ABCB1 and ABCG2 mRNA has not shown any significant association with DFS in GBC. Also, the SNP, methylation and protein expression in ABCB1 did not show any significant association with DFS in GBC patients.

The results of multivariate analysis revealed that, for OS the important predictive factors were LN metastasis (p=0.014) and presence of gallstones (p=0.052). In DFS; the tumor differentiation (p=0.055) and stage (p=0.037) has maintained their significance.

Among the cohort of 54 patients underwent curative resection, 48 patients were suitably eligible for adjuvant chemotherapy treatment. Twenty-seven patients of them had received adjuvant chemotherapy and kept in chemo group whereas 21 patients did not receive any adjuvant treatment (non-chemo group). In both the groups the expression status of ABCB1 and ABCG2 was compared with the survival. The up-regulation of ABCB1 genes showed similar incidence of death events 6/16 (37%) compared to 4/11 (37%) in patients with down-regulation. The up-regulation of ABCG2 showed lower incidence of death events 6/22 compared to 4/5 in patients with down-regulated. Kaplan Meier curve showed better overall survival pattern in patients with up-regulated ABCG2 with time. However, in non-chemo group the event of death was higher in cases with down-regulation as compared to cases with up-regulation of ABCB1(4/11 vs 4/16). In ABCG2 the event of death was higher in cases with up-regulation compared to those with down-regulation (7/18 vs 1/9). However, none of these changes were statistically significant. The above findings suggest that there could be interplay of other confounding factors affecting the function of ABC transporter in gallbladder cancer.