EGFR AND SMAD4 NEGATIVELY CORRELATED IN THE PROGRESSION OF GALLBLADER CANCER IN EASTERN INDIAN PATIENTS

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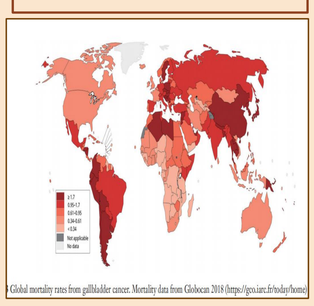
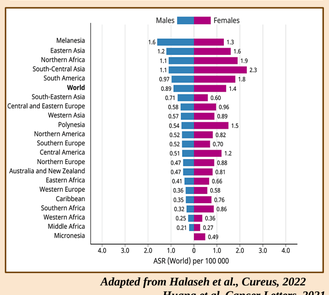
EPIDEMIOLOGY OF GALLBLADDER CANCER

The ASR of mortality was 1.7 per 100,000 persons (worldwide average) and the highest mortality rates were observed in Eastern Asia (2.4), South America (2.0), whilst the lowest rates were found in West Africa (0.28), and middle Africa (0.29).

There were around 116,000 new cases of GBC in 2020, with an average incidence rate of 1.4 per 100,000 in females and 0.9 per 100,000 in males. The incidence of GBC stands highest amongst native Chilean women with a rate of 27.3 per 100,000 persons.



Global mortality rates from gallbladder cancer. Mortality data from Globocan 2018 (https://gco.iarc.fr/today/home)



Adapted from Halaseh et al., Cureus, 2022
Huang et al. Cancer Letters, 2021

ETIOLOGICAL RISK FACTORS AND EPIDEMIOLOGY OF GBC IN INDIAN SCENARIO

GLOBOCAN 2020 data reports 15,333 incidences of GBC in India in a year, and accounts for one of the three leading cancers for women in North and North-East India. The age standardized rate (ASR) for GBC in women of North and north-east India are 11.8/100,000 population and 17.1/100,000 population respectively.

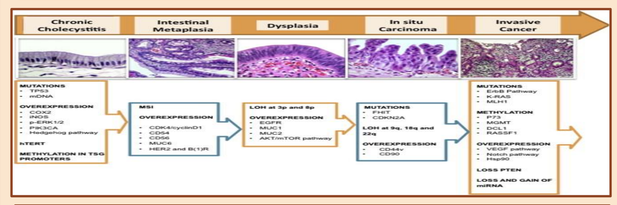
Patient predisposition	Environmental factors	Patient factors/conditions
Female sex	Chronic bacterial infections	Diabetes
Age	Aflatoxins	High body mass index
Race/ethnicity	Ochratoxin	Primary sclerosing cholangitis
Genetics (variants)	Arsenic	Porcelain gallbladder
	Liver fluke	Gallbladder polyps
	Geography	Crohn's disease
		Anomalous biliary ductal insertion
		Gallstones
		Sjogren's syndrome

Adapted from Dutta et al. Chinese Clinical Oncology, 2019

GENETIC ABERRATIONS LEADING TO GBC

- Most frequently mutated genes in GBC: *Tp53*, *SHH*, *ELF3*, *ARID1A*.
- Most frequently amplified oncogenes in GBC: *CDK4, ERBB2, FRS2, MDM2*, AND *MYC*.
- Most frequently deleted tumor suppressor genes in GBC: *CDKN2A, CDKN2B*.
- Most frequently dysregulated genes in GBC: *EGFR, ERBB2, CDKN2A, MUC1, MUC5A, p16, Cyclin E, Cyclin D1*.
- Loss of heterozygosity (LOH) has been reported in various chromosomal regions in GBC including 2p, 3p, 5q, 7q, 8p, 9p and 22q.
- Single nucleotide polymorphisms (SNPs) in genes *ABCB1*, *ABCB4*, *ABCG8*, *DCC*, etc.
- Epigenetic factors, mainly hypermethylation induces silencing of TSGs like *SHP1, CDH13, CDH1, APC*, etc in GBC.
- High degree of microsatellite instability infrequently observed in GBC (Weighted average = 3.5%).
- All genetic risk factors vary widely across different geographical and ethnic backgrounds.

FUNCTION OF GENES INVOLVED IN OUR STUDY



- ❖*EGFR/ERBB2* - *EGFR* and *Her2/neu*(or *ERBB2*) are members of the *ERBB2* receptor tyrosine kinase family associated with cell adhesion, differentiation and migration.
- ❖*CCND1* - *CCND1* (Cyclin D1) expression is a key mediator in the transition from G1 to S phase of the cell cycle.
- ❖*MYC* - *MYC* encodes for transcription factors that activate expression of many pro-proliferative genes.
- ❖*CDKN2A* – Inhibits interaction of CDKs with Cyclin D1.
- ❖*SMAD4* - Arrests cell cycle at the G1/S cell-cycle checkpoint.
- ❖*KRAS* - Encodes protein K-ras, which relays proliferative signals from outside the cell to cell's nucleus, and mutated K-ras have been implicated in many cancers.

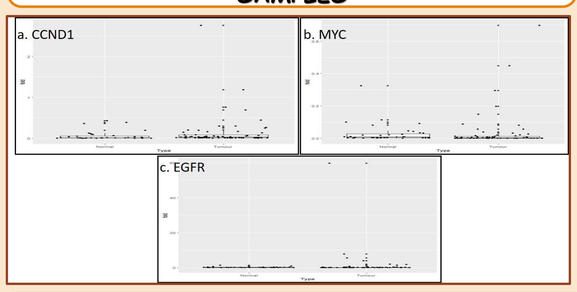
AIMS AND OBJECTIVES

- To study the differential expression of 6 frequently dysregulated genes in GBC in East Indian population.
- To estimate the amplification pattern of *ERBB2/Her2-neu* gene.
- To estimate the mutational frequency of *KRAS* codon 12 in gallstone disease (GSD) and GBC patient samples by different techniques.
- To draw a correlation between the different gene expression patterns, and associated clinico-pathological parameters.
- To validate *EGFR-SMAD4* expression correlation, *ERBB2* amplification, and *KRAS* codon 12 mutational frequency in independent GBC patient cohort.
- To validate protein expression of Egfr, Smad4 and Erbb2 from tissue blocks of patient samples.

MATERIALS AND METHODS

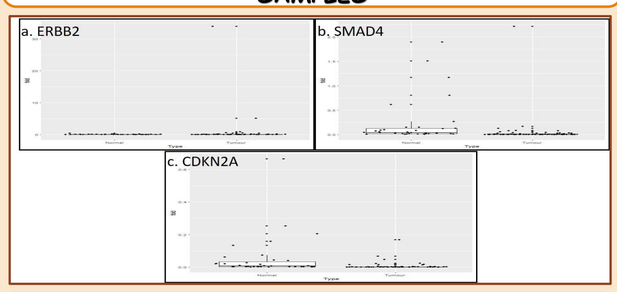
➢GBC tissue alongwith adjacent normal samples and GSD tissue samples were collected from 2 different government hospitals in Kolkata megacity followed by IRB Institutional ethical rules.
➢Real Time PCR analysis conducted to study differential gene expression.
➢Distributions of $2^{-\Delta ct}$ values for the respective genes were checked by Anderson–Darling test for both matched tumor and adjacent normal groups. Wilcoxon signed rank test was performed for statistical analysis.
➢Taqman Copy Number Assay was done to study *ERBB2* amplification.
➢Egfr, Smad4 and Erbb2 protein expressions were estimated by Immunohistochemistry methods.
➢Gene expression data and clinico-pathological data were correlated with Pearson's correlation.
➢RFLP, Allele Specific PCR and Sanger Sequencing were conducted to detect *KRAS* codon 12 mutations.

DIFFERENTIAL GENE (*CCND1, MYC* and *EGFR*) EXPRESSION STATUS IN GBC SAMPLES



Gene expression status of (a) *CCND1* ***(p value = 0.50)***, (b) *MYC* ***(p value = 0.07)*** and (c) *EGFR* ***(p value = 0.0005)*** in 68 tumor samples with adjacent normal samples.

DIFFERENTIAL GENE (*ERBB2, SMAD4* and *CDKN2A*) EXPRESSION STATUS IN GBC SAMPLES



Gene expression status of (a) *ERBB2* ***(p value = 0.00001)***, (b) *SMAD4* ***(p value = 0.009)*** and (c) *CDKN2A* ***(p value = 0.003)*** in 68 tumor samples with adjacent normal samples.

CORRELATION STUDIES

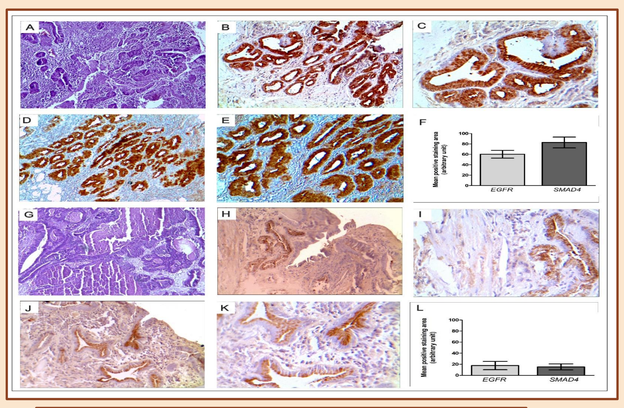
		CCND1	MYC	EGFR	ERBB2	CDKN2A	SMAD4
GallStone	Pearson Correlation	-.062	.050	1.000**	-.047	-.258	-.375*
	Sig. (2-tailed)	.696	.753	**.000**	.777	.104	**.016**
	N	42	42	42	39	41	41
Lymph Node	Pearson Correlation	.271	-.207	-.047	1.000*	.272	.127
	Sig. (2-tailed)	.091	.200	.777	**.000**	.094	.442
	N	40	40	39	40	39	39
Stage	Pearson Correlation	.416**	,716*	-.141	.025	-.038	-.083
	Sig. (2-tailed)	**.006**	**.000**	.372	.877	.809	.599
	N	43	43	42	40	42	42
Grade	Pearson Correlation	.111	.087	.479**	.013	-.129	-.250
	Sig. (2-tailed)	.488	.588	**.002**	.937	.427	.120
	N	41	41	40	39	40	40

Clinicopathological parameters

		CCND1	MYC	EGFR	ERBB2	CDKN2A	SMAD4
CCND1	Pearson Correlation	1	.497**	-.062	.271	-.179	-.106
	Sig. (2-tailed)		**.001**	.696	.091	.256	.505
	N	43	43	42	40	42	42
MYC	Pearson Correlation	.497**	1	.050	-.207	-.050	-.139
	Sig. (2-tailed)	**.001**		.753	.200	.753	.381
	N	43	43	42	40	42	42
EGFR	Pearson Correlation	-.062	.050	1	-.047	-.258	-.375*
	Sig. (2-tailed)	.696	.753		.777	.104	**-.016**
	N	42	42	42	39	41	41
ERBB2	Pearson Correlation	.271	-.207	-.047	1	.272	.127
	Sig. (2-tailed)	.091	.200	.777		.094	.442
	N	40	40	39	40	39	39
CDKN2A	Pearson Correlation	-.179	-.050	-.258	.272	1	.583**
	Sig. (2-tailed)	.256	.753	.104	.094		**.000**
	N	42	42	41	39	42	42
SMAD4	Pearson Correlation	-.106	-.139	-.375*	.127	.583**	1
	Sig. (2-tailed)	.505	.381	**-.016**	.442	**.000**	
	N	42	42	41	39	42	42

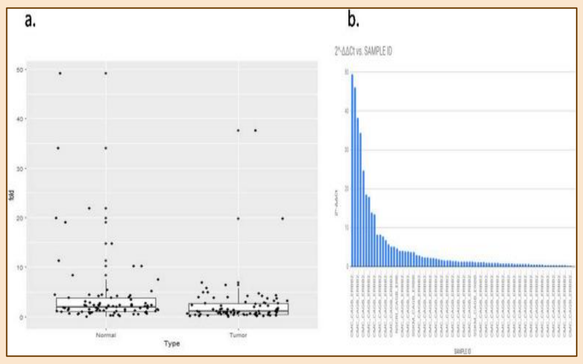
Gene expression

IMMUNOHISTOCHEMICAL STUDIES OF *EGFR* AND *SMAD4* EXPRESSION IN GBC PATIENT SAMPLES



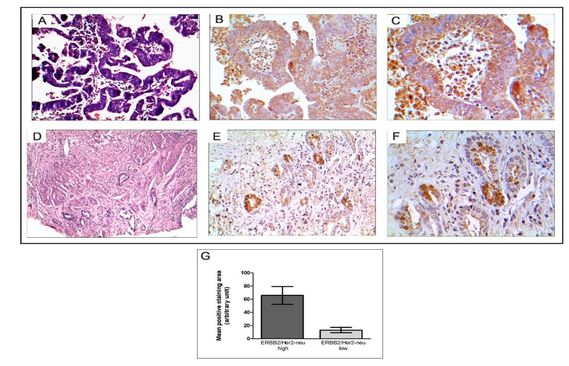
Total 15 tumor and normal paired tissue blocks were taken for IHC studies

ERBB2/HER2-NEU GENE AMPLIFICATION AS OBSERVED BY TAQMAN COPY NUMBER ASSAY



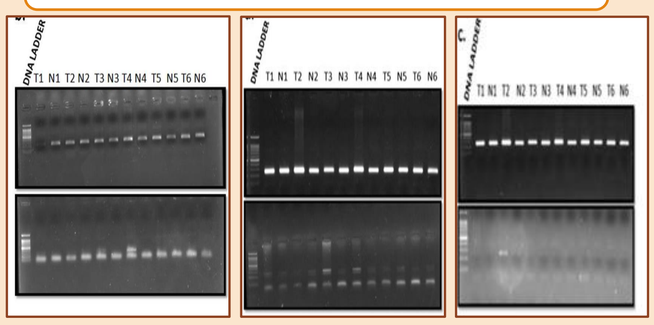
a Amplification of *ERBB2/Her2-neu* gene in GBC samples ***(p value = 0.03)***. **b** Distribution of *ERBB2/Her2-neu* gene amplification in GBC by Taqman copy number assay.

IMMUNOHISTOCHEMICAL STUDIES OF *ERBB2* EXPRESSION IN GBC PATIENT SAMPLES



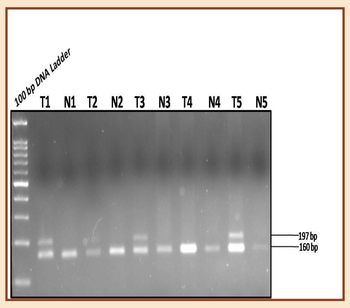
Total 15 tumor and normal paired tissue blocks were taken for IHC studies

VALIDATION OF *KRAS* CODON 12 MUTATIONS DETECTED BY ASPCR

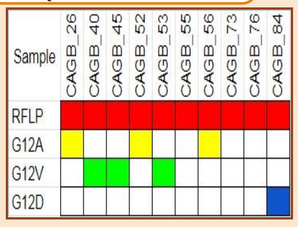


Visualization of Agarose Gel Electrophoresis after run of Allele Specific PCR for detection of (a) *KRAS G12A* mutations (b) *KRAS G12V* mutations (c) *KRAS G12 D* mutations

DETECTION OF DIFFERENT TYPES OF *KRAS* CODON 12 MUTATIONS AMONG GBC SAMPLES BY RFLP TECHNIQUE



KRAS codon 12th mutation detected by PCR-RFLP method.



11.5% of 68 GBC samples recorded codon 12 mutations, while no codon 12 mutations have been recorded in GSD samples. We did not observe any codon 13 and codon 61 mutations in GBC as well as GSD patient samples.

CONCLUSION

❑Overexpression of *EGFR* and *ERBB2* and downregulation of *CDKN2A* and *SMAD4* have been found to be statistically significant in the population.

❑ A negative Pearson correlation has been observed between *EGFR* and *SMAD4* expression levels in Indian GBC samples and validated also in another independent cohort.

❑*ERBB2/Her2-neu* amplification have been recorded as statistically significant in the GBC samples.

❑The above two observations have been validated in tissue samples through IHC studies.

❑Strong positive correlations have been observed between *EGFR* overexpression and recorded gallstone history, and between *ERBB2* overexpression and lymph node metastasis after Pearson correlation analysis.

❑Low percentage (11.5%) of *KRAS* codon 12 mutation have been observed and validated among Indian GBC patients but not in GSD patient samples.

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