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ORIGINAL ARTICLE

Role of CYCLIN D1, E-Cadherin, EGFR, Her-2, KI67, and P53 Expressions as Prognostic Markers in Gall Bladder Cancer

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ABSTRACT

Introduction: Worldwide gall bladder cancer (GBC) is known to be the commonest malignant tumour of the biliary tract .It is the most aggressive carcinoma of the biliary tract with short median survival from the time of diagnosis. The aggressive biologic behavior of the carcinoma and non-availability of sensitive screening tests for early detection may be responsible for the poor prognosis associated with GBC. Owing to the delayed diagnosis at an advanced stage, only 10% of the patients are found to be eligible for a curative surgical resection.

Material and Methods: All consecutive patients diagnosed with neoplastic and non-neoplastic gallbladder lesions in the Department of Pathology, Subharti Medical College were included in the study between the year 2017 -2019. The hematoxylin and Eosin stained biopsies of 320 patients were assessed and out of them 100 patients were chosen as the sample for the study. The clinicopatholgical data of the 100 patients were compiled into a data base and de-identified.

Results: Age distribution of Gall Bladder lesion cases in our study was from 30 years to more than 60 years of age. 46.20% of females in the age group of 45 years to 60 years presented with mass in the gall bladder. There was significant difference in the presence of mass between the Neoplastic and non-neoplastic group among 45-60 years of age ($p<0.001$). It was analyzed that there had been a significant difference between the neoplastic and non neoplastic tumour morphology and age distribution among males. The neoplastic tumours were highest in >60 years age group while neoplastic tumours were highest among 45-60 years age group . The presence of E-cadherin, Ki67 and P53 together suggested the presence of histological grade of carcinoma. There was no significant association between the presence of metastasis and biomarkers concentrations.The presence of E-cadherin, Ki67 and P53 together suggested the presence clinical stage of carcinoma .

Conclusions: The minimal response of advanced cases of GBC to traditional treatments calls for new prognostic and treatment perspectives to be identified. Novel prognostic biomarkers could bring about the needed breakthrough in this regard as they will help in the identification of patients who will benefit tremendously from adjuvant and targeted therapies.

Keywords: Cyclin D1, E-cadherin, EGFR, HER- 2, Ki67, p53 tumor marker ,neoplastic ,non -neoplastic Gall bladder lesions

INTRODUCTION

Gallbladder cancer [GBC] is a rare and invasive type of carcinoma. A common form of Biliary tract cancer, GBC develops in the epithelial lining of the gallbladder [1,2]. It is one of the lethal forms of carcinoma with a higher recurrence rate [1] and poor survival rate [2,3,4]. It encompasses a variety of risk factors such as geographical variations, environmental variables, age, gender, food habits, and lifestyle along with genetic predisposition, appear to have a bearing on its global incidence [4,5]. Benign diseases like cholelithiasis, cholecystitis, porcelain gallbladder, polyps etc., cause chronic inflammation of the gallbladder epithelia and thus contribute to the risk of developing GBC [1,2].

At present, early surgical resection presents a positive prognosis for patients, but early diagnosis poses a significant challenge since GBC is characterised by delayed symptom expression. Further, the ability to aggressively invade and metastasize to local organs and outlying lymph nodes contributes to the higher mortality associated with GBC. This results in a delayed diagnosis of most patients and response to traditional therapies remaining dismal with disease progression [1,4].

In order to improve treatment efficiency, there is a need for accurate and early diagnosis followed by targeted therapy options. Identifying specific prognostic biomarkers and prospective candidates for targeted therapies has become imperative in treating GBC [4,5]. Several molecular markers have been studied to identify their role in discerning histological grade, wall infiltration extent and metastasis [5], their potential as exclusive markers for GBC are yet to be established as carcinogenesis itself is a complex multistep process. Prognostic markers help establish possible disease prognosis [6] and to recognise clinical endpoints and patient response to an individual therapy method [3]. Recent understanding of Gallbladder carcinogenesis has opened up avenues to develop precision prognostic markers that could help identify disease progression and help patients receive personalised and targeted treatment.

Identifying the molecular and genetic factors that bring about the transition of benign inflammatory conditions into carcinomas can be used to develop an effective early detection and staging system. A biomarker developed for the detection of early molecular changes or risk indicators of carcinogenesis could change the prognosis in a positive direction [7]. An exclusive marker for GBC like that of CA 125 in ovarian cancer and PSA in prostate cancer [6] could bring about a change in how GBC will be diagnosed and treated.

In this aspect, we have strived to do a present study to analyse as how the expression of Cyclin D1, E-Cadherin, EGFR, HER-@, KI67 and P53 impacts the progression of GBC and if their respective role in the carcinogenesis of GBC can be exploited to their clinical applicability as prognostic tools.

MATERIAL AND METHODS

All consecutive patients diagnosed with neoplastic and non-neoplastic gallbladder lesions in the Department of Pathology, Subharti Medical College were included in the study between the year 2017 -2019. The hematoxylin and Eosin stained biopsies of 320 patients were assessed and out of them 100 patients were chosen as the sample for the study. The clinicopatholgical data of the 100 patients were compiled into a data base and de-identified.

RESULTS

Age distribution of Gall Bladder lesion cases in our study was from 30 years to more than 60 years of age. 46.20% of females in the age group of 45 years to 60 years presented with mass in the gall bladder. Table 1 and Figure 1 shows the difference in distribution of Mass between Neoplasia & Non Neoplasia across Age group. Related to Mass presence, there was significant difference between the Neoplastic and non-neoplastic group among >60 years of age group. None of the cases with Gall bladder lesion had mass in the 30-44 years of age between the neoplasia and non-neoplasia group. There was significant difference in the presence of mass between the Neoplastic and non-neoplastic group among

45-60 years of age (p<0.001). About 54.2% males in neoplastic and only 5% males in non-neoplastic group had mass present and there was significant difference found between the Neoplastic and non-neoplastic group. About 85.7% females in neoplastic and only 6.70 females in non-neoplastic group had mass present and there was significant difference between the Neoplastic and non-neoplastic group. (Table 2 and Figure 2). There was also significant difference between the Neoplastic and non-neoplastic in female (p=0.02). (Table 3 and Figure 3). Regarding the mean value of biomarker distribution related to the neoplastic lesions. The mean cadherin value was highest in Adenosquamous Carcinoma (3.0) followed by Invasive Paillary Adenocarcinoma (2.8). The Mean Cyclin D1 value was highest as 3.0 and had been found in Adenosquamous Carcinoma, Infiltrating Adenocarcinoma, The mean Ki67 value was highest in Adenosquamous Carcinoma (3.0), followed by Undifferentiated Adenocarcinoma (2.8), Mucinous Carcinoma (2.5). The mean p53 was highest as 3.0, in Adenosquamous Carcinoma, Infiltrating Adenocarcinoma, Mucinous Carcinoma and Undifferentiated Adenocarcinoma. It was analyzed that there had been a significant difference between the neoplastic and non neoplastic tumour morphology and age distribution among males. The neoplastic tumours were highest in >60 years age group while neoplastic tumours were highest among 45-60 years age group (Table 4 and Figure 4). In the Model summary of biomarkers severity and its association with the Histological Grade of Carcinoma. The maximum R Square change was with model 2 followed by model 3. Model 3 explained the most of the association (51%) with the biomarkers and histological grade of carcinoma. The presence of E-cadherin, Ki67 and P53 together suggested the presence of histological grade of carcinoma. (Table 5). The model summary of biomarkers severity and its association with the clinical staging of Carcinoma in Table 6 showed that the maximum R Square change was with model 2 followed by model 3. Model 3 explained the most of the association (58%) with the biomarkers and clinical stage of carcinoma. The presence of E-cadherin, Ki67 and P53 together suggested the presence clinical stage of carcinoma.There was no significant association between the presence of metastasis and biomarkers concentrations (Table 7).

Table 1: Difference in distribution of Mass between Neoplasia & Non Neoplasia across Age group among Gall Bladder lesion cases

Age Category	Tumor Morphology		Neoplastic	Non-Neoplastic	Total	χ2-value	p-value
>60	Mass	Number	17	0	17	13.55	<0.001
		Percent	85.00%	0.00%	68.00%		
	NA	Number	3	5	8		
		Percent	15.00%	100.00%	32.00%		
	Total	Number	20	5	25		
		Percent	100.00%	100.00%	100.00%		
30 to 44	NA	Number	3	12	15	NA	
		Percent	100.00%	100.00%	100.00%		
	Total	Number	3	12	15		
		Percent	100.00%	100.00%	100.00%		
45 to 60	Mass	Number	19	3	22	24.57	<0.001
		Percent	70.40%	9.10%	36.70%		
	NA	Number	8	30	38		
		Percent	29.60%	90.90%	63.30%		
	Total	Number	27	33	60		
		Percent	100.00%	100.00%	100.00%		
Total	Mass	Number	36	3	39	42.55	<0.001
		Percent	72.00%	6.00%	39.00%		
	NA	Number	14	47	61		
		Percent	28.00%	94.00%	61.00%		
	Total	Number	50	50	100		
		Percent	100.00%	100.00%	100.00%		

Chi Square Test, Sig 2 tailed, p<0.05

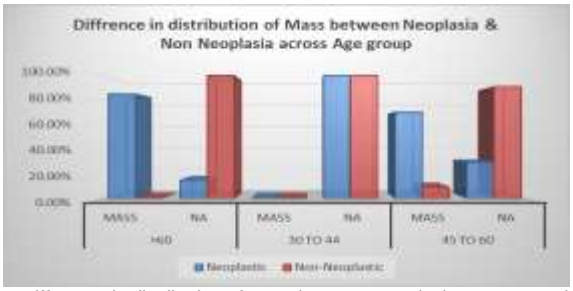

Fig 1: Difference in distribution of Mass between Neoplasia & Non Neoplasia across Age group among Gall Bladder lesion cases

Table 1 & Figure 1 shows the difference in distribution of Mass between Neoplasia & Non Neoplasia across age group. Related to Mass presence, there was significant difference between the Neoplastic and non-neoplastic group among >60 years of age group. None of the cases had mass in the 30-44 years of age group between the neoplasia and non-neoplasia group. There was significant difference in the presence of mass between the Neoplastic and non-neoplastic group among 45-60 years of age group (p<0.001).

Table 2: Difference in distribution of Mass between Neoplasia & Non Neoplasia across Gender among Gall Bladder lesion cases

Gender	Tumor Morphology		Neoplastic	Non-Neoplastic	Total	χ2-value	p-value
Male	Mass	Number	12	1	13	8.551	0.002
		Percent	54.50%	5.00%	31.00%		
	NA	Number	10	19	29		
		Percent	45.50%	95.00%	69.00%		
	Total	Number	22	20	42		
		Percent	100.00%	100.00%	100.00%		
Female	Mass	Number	24	2	26	11.124	0.036
		Percent	85.70%	6.70%	44.80%		
	NA	Number	4	28	32		
		Percent	14.30%	93.30%	55.20%		

	Total	Number	28	30	58		
		Percent	100.00%	100.00%	100.00%		
Total	Mass	Number	36	3	39	27.55	<0.001
		Percent	72.00%	6.00%	39.00%		
	NA	Number	14	47	61		
		Percent	28.00%	94.00%	61.00%		
	Total	Number	50	50	100		
		Percent	100.00%	100.00%	100.00%		

Chi Square Test, Sig 2 tailed, p<0.05

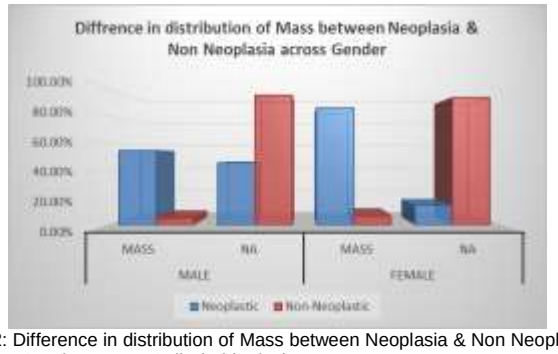

Fig 2: Difference in distribution of Mass between Neoplasia & Non Neoplasia across Gender among Gall Bladder lesion cases

Table 2 & Figure 2 shows the difference in distribution of Mass between Neoplasia & Non Neoplasia across the gender among Gall Bladder lesion cases. About 54.2% males in neoplastic and only 5% males in non-neoplastic group had mass present and there was significant difference between the Neoplastic and non-neoplastic group. About 85.7% females in neoplastic and only 6.70 females in non-neoplastic group had mass present and there was significant difference between the Neoplastic and non-neoplastic group.

Table 3: Difference in distribution of Presence of Stone between Neoplasia & Non Neoplasia across Gender among Gall Bladder lesion

Gender	Presence of Stone		Neoplastic	Non-Neoplastic	Total	χ2-value	p-value
Male	Present	Number	2	12	14.00	11.544	0.007
		Percent	9.10%	60.00%	33.30%		
	Absent	Number	20	8	28.00		
		Percent	90.90%	40.00%	66.70%		
	Total	Number	22	20	42.00		
		Percent	100.00%	100.00%	100.00%		
Female	Present	Number	18	28	46.00	15.22	0.02
		Percent	64.30%	93.30%	79.30%		
	Absent	Number	10	2	12.00		
		Percent	35.70%	6.70%	20.70%		
	Total	Number	28	30	58.00		
		Percent	100.00%	100.00%	100.00%		
Total	Present	Number	20	40	60.00	20.25	<0.001
		Percent	40.00%	80.00%	60.00%		
	Absent	Number	30	10	40.00		
		Percent	60.00%	20.00%	40.00%		
	Total	Number	50	50	100.00		
		Percent	100.00%	100.00%	100.00%		

Chi Square Test, Sig 2 tailed, p<0.05

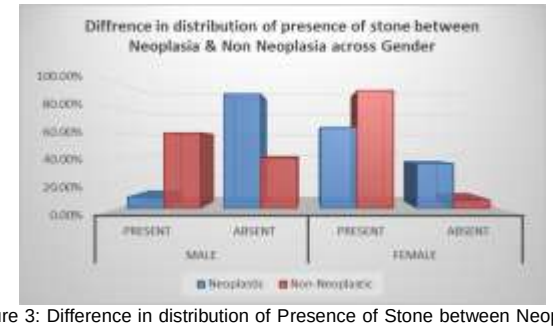

Figure 3: Difference in distribution of Presence of Stone between Neoplasia & Non Neoplasia across Gender among Gall Bladder lesions

Table 3 & Figure 3 shows the difference in distribution of presence of stone between Neoplasia & Non Neoplasia across gender. Related to presence of stone, there was significant difference between the Neoplastic and non-neoplastic group among male (p=0.007). There was significant difference between the Neoplastic and non-neoplastic in female (p=0.02).

Table 4: Tumour Morphology and age distribution among Gall Bladder lesion cases(Female)

Tumor Morphology	Female	30 to 44	45 to 60	>60	Total	χ2-value	p-value
NEOPLASTIC	Number	1	12	15	28		
	Percent	25.00%	35.30%	75.00%	48.30%	9.88	0.046
NONNEOPLASTIC	Number	3	22	5	30		
	Percent	75.00%	64.70%	25.00%	51.70%		
Total	Number	4	34	20	58		
	Percent	100.00%	100.00%	100.00%	100.00%		

Chi Square Test, Sig 2 tailed, p<0.05

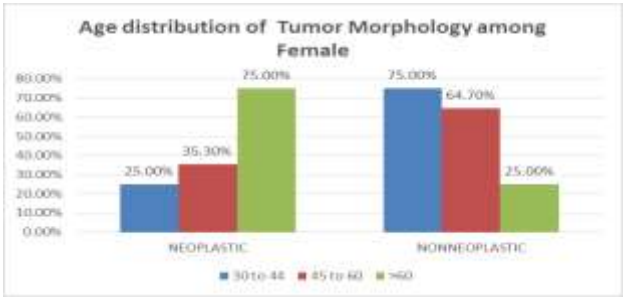

Figure 4: Tumour Morphology and age distribution among Gall Bladder cases(Female)

Table 4 & Figure 4 shows the significant difference between the neoplastic and non neoplastic tumour morphology and age distribution among females. The neoplastic tumours were highest in >60 years age group (75%), while neoplastic tumours were highest among 45-60 years age group.

Table 5: Relationship between the different types of Biomarkers in the neoplastic lesions of Gall Bladder

Neoplasia	Correlation	E-Cadherin	Ki67	P53	EGFR	HER2	Cyclin D1
E-Cadherin	r-value	1	.606**	.525**	.611**	.576**	.526**
Ki67	r-value	.606**	1	.462**	.396**	.646**	.474**
P53	r-value	.525**	.462**	1	.429**	.475**	.557**
EGFR	r-value	.611**	.396**	.429**	1	.414**	.488**
HER2	r-value	.576**	.646**	.475**	.414**	1	.425**
Cyclin D1	r-value	.526**	.474**	.557**	.488**	.425**	1

Spearman's correlation, Sig 2 tailed, p<0.05

Table 5 shows the Relationship between the different types of Biomarkers in the neoplastic lesions and there were moderate to good correlation was present between the each biomarkers. The positive correlation among the biomarkers suggested the presence and concentration were existing correlated.

Table 6: Relationship between the Biomarkers presence and histological grade, Clinical staging and Metastatic in the neoplastic lesions of Gall Bladder

Neoplasia	Correlation	E-Cadherin	Ki67	P53	EGFR	HER2	Cyclin D1
Histological grade	r-value	.520**	.601**	.640**	.441**	.605**	.516**
Clinical Staging	r-value	.602**	.630**	.689**	.358*	.624**	.573**
Metastasis	r-value	-.486**	-.565**	-.437**	-.485**	-.386**	-.419**

Spearman's correlation, Sig 2 tailed, p<0.05

Table 6 shows the Relationship between the Biomarkers presence and histological grade, Clinical staging and Metastatic in the neoplastic lesions. The Histological grade was significantly correlated with the concentration of biomarkers and was highest for the P53 biomarkers. The clinical staging grade was significantly correlated with the concentration of biomarkers and was highest for the P53 biomarkers. The metastasis was significantly negatively correlated with the concentration of biomarkers and was highest for the E cadherin biomarkers.

Table 7: Relationship between the different types of Biomarkers in the Non-neoplastic lesions of Gall Bladder

Non neoplasia	Correlation	E-Cadherin	Ki67	P53	EGFR	HER2	Cyclin D1
E-Cadherin	r-value	1	.701**	.685**	.564**	.510**	.758**
Ki67	r-value	.701**	1	.625**	.646**	.632**	.790**
P53	r-value	.685**	.625**	1	.646**	.485**	.725**
EGFR	r-value	.564**	.646**	.646**	1	.525**	.646**
HER2	r-value	.510**	.632**	.485**	.525**	1	.513**
Cyclin D1	r-value	.758**	.790**	.725**	.646**	.513**	1

Spearman's correlation, Sig 2 tailed, p<0.05

DISCUSSIONS

Although the overall incidence is low, it is the most aggressive carcinoma of the biliary tract with short median survival from the time of diagnosis. The aggressive biologic behavior of the carcinoma and non-availability of sensitive screening tests for early detection may be responsible for the poor prognosis associated with GBC. Anatomically complex porto-biliary-hepatic system further increases the mortality and morbidity following surgical intervention. Moreover, the chances of tumoral spread subsequent to tumor manipulation and increased risk of tumor recurrence further adds to the disease burden .Hence there can be a great role of molecular markers iin detecting the GBC at an early stage. Few important immunomarkers which may play a significant role in its diagnosis and prognosis are as follows:

Cyclin D1: Cyclin D1 is a 295 amino acid protein encoded by the 13,388 base pairs long CCND1 gene located on the long arm of chromosome 11. While Cyclin D1, D2, D3 have homogenous regulatory roles, only Cyclin D1 is significantly overexpressed in cancerous tumours [8]. Higher levels of Cyclin D1 can occur either due to the amplification or chromosomal rearrangements of its encoding gene CCND1 or interruption to the transport and proteolysis of the protein itself [8,9]. The important role of Cyclin D-RB-E2F pathway in the development of many forms of human cancer has been established [10]. Mutations and amplification of the CCND1 gene and overexpression of its protein Cyclin D1 has been observed in most human cancers [10,11]. Disruption to the normal transcription, increased levels and ubiquitination of Cyclin D1 along with the assembly and hyperactivation of its cognate CDK all result in uncontrolled cellular proliferation [12]. In addition to these factors, cyclin D1 overexpression can be caused by alterations of associated signalling intermediates, including the RAS–MEK–ERK and PI3K pathways [13]. While several studies on drugs targeting Cyclin D1 regulation have been made [14,15,16], its role as a prognostic marker in GBC is yet to be established and warrants further investigation. Assessment of the complex contributory role played by Cyclin D1 in carcinogenesis and tumour progression could enable the development of effective personalized therapies through proper prognosis and accurate staging of GBC.

Hui et al suggested that CCND1 amplification and CyclinD1 overexpression could be independent events impacting carcinogenesis. Their study correlated the amplification of the CCND1 gene with increased mortality among GBC patients. In another study, Cyclin D1 overexpression was frequently observed in surgically resected samples of adenocarcinomas and adenomas, but no overexpression was observed in the normal epithelium or adenomyoma of the gallbladder. Based on this, it was proposed that Cyclin D1 overexpression might be an early-stage event in GBC [11]. Reports from another study indicated that cyclin D1expression in gallbladder carcinoma and adenoma were comparatively higher than those in chronic cholecystitis, although, no significant difference was observed in the expression levels of Cyclin D1 in carcinoma and adenoma samples [17].

Further, Doval et al noted raised levels of cyclin D1 in poorly differentiated tumours and distant metastasis, although this result was deemed to be statistically insignificant by the authors [18]. It has been postulated that in cancers with lower levels of Cyclin D1, deactivating mutations on its inhibitors or in its downstream substrates might result in deregulation without the necessity for its overexpression. As such, it is not Cyclin D1 alone but a combination of other altered checkpoints that contribute to malignancy in GBC [9].

On observation, studies conducted on the role of Cyclin D1 in GBC show significant differences in their conclusions. This can be attributed to the complex pathways that drive the Cyclin D1 regulation, smaller sample number and difference in assays used. Despite the different results obtained in these studies, all of them unilaterally confirm the dominant role played by Cyclin D1 plays in the development of GBC.

We might be able to better utilise Cyclin D's role in carcinogenesis and tumour progression by exploring the associative events and the proteins that interact with Cyclin D1[12]. If this is achieved, a specific and individualised prognosis can be obtained by using Cyclin D1 levels in conjunction with other markers to predict the outcome of the disease effectively.

E-Cadherin: E-cadherin [Epithelial-Cadherin] is a 120KDa glycoprotein, encoded by the CDH1 gene located on Chromosome 16 [19]. E-cadherin, in normal epithelial cells, functions to establish and maintain the Adherens Junctions between cells through calcium mediation [20,21]. E-cadherin gene is a tumour suppressor gene [22] and loss of E-cadherin protein increased metastatic potential and apoptosis resistance in tumour cells [23]. It is one of the extensively studied biomarkers as several human cancers occur through the transformation of epithelial cells.

Xu et al postulated that the loss of expression of E-cadherin is an important event in the progression, spread and prognosis of GBC. Their study concluded that loss of E-cadherin indicated lymph node metastasis and proffered poor prognosis in GBC [24].

Inactivation of E-cadherin can be compiled into two general categories where mutations can result in the translation of a non-functional protein product or cause the complete absence of the E-cadherin molecule itself. While complete loss of E-cadherin resulted in epithelial-to-mesenchymal transition (EMT), point mutations that preserved the E-cadherin cytoplasmic tail while altering its extracellular domain did not result in EMT [23]. Decreased E-cadherin levels in malignant cells can be a result of gene mutation, promoter methylation, transcriptional repression, or posttranslational modification of the cadherin/catenin complex [20]. Further, methylation of the CDH1 gene has been associated with the incidence of distant metastasis in GBC [25]

Puhalla et al demonstrated difference in the membranous and cytoplasmic E-cadherin levels between normal gallbladder epithelia, inflamed tissue and GBC. They also noted lower E-cadherin levels in undifferentiated tumours [26].

Costa et al, suggested that decreased E-cadherin expression could be associated with an increase in the proportion of undifferentiated tumours, metastases and the extent of wall invasion in GBC. However, they failed to establish a prognostic correlation between E-cadherin expression and patient survival could be established [27].

Na et al demonstrated that the activation of E-cadherin at the cell surface using activating monoclonal antibodies [mAbs] resulted in the inhibition of metastasis progression. They used endogenous genetically driven mouse mammary tumour cells. They concluded that activation of E-cadherin can inhibit metastasis at different stages through various pathways. [28].

E-cadherin has been established as a prognostic marker in squamous cell carcinoma of the head and neck [29], associated with poor prognosis in prostate cancer [30] and the presence of increased levels of serum soluble E-cadherin an 80KD degradation product of cellular E-cadherin has been linked to poor prognosis in gastric cancer [31].

It has also been noted that E-cadherin expression in some highly metastatic cell lines tend to remains unaltered. This suggests that EMT might not always be the reason for invasiveness of carcinomas. To finely understand E-cadherin's prognostic role in GBC, its interaction with other associative genes and pathways needs to be studied as well [32]. AEG-1 expression [19], β- catenin expression [31] and SDC1 expression [33] have been found to influence E-cadherin expression and/or functionality.

E-cadherin's role in metastasis and its differing levels or loss could be exploited to understand and evaluate the disease prognosis among GBC patients. However, this warrants further analysis and understanding of the complex pathways that contribute lower levels of E-cadherin or its loss thereof including its interactions with partner molecules during carcinogenesis, EMT and metastases.

EGFR: Epidermal Growth Factor Receptor (EGFR), a transmembrane receptor tyrosine kinase belonging to the ErBb family, plays an important role in the signal transduction pathways involved in several cellular functions like metabolism, differentiation, progression of cell-cycle and apoptosis [34]. Being one of the first tyrosine kinases to be described it was studied extensively as a target for novel drugs as the ATP-binding site of protein kinases could be effectively blocked by inhibitors [35]. Tyrosine kinase domain of EGFR which is essential for cell proliferation and differentiation is located in the cytoplasmic carboxy-terminal. Abnormal cellular proliferation can be brought about by amplification of EGFR, and its ligands, mutations of its gene [36].

Leone et al were able to demonstrate the presence of mutations in the EGFR gene and the subsequent activation of downstream signalling pathways in GBC. They classified these mutations as heterozygous (amplification of wild-type sequence on the second allele) and homozygous/hemizygous (amplification of the mutated sequence only) [37].

EGFR overexpression in human cancers has been reported through several studies [23, 38,39]. Gene mutation, amplification or translational upregulation of EGFR could be behind the higher EGFR levels detected in tumour cells.

In general overexpression of EGFR is observed in poorly differentiated tumours. It has also been noted in poorly differentiated tumours that are resistant to conventional therapies [23]. EGFR levels in well-differentiated adenocarcinoma of the gall bladder were noted to be lower than that of poorly differentiated malignancies [40].

Kaufman et al, in their study, established a negative correlation between histological differentiation of GBC and EGFR overexpression. They suggested that on assuming poorly differentiated tumours behaved more aggressively, EGFR expression levels may suggest the extent of aggressiveness of GBC [34]. Elevated E-cadherin levels in GBC can be considered as an independent prognostic variable among patients and can be an indication of adverse prognosis [40,41].

Kawamato et al, in their study, found that EGFR was overexpressed in 16% of GBC samples they studied but was absent in extrahepatic bile duct cancer and intrahepatic bile duct cancer samples [42].

Li et al, in their study, demonstrated how EGFR nuclear translocation impacted upregulation of iNOS, which in turn could lead to the aggressive invasion characterised by GBC. This could also explain why certain patients show resistance to EGFR targeted therapies. Hence, considering EGFR levels and their role in both cytoplasm and nucleus might prove useful for potent drug design and accurate prognosis [43]. Also, EGFR gene amplification alone might not have a significant impact on patient prognosis [42,44].

A standardized scoring system for EGFR overexpression in breast cancer was evaluated by Lee et al [45]. GBC too could benefit from similar studies. EGFR levels at various stages of GBC development, wall invasion, lymph node metastasis and distant metastasis could be analysed to arrive at a GBC specific profile.

HER2: The c-erbB-2/HER2/neu protooncogene is translated into an185-kDa transmembrane tyrosine kinase receptor protein. EGFR and HER2 share structural homology and are modulated through the same downstream signal transduction systems [46,47]. HER2 overexpression has been detected and correlated to the progression of several human cancers [6,47]. HER2 overexpression in GBC has also been established in several studies [47,48,49]. However, HER2 overexpression need not be essentially as the result of HER2 gene amplification alone [47]. HER-2 protein overexpression could be due to gene deregulation while, HER-2 gene amplification may be an independent prognostic factor for survival in a selective patients with lymph node metastases [50,51]. HER-2 overexpression in 31.2% of gallbladder cancers, while gene amplification was reported positive in only 20.9% [52]. It was noted that HER2 gene amplification observed among GBC patients have been found to reflect that which has been also observed among breast cancer patients.

Toledo et al suggested that HER2 overexpression might be linked to precursor lesions than the invasive malignant tumour itself. No HER2 expression was found in normal gallbladder epithelia while significant levels of expression were observed in both the carcinoma and precursor lesions [50].

HER2's role as a prognostic marker has been studied in other cancers as well. In their review, Jørgensen et al observed severe variations in the positivity rates reported among gastric cancer patients. However, they were able to associate HER2 expression in gastric cancer with a negative prognosis [53].

The impact of geographical variations in HER2 amplification among different populations with gastric cancer has been observed and studied. It could be further investigated if these differences are applicable in GBC as locational variability has been observed as one of the major risk factors influencing the incidence of GBC [48]. Doval et al found that only 4% of the GBC patients tested positive and implied that Her-2 overexpression is a rare event among the Indian population [18].

Marked variations in HER2 expression was found in several studies. This could be attributed to the different assays and scoring systems used to define and measure HER2 overexpression in GBC [49]. Neyaz et al suggested the use of HER2/neu scoring system using gastric criteria can be applied to GBC as well. They observed the presence of intratumoral heterogeneity [ITH] in GBC with respect to HER/Neu expression and its correlation to the presence of papillary exophytic growth [54]. Evaluating HER2 overexpression in GBC might help understand the process to malignancy from precancerous lesions. With some studies reporting higher HER2 levels in precancerous lesions in comparison to the malignant tumour itself, its levels might be studied and understood to effectively identify benign lesions that have malignant potency.

KI-67: The ki-67 antigen is a nuclear protein which is used as a reliable indicator of cellular proliferation [55,56]. It has two isoforms of 345KDa and 395KDa and its encoding gene is present on chromosome 10 [56]. The concentration of Ki-67 dynamically changes through the cell cycle progression in proliferating cells and is absent in their normal state [57]. Sobecki et al, elaborated the role of ki-67 in the maintenance of the compact nature of heterochromatin while facilitating interactions of different regions of the genome to modulate the rates of transcription of various genes involved in the cell cycle [58]. Ki-67's influence on carcinogenesis can be extrapolated by understanding the regulatory dynamics of chromatin organisation during gene expression to facilitate increased or decreased expression of certain proteins [61]. Toledo et al used Ki-67 expression to prove increased cell proliferation in epithelial cells with metaplasia and carcinoma in situ of the gallbladder [50].

Ki-67 Labelling Index [LI] is the percentage of Ki-67 antigen-positive cells in a given sample and is generally used to describe Ki-67 expression levels in any given sample [62]. Ki-67 LI has been understood to be an independent prognostic factor in cancer [56] and mean Ki-67 LI has been found to increase with tumour grade with the lowest level among well-differentiated and highest levels observed among poorly differentiated malignancies [61]. The ki-67 expression has been correlated with patient survivability in other types of cancer [52, 60]. MIB1 is a monoclonal antibody with a higher affinity to Ki-67 has been used to evaluate its levels [62,63]. Higher MIB1 LI is linked to poorly differentiated tumours, lymph node metastasis and poor survival rate [63,56]. Increased Ki-67 LI among poorly differentiated tumours observed could be due to their tendency to rapidly proliferate [18,56]. Grau et al in their study could not corroborate the higher expression of Ki-67 observed and reported in previous studies of moderately differentiated tumours in comparison to poorly differentiated tumours [64]. The expression of Ki-67 in gallbladder had no bearing on patient survival, histological differentiation or gall bladder wall invasion [65].

In gastric cancer, postoperative tissue samples were characterized by high Ki-67 and the prognosis for patient survival improved when chemotherapy was used as adjuvant therapy. This proposed the idea where Ki-67 LI can be used to determine the need for adjuvant chemotherapy after surgical resection and improve patient prognosis [60].

A significant difference in the Ki-67 levels between malignant and benign lesions was observed in GBC [66]. Studies also have established a minor connection between Ki-67 expression, age and gender of the patient. It was found that Ki-67 overexpression is higher in patients belonging to the age group< 40 years [60,67]. Since only rapidly proliferating cells express Ki-67 antigen, it remains to be seen if aggressive tumours can be identified and targeted with immunotherapy involving antibodies specific to Ki-67.

p53: Located in the small arm of Chromosome 17, the TP53 gene translates into a 53-kDa nuclear phosphoprotein that acts to preserve the integrity and coherence of the genome by acting as a tumour suppressor [68]. Under normal circumstances, p53 inhibits proliferation of stressed and damaged cells thereby effectively stopping tumour development [69]. Mutations of the TP53 gene is one of the common genetic abnormalities found in most human cancers [70,71]. The most common mechanism of inactivation of p53 arises from point mutation that results in a translational product that has altered conformation and defective functionality. In addition, either a missense mutation or allele deletion often results in the absence of wild-type p53. Both these scenarios result in loss of tumour suppression functionality. Nonsense mutation or methylation of the p53 gene can result in total loss of p53 protein [68]. A significant amount of TP53 mutations identified so far are missense which extends the half-life of protein by several hours causing intranuclear accumulations and the same can be observed through immunohistochemistry [6,71]. TP53 amplification and p53 accumulation could be independent events as overexpression of wild-type protein were noted in studies and this could be due to stabilisation or disruption of its proteolysis by other interacting molecules involved in GBC carcinogenesis [73].

Wang et al in their study found statistically significant variations in the overexpression of p53 protein between precursor lesions and carcinomas of the gallbladder and between normal epithelia and carcinomas of the gallbladder. Based on these findings they stipulated that gallbladder adenomas lacked the

abnormalities of p53 often seen in GBC samples. They also went further in suggesting that p53 overexpression may not play an important role in the adenoma to carcinoma pathway in GBC carcinogenesis [74]

Zhang et al found evidence of correlation between the presence of NO and mutative P53 being expressed in chronic cholecystitis and chronic cholecystitis with adenomyoma and postulated that NO is one of the important factors in gallbladder cancer development. They suggested that NO can influence the expression levels of P53 [75]. Grau et al did not observe any difference in the survival times of GBC patients with or without p53 overexpression [64].

The significance of immunostaining of p53 in GBC has been studied widely with varying results. Oohashi et al used immunostaining effectively to distinguish between malignant and benign lesions of the gall bladder. They concluded that the overexpression of p53 is an early event in ~70% of well-differentiated adenocarcinomas of the gall-bladder, and this change is sustained through the progression towards invasive neoplasm from intramucosal neoplasm [65].

Using a single case study Takano et al, elaborated how p53 overexpression can be utilised to predict recurrence after surgical resection of the primary tumour. In their case study, though the remnant of the cystic duct after surgery exhibited no signs of neoplasm when analysed with H&E staining, significant p53 overexpression was observed using immunostaining. The patient suffered a recurrence in the bile duct after 2.5 years [71].

While most studies correlate the role of altered p53 protein in the progression of gall bladder cancer, contradictory studies have also emerged and its prognostic role has remained contentious. Uncontrolled expression of p53 or its loss there off has been correlated with poor prognosis in GBC by Kim et al in 2013. They had studied changes in p53 expression among Gall Bladder adenocarcinoma samples by observing the percentage of stained cancer cells. Cases that have significantly progressed showed increased p53 expression indicating that aberrant expression of p53 is a late event in the carcinogenesis process [5].

Kaur et al, through their study, postulated that p53's role was largely limited to the progression of the malignant tumour from lower to a higher grade but not in metastasis. They were able to correlate p53 overexpression was inversely linked to the grade of the tumour [73]. Costa et al suggested that p53 in GBC can be correlated with a late event in carcinogenesis as, in their study, p53 positivity was found to be higher in advanced cases [27].

Studies also vary ambiguously concerning the timing of TP53 mutations in tumour development based on the population examined, and analysis methods. The various pathways in which p53 mutations impact carcinogenesis may be further influenced by genetic predisposition, oncogenic stress, carcinogen exposure etc. Identifying the status of p53 in GBC samples might prove useful in early recognition and surveillance to identify recurrence. In an extended sense, it can help in predicting a case-suitable targeted treatment regimen .

CONCLUSION

Gallbladder cancer patients are usually are diagnosed in later stages when conventional treatments are ineffective, resulting in higher mortality rates . The minimal response of advanced cases of GBC to traditional treatments calls for new prognostic and treatment perspectives to be identified. Novel prognostic biomarkers could bring about the needed breakthrough in this regard as they will help in the identification of patients who will benefit tremendously from adjuvant and targeted therapies.

Despite the available data and years of research, a prognostic marker that is 100% specific and sensitive to GBC is not yet available .A diverse number of molecular markers has been studied for their potential to be prognostic markers in GBC. Of these p53 and HER2 have been studied very extensively and have shown promise. Though these can be used as prognostic markers in GBC, current data available is insufficient for their efficient clinical use to demarcate GBC from other forms of GI cancers and benign conditions that mimic malignancy [6].

The deregulation and accumulation of the molecular markers we have discussed so far impact carcinogenesis of the gall bladder significantly. Further analytical studies on the concentration levels of these markers in normal vs precancerous vs cancerous tissues should be carried out with standardized assays to achieve clinically applicable results. [71]. Multivariable analysis that includes geographical variations, genetic predisposition, gender, co-expression of oncogenes etc., also needs to be explored in detail. Highly specific prognostic markers can help individualise treatment options and bring down the mortality rate in GBC.

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