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Aberrant signature patterns of ATM, γ-H2AX and p53 proteins in the patients exposed to methyl isocyanate diagnosed with gallbladder cancer

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ABSTRACT

Cancer of gallbladder is a hidden phenomenon and highly malignant with underprivileged diagnosis and poor survival. Study of cancer patterns amongst victims of Bhopal gas tragedy exposed to methyl isocyanate revealed higher incidence of gallbladder cancer that necessitated a more objective elucidation of the disease at its molecular level. Tissues of 92 cases of gallbladder cancer patients were taken in the study (31 men and 61 women, age range 16–85 years, mean age 45.8±1.50 years). Mutations of ATM, γ-H2AX and p53 were predominantly seen in the methyl isocyanate exposed cohort diagnosed with adenocarcinoma, with 61.4% (43/70), 54.3% (38/70) and 73% (51/70) respectively, involving infiltration into the papillary and mucinous region/cell types of the gallbladder. Out of these, the expression frequency of all the above three genes was higher in moderately differentiated adenocarcinoma in comparison to poorly and well-differentiated ones. The results of the present study support the hypothesis that ATM and p53 mutations provide fundamental genetic signatures influencing tumor behavior across patient subsets and invasiveness of the disease, while γ-H2AX is apparently an ordinary pathway involved in the genesis of tumors.

Key words: ATM, p53, γ-H2AX, gallbladder adenocarcinoma, adenosquamous carcinoma, adenoma with dysplasia

Introduction

On a global scale, the cancer of gallbladder (CAGB) is fifth most aggressive cancer of the gastrointestinal tract (Jones, 1990) with incidence three times greater in the female population of Chile, India and Japan when compared to men (Toledo et al., 2012). There is a prominent worldwide geographic and ethnic variability of gallbladder cancer (GBC) incidence (Wistuba & Gazdar, 2004). The highest CAGB incidence rate is reported in women (21.5/100 000) in India (Goldin & Roa, 2009). A progressive increase in incidence and mortality for CAGB has been reported worldwide (Gatto et al., 2010). A huge number of studies have been carried out to recognize diverse genetic and non-genetic risk factors for CAGB, but the problem of diagnosis, prognosis or therapy of CAGB has still remained an mystery and hitherto not to be clearly understood. So, when gallbladder cancer is diagnosed, surgically resection remains the only way to rescue (Lazcano-Ponce et al., 2001). Therefore, it is desirable to understand the tumor biology of CAGB, which may help to identify prognostic biomarkers for the development of more successful strategies for early diagnosis and targeted therapies.

The ATM is a nuclear protein kinase, a member of the phosphatidylinositol-3 kinase family of proteins. It is known to be involved in the cellular response to DNA double-strand breaks (DSBs) at several levels by phosphorylating key substrates involved in DNA repair and/or cell cycle control (Rotman & Shiloh, 1998; Gamper et al., 2012). This protein was discovered as mutated proteins in patients with ataxia-

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telagiectasia (A-T), a severe genetic disorder characterized by series of dysfunctions and disorders (Savitsky et al., 1995). Gamma-H2AX is a histone H2A variant that is distributed throughout most of the genome. It is a vital substrate for the DNA repair machinery (Burma et al., 2001; Stiff et al., 2004). Moreover, p53 is a gene that codes a protein regulating the cell cycle and hence functions as tumor suppression. It is very important for the cells in multicellular organisms to suppress malignancies by preventing genome mutations (Strachan & Read, 1999). Hence, DNA DSBs activate several signaling molecules, including ATM, a member of PIKK family (Abraham, 2004), which further trigger autophosphorylation of ATM at Ser1981 that results in the subsequent phosphorylation of γ-H2AX at Ser139 and initiates cell signaling events to induce cell cycle arrest or apoptosis through phosphorylation of p53 at Ser15 (Kang et al., 2005). A large number of studies have been carried out to identify different genetic and non-genetic risk factors for gallbladder malignancy, including epigenetic alterations, expression profiling, single nucleotide polymorphisms (SNPs), loss of heterozygosity (LOH), biochemical studies, dietary habits, environmental factors, family history, etc., but the problem of diagnosis, prognosis or therapy of gallbladder malignancy has still remained a mystery. By the time gallbladder cancer is diagnosed, resection remains the only way to rescue. About 32% of 5 year survival rate is reported for lesions confined to the gallbladder mucosa, but only 10% of 1 year survival rate for more advanced stages (Lazcano et al., 2001).

The Bhopal gas tragedy taken place in India is among the world's worst known industrial disasters, which led to the leakage of methyl isocyanate (MIC) and its related toxic gas products resulting in mortality of 2500-6000, and debilitating over 200 000 people. The severity of exposure to MIC was extreme and the survivors continue to experience increased multi-systemic morbidity (Dhara & Dhara, 2002; Mishra et al., 2009a), including cancer (Ganesh et al., 2005), which clearly establishes a genetic link of cancer with MIC exposure (Dikshit & Kanhere, 1999; Senthilkumar et al., 2012).

The exposure they also possess the capability to modulate the biomolecules resulting in series of biotransformation events, which in turn may cause a variety of health problems (Mishra et al., 2008). Isocyanates are thus becoming of interest in the field of genetic toxicology as they may react with DNA to produce DNA damage (Shelby et al., 1987; Tamura et al., 1992). The sequence of molecular changes leading to neoplastic transformation in the gallbladder remains elusive. Although the capability of isocyanates to induce carcinogenesis had been addressed in the past, the detailed molecular repercussions underlying their genetic hazards upon occupational/accidental exposures remain an intricate issue and were hitherto unknown. Gallbladder malignancy has been associated with genetic and environmental risk factors, but there is limited information about the molecular changes involved in its pathogenesis. The incidences of gallbladder cancer in gastrointestinal tract are found in higher ratio in Bhopal gas victims, but they are far from conclusive.

Therefore, mechanistic understanding of causal relationship between gene environment interactions and chronic cholecystitis influenced progression towards gallbladder malignancy has to be elucidated to develop effective early diagnostics and novel targeted therapeutic strategies. However, the relationship between ATM, γ-H2AX, p53 and angiogenesis, lymphangiogenesis, as well as the pathogenesis and prognosis of CAGB have not yet been identified in a cohort exposed to MIC. In this study, the aberrant expressions of ATM, γ-H2AX and p53 in tissue sections with adenocarcinoma, adenosquamous carcinoma and adenoma with dysplasia of CAGB were examined using immunohistochemistry.

Materials and Methods

Sample selection

In the present investigation, cancer tissues of the gallbladder were collected from Bhopal Memorial Hospital and Research Centre (BMHRC), Raisen Bypass Road Bhopal M.P. India. CAGB is higher cancer incidences of the gastrointestinal tract noticed in the survivors of the Bhopal gas tragedy taken place in 1984. These specimens were collected after obtaining patient's informed consent. The study was approved by the Institutional Review Board of BMHRC. Only subjects from 36 municipal wards considered "MIC affected" were selected for the study.

Tissue specimens

The study was performed on 92 surgically resected cancer tissues of gallbladder. Out of these, 31 patients were men and 61 were women. Age range 16–85 years, mean age 45.83±1.5 years) with 70 adenocarcinoma (13 well-differentiated, 48 moderately differentiated and 9 poorly differentiated), 10 adenosquamous carcinoma and 12 gallbladder adenoma were examined. A gallbladder adenoma was defined as any

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outgrowth that histologically showed gland proliferation and some grade of dysplasia. Gallbladder adenocarcinoma was defined as an outgrowth with cells showing atypicity, forming glands and originating on superficial epithelia that can be observed microscopically. The definitions of histological classification and stage grouping were made according to the WHO International Histological Typing of Tumors (Albores-Saavedra et al., 1992) and the International Union against Cancer (Beahrs et al., 1992), respectively.

All surgically resected specimens of gallbladder cancer were initially fixed in 10% neutral buffered formalin and then embedded in paraffin-wax after further dehydration processing. Then, the cut sections were stained with hematoxylin and eosin. Diagnosis was based on clinical suspicion and histopathological confirmation in each patient.

Immunohistochemical staining

ATM, γ-H2AX and p53 expression was examined by an immunohistofluorescence method using a spectral bio-imaging system (Applied Spectral Imaging, Germany). Briefly, 3 to 5 µm sections were cut from the paraffin embedded tissue blocks and placed on poly-L-lysine coated slides. Immunohistochemcial staining of ATM, γ-H2AX and p53 was performed using the indirect immunohistofluorescence method. In brief, the sections were deparaffinized with xylene rinse and rehydrated into distilled water through a graded alcohol series (100, 90, and 70%). The mounted tissue was then rinsed with phosphate-buffered saline on the slide. The slides were then incubated with primary mouse monoclonal anti-ATM/anti-γ-H2AX/anti-p53 antibody (Santa Cruz Biotechnology Inc., USA) at a dilution of 1:1000 in a humidified chamber for 2-3 h at room temperature. The slides were washed three times in phosphate-buffered solution and further incubated with fluorescein isothiocyanate/Texas Red conjugated secondary antibody (Santa Cruz Biotechnology Inc.) at 1:40 dilution for 1 h at room temperature. Tissue nuclei were counterstained with DAPI (4,6-diamidinophenylindole) and incubated for 15-20 min. Finally, the slides were mounted with antifade solution and then examined using a spectral bio-imaging system (Applied Spectral Imaging).

Criteria for positive immunohistochemical staining

Through immunohistochemical staining of the tissue samples results were quantitatively and qualitatively evaluated. ATM, γ-H2AX and p53 immunoreactivity was considered to be positive when more than 10% of cells with nuclear, and membrane-bound or cytoplasmic staining were observed, respectively. Cytoplasmic staining for p53 was disregarded. Intensity of staining was graded as weak, moderate and strong (Kim et al., 2001). Data were processed using Excel, and the SPSS 10.0 software (SPSS Inc., USA).

Results

The 92 GBC cases in our study were of the following types: 61 (66.3%) were females and 31 (33.7%) were males, and their histological classification was 70 adenocarcinoma, 10 adenosquamous carcinoma, and 12 adenoma with dysplasia (Table 1). We noted that aberrant expression of ATM, γ-H2AX and p53 was predominantly seen in the MIC exposed cohort diagnosed with adenocarcinoma and frequency of 61.4% (43/70), 54.3% (38/70) and 73% (51/70), respectively involving infiltration into the papillary, and mucinous region/cell types of the gallbladder (Figures 1, 2, 5, 6, Table 1).

Table 1. *Correlation of ATM, γ-H2AX and p53 over expression in gallbladder carcinoma showing the value for positive and negative expression.*

Histological subtype	Number of samples	ATM		γ-H2AX		p53		Chi-square values
		P	N	P	N	P	N	
Adenocarcinoma	(N = 70)	43 (61.4%)	27 (38.6%)	38 (54.3)%	32 (45.7%)	51 (71%)	19 (29%)	1.9
Adenosquamous carcinoma	(N = 10)	5 (50%)	5 (50%)	3 (30%)	7 (70%)	4 (40%)	6 (60%)	0.5
Adenomas with dysplasia	(N = 12)	5 (41.7%)	7 (58.3%)	4 (33.3%)	8 (66.7%)	2 (16.6%)	10 (83.3%)	1.3

Note: P –Positive, N- Negative

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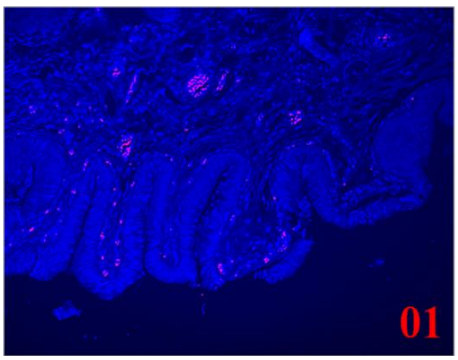

Figure 1. *Immunohistofluorescence detection of **ATM** protein in the nuclei of adenocarcinoma of gallbladder cancer tissue (Pink spots: positive nuclear staining in background of DAPI counter-stained tissue) (original magnification 40X).*

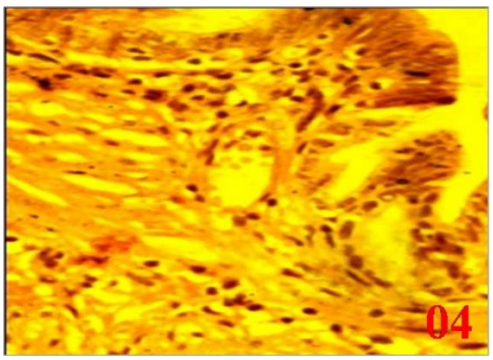

Figure 4. *Photomicrograph representing hematoxylin and eosin image of ATM overexpression (original magnification 40X).*

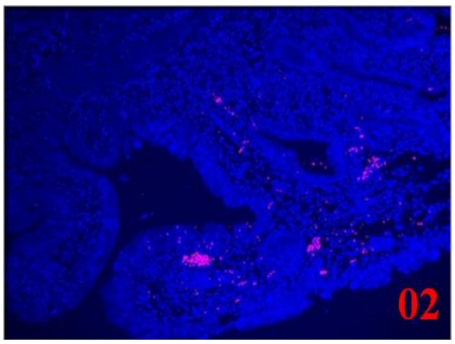

Figure 2. *Immunohistofluorescence detection of **ATM** protein in the nuclei of adenosquamous carcinoma of gallbladder cancer tissue (Pink spots: positive nuclear staining in background of DAPI counter-stained tissue) (original magnification 40X).*

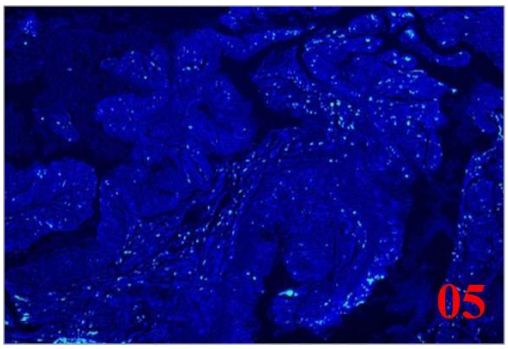

Figure 5. *Photomicrograph representing immunohisto-fluorescence detection of γ-H2AX protein expression in the nuclei of moderately differentiated adenocarcinoma (fluorescent spots: positive nuclear staining in background of DAPI counter-stained tissue) (original magnification 40X).*

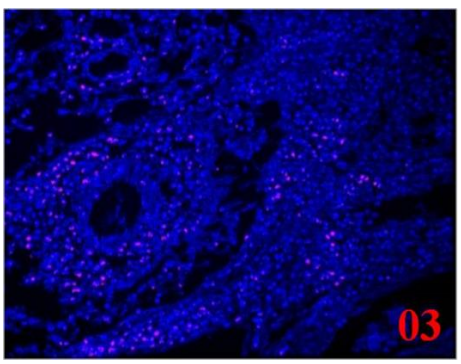

Figure 3. *Immunohistofluorescence detection of **ATM** protein in the nuclei of adenoma with dysplasia of gallbladder cancer tissue (Pink spots: positive nuclear staining in background of DAPI counter-stained tissue) (original magnification 40X).*

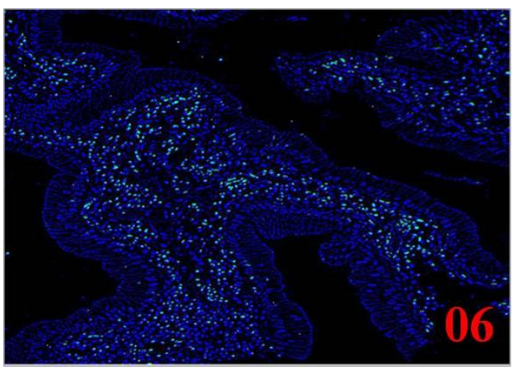

Figure 6. *Immunohistofluorescence detection of p53 protein overexpression in the nuclei of moderately differentiated adenocarcinoma (fluorescent spots: positive nuclear staining in background of DAPI counter-stained tissue) (original magnification 40X).*

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Table 2. *Representation of ATM, γ-H2AX and p53 expression with clinicopathological features like age, gender and tumor differentiation.*

Feature	Total samples (N=92)	ATM		γ-H2AX		p53	
		P	N	P	N	P	N
Age (Years)							
≤ 65	N =29	16 (55.1%)	13 (44.9%)	14 (48.3%)	15 (51.7%)	17 (58.6%)	12 (41.4%)
≥ 66	N =63	37 (58.7%)	26 (41.3%)	31 (49.2%)	32 (50.8%)	40 (63.5%)	23 (36.5%)
Gender							
Male	N =31	17 (54.8%)	14 (45.2%)	15 (48.4%)	16 (51.6%)	18 (58.0%)	13 (42%)
Female	N =61	36 (59.0%)	25 (41.0%)	30 (49.2%)	31 (50.8)	39 (64.0%)	22 (36%)
Grade differentiation [Adenocarcinoma (N = 70)]							
Well differentiated	N =13	8 (61.5%)	5 (38.5%)	7 (53.9%)	6 (46.1%)	9 (69.2%)	4 (30.8%)
Moderately differentiated	N =48	31 (64.6%)	17 (35.4)	26 (54.1%)	22 (45.9%)	39 (81.0%)	9 (19%)
Poorly differentiated	N =09	4 (44.4%)	5 (55.6%)	3 (33.3%)	6 (66.7%)	3 (33.3%)	6 (66.7%)

Note: P –Positive, N- Negative

However, aberrant expression of γ-H2AX was confined to adenosquamous carcinoma and adenoma with dysplasia with 3/10 (30%) and 4/12 (33.3%) cases, respectively. In contrast, the expression of ATM (Figures 2, 3) and p53 was relatively moderate in adenosquamous carcinoma and less in adenomas with dysplasia, while γ-H2AX expression is low in all types of CAGB in comparison of ATM and p53 (Table 1). The standard Chi-square tabular value at degree of freedom 2 on P=0.001 is 13.82, while the obtained positive values for adenocarcinoma, adenosquamous carcinoma and adenoma with dysplasia were 1.95, 0.5 and 1.27, respectively. This indicates that the obtained values are lower than the standard Chi-square tabular value. The interpretation drawn from Table 1 describes that all the expected and observed frequencies are significantly similar. Thus, the hypothesis is up to the standard.

Furthermore, according to the differentiation grade, gallbladder adenocarcinomas were sub-grouped into well, moderately and poorly differentiated adenocarcinomas. The expression of ATM were 8 (61.5%), 31 (64.6%) and 4 (44.4%); γ-H2AX were 7 (53.9%), 26 (54.1) and 3 (33.3%) and p53 were 9 (69.2%), 39 (81%) and 3 (33.3) in well, moderately and poorly differentiated adenocarcinomas, respectively (Table 2). Although there was not significant, a correlation was seen between ATM, γ-H2AX and p53 overexpression and differentiation grade. Moreover, ATM, γ-H2AX and p53 overexpression was relatively stronger in the moderately differentiated adenocarcinoma cases when compared with moderate expression of well and poorly differentiated cases (Table 2).

Discussion

The development of a normal cell into a tumor cell appears to depend in part on mutations in genes that normally control cell cycle and cell death, thereby resulting in inappropriate cellular survival and tumorogenesis (Morgan & Kastan, 1997). ATM, γ-H2AX and p53 are the gene products that are believed to play a major role in maintaining the integrity of the genome. As the MIC being the primary cause of world's worst known industrial disaster, it has been documented to have serious genotoxic implication leading to deregulation genetic stability. These types of agents are very harmful to our genetic material and can create genomic instability being associated with various types of malignancies (Toyooka & Ibuki, 2007).

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In the current study, the ATM, γ-H2AX and p53 expression were analyzed through immunohistofluorescence in gallbladder cancer tissue specimens and determined the effects of MIC. The investigation of above mentioned genes expression brings important data regarding the carcinogenesis process in the gallbladder, initiated on the background of MIC exposed population at Bhopal, India.

Since most of human carcinogens are genotoxins (Williams & Weisburger, 1991; Smart, 1994), considerable resources have been and are being expended in efforts to understand the mechanism of genotoxin-induced carcinogenesis, thus leading to a better prevention or even the treatment of cancer. Although gallbladder carcinoma is a highly malignant neoplasm, there is very limited information about the molecular changes involved in its pathogenesis (Wistuba et al., 2001). Among the pathogenic models that explain the neoplastic transformation of gallbladder epithelium are the metaplasia-dysplasia-carcinoma and the adenoma-carcinoma pathways (Goldin & Roa, 2009). In the present study data have shown that the women are at high risk for CAGB with maximum cases of adenocarcinoma (76%). Out of these, the highest numbers of cases has been diagnosed for moderately differentiated adenocarcinoma (68.6%), which evidence lack of proper diagnosis of the disease as reported in the literature. Since the sensing of DNA damage is one of the earliest steps in the cellular response to gentoxic stress, identification of these "sensors" is the most prominent challenge (Yang et al., 2003).

The role of various oncogenic mutations in gallbladder cancer is an area of active research since no promising biomarker has been found that can distinguish GBC at an early stage (Mishra et al., 2009b). Several studies have reported the associations between genetic variants of ATM and risk of cancer development (Zhao et al., 2012), and the dynamics in the values for Ki-67, p53 and MDV followed from premalignant lesions to carcinoma sustains the hypothesis of the metaplasia-dysplasia-carcinoma sequence (Stancu et al., 2007). These findings are supported by the current qualitative screening experiments on gallbladder cancer tissue specimens for the expression of DNA damage factors viz., ATM, γ-H2AX and p53 phosphorylation states, which increased in exponential fashion. In other words, the obtained data of the present study signify the extent of DNA damage that has occurred due to the toxic exposure to MIC and also these cellular events are indirectly promoting genomic instability that is the hallmark of various solid tumors.

Although ATM kinase plays a key role in initiating several DNA repair pathways as it is a master controller of cellular pathways and networks, orchestrating the responses to a specific type of DNA damage: the double strand break (DSBs) (Pandita, 2002) as it plays a key role in the recognition, signaling and repair of DNA DSBs (Ewald et al., 2008). Misregulation of ATM may lead to broad dysfunction in DNA repair and the accumulation of genome alterations (Lee et al., 2011). Homozygous mutations in the ATM gene can cause human genetic disorder ataxia-telangiectasia, which is characterized by cerebellar degeneration, immunodeficiency, cancer predisposition, and acute sensitivity to ionizing radiation (Ejima et al., 2000). DNA DSBs caused by exposure to DNA damaging agents initiate rapid and highly coordinated series of molecular events triggering DNA damage repair. One of the earliest of such events includes the formation of γ-H2AX by phosphorylation of the Ser139 of histone H2AX (Seo et al., 2012), which is considered a surrogate marker of DSBs. Our results support the hypothesis that the mutated expression of ATM and γ-H2AX in the adenocarcinoma plays its role in the gallbladder carcinogenesis as it misregulate the cell cycle. This perspective has concentrated on ATM as a potentially useful tool to further human health. Furthermore, quick and inexpensive methods using ATM phophorylation and γ-H2AX formation for DSBs detection in blood, skin or other tissues that are obtained by minimally invasive procedure could be a valuable tool, permitting clinicians to monitor whether an agent is causing DNA damage in the patient.

Protein p53 is a regulator of the cell cycle and one of the most frequently altered targets in the majority of human neoplasis. Its main function is to maintain the reliability of DNA and induce apoptosis of those cells with an abnormal DNA. It has been shown that mutated p53 is involved in the pathogenesis of carcinoma of the gallbladder and appears to be one of the factors involved in the genesis of this process (da Rocha et al., 2004). Aberrant expression of p53 in the gallbladder has been reported mainly in adenocarcinomas (35-65%) (Roa et al., 2000), but it has also been found in adenomas (0-17%) (Wang et al., 2006). The results of the current study are in accordance with those reported in the literature, since we found p53 overexpression in 74% of the cases of adenocarcinomas and only in 16.6% of the adenomas with dysplasia. This variable expression of p53 further suggests that adenomas express p53 very late in their development to adenocarcinoma and also that the molecular events and origins of p53 are different in adenocarcinomas

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and adenomas (Wistuba et al., 1999). The distribution of p53 protein in invasive carcinomas and the adjacent dysplastic and preinvasive lesions suggests that it is more commonly expressed than previously reported literature. The fact that p53 protein is also expressed in cases of adenoma with dysplasia and adenosquamous cell carcinomas is associated with invasiveness of the disease offers further support to the contention that p53 gene mutations may have a role in the pathogenesis of gall bladder malignancy.

The study conclude that the expression of p53 and ATM protein may possibly be an indication of likely disease progression from dysplasia to carcinoma and invasive disease, whereas the γ-H2AX seems to be a minor in comparison to ATM and p53. The above discussions indicate that the increased ATM and p53 expression were associated with MIC exposure and tumor dedifferentiation. These results demonstrated that the monitoring of DSB responses through γ-H2AX formation (Ismail et al., 2007; Tanaka et al., 2007). ATM phosphorylation and p53 overexpression could be an excellent potential for judging/developing therapeutic progress and diagnosis of cancer progression. Further investigations are in progress to undertake similar studies on archived tumor tissues of varied origins and forms to examine and validate the value of these proteins as potential biomarkers for early diagnosis of the disease hitherto unreported.

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