National Journal of Medical and Allied Sciences

[ISSN Online: 2319 – 6335, Print: 2393 – 9192|Original article |Open Access]

Website:-www.njmsonline.org

PREVALENCE OF SALMONELLA TYPHI (S.TYPHI) CARRIER STATE AND ALTERED P53 GENE EXPRESSION IN CHRONIC CHOLECYSTITIS & CHOLELITHIASIS PATIENTS IN EASTERN INDIA

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ABSTRACT

Background- The over-expression of the p53 gene and K-ras gene have been well observed in CaGB cases**.** The aim was to establish the percentage of Salmonella Typhi positivity in the operated samples of chronic cholecystitis and cholecystitis and cholelithiasis patients and among those patients alteration of p53 expression was studied.

Material & Methods- The S.Typhi carrier state in Chronic Cholecystitis and Cholelithiasis patients was analyzed by the presence of S.Typhi DNA isolated and demonstrated from gall bladder tissues by Polymerase chain reaction (PCR). p53 expression alteration was also studied in these cases by immunohistochemistry. After analysis, there was a significant number of positive expressions of the p53 gene through IHC examination were found in the S.Typhi carrier state of these samples.

Results-From the samples examined in this study a significant positivity of S. Typhi carrier state was found(~10%) and among those S. Typhi, positive samples alteration in p53 expression was quite high(~70%).

Conclusion-In this study the prevalence of S.Typhi carrier state in eastern India is high than the worldwide prevalence observed. Again the alteration of p53 expression is also significant in S.Typhi positive chronic cholecystitis and cholelithiasis cases.

Keywords- S.Typhi, p53, chronic cholecystitis, cholelithiasis.

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INTRODUCTION

Typhoid fever is a systemic infection caused by Salmonella enterica serotype Typhi (S. Typhi). It is a human-specific disease. It occurs worldwide, primarily in developing nations whose sanitary conditions are poor. The disease remains an important public health problem in developing countries [1]. Typhoid fever is endemic in Asia, Africa, Latin America, the Caribbean, and Oceania, but 80% of cases come from Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, or Vietnam [1]. Within those countries, typhoid fever is most common in underdeveloped areas. Typhoid fever infects roughly 21.6 million people (incidence of 3.6 per 1,000 population) and kills an estimated 200,000 people every year [2]. Northern India has the maximum prevalence of S.typhi cases & carriers [3]. Eastern India is among the endemic zone of S.typhi infection [4]. The main source of infection is the feces & urine of the case & carrier. The carrier may be a convalescent carrier or chronic carrier. A convalescent carrier excretes bacilli for 6 to 8 weeks after which the number of bacilli decreases rapidly. And the chronic carrier excretes bacilli for more than a year after a clinical attack [4]. The clinical diagnosis of typhoid cases & carriers has been a medical challenge due to its similarity to many other infective illnesses.

The outcome of blood culture is limited by the previous use of antibiotics and a low bacterial load [5]. The continued high incidence of typhoid is due to the dissemination of the disease via typhoid carriers. Widal test, the commonly used serological test is positive only in the latter part of the disease. Moreover, its interpretation in endemic areas is fraught with disadvantages [6]. Hence there is an urgent need to increase the chance of detecting the carriers to decrease the risk that they pose to the communities. Polymerase chain reaction (PCR) is a sensitive and specific method used for the diagnosis of several infectious diseases. In typhoid carrier states, it can be an effective tool because it can be used even in cases where antibiotic therapy has been started or the pathogen load is very low. A nested PCR makes the detection more sensitive and can detect the presence of even 3-5 bacilli [7]. Therefore, nested PCR in the laboratory can be used as a diagnostic tool in culture-negative cases of typhoid fever and carrier. Carcinoma of the gall-bladder (CaGB) is the sixth most common gastrointestinal tract cancer and is endemic in several countries [8]. It is the most common malignant tumor of the biliary tract and a particularly high incidence is observed in Chile, Japan, and India. In the northern and eastern zones of India, it is the third most common gastrointestinal malignancy [9]. Gallbladder cancer is one of the most aggressive malignancies with an extremely poor prognosis. Surgical resection remains the only chance of cure but is possible in only a small percentage of patients with CaGB. The 5-year survival rate for cancers confined to the gallbladder is 32% and for advanced-stage cancers is 10% [10, 11]. This emerges the necessity for identifying predisposing factors and the tools for early diagnosis and treatment. CaGB has been co-related with various predisposing factors. Genetic disorders such as multiple familial polyposes (Gardner`s syndrome), Peutz-Jegher`s syndrome, porcelain gall bladder, and anomalous pancreaticobiliary ductal communication as seen in the choledochal cyst. Long-standing gall stones also predispose to the development of gall bladder cancer [12, 13]. The association between various chronic infections and the development of a malignant state is a fact. Various mechanisms have been postulated to explain this association. In the case of the gall bladder, a chronic typhoid carrier state is established to be a high-risk factor for the development of carcinoma of the gall bladder due to chronic infection and inflammatory sequelae [14, 15]. Because of genetics, mutation of p53 and K-ras gene is seen in gall bladder cancer. Mutant p53 is found in about 92% of invasive CaGB and 28% of dysplastic epithelium. K-ras mutations are seen in 39% of CaGB. The over-expression of the p53 gene and K-ras gene have been well observed in CaGB cases. This can be demonstrated by alteration of gene expression in the immunohistochemical examination. [16, 17].

In this study, we evaluated the percentage of positivity of S.typhi carrier state in chronic cholecystitis and cholelithiasis cases in Eastern India. Also, we evaluated the association with alteration of the p53 gene expression in chronic cholecystitis and cholelithiasis cases in Eastern India.

MATERIAL & METHODS

Gall bladder samples from the patient of Chronic Cholecystitis and Cholelithiasis were collected A thorough histological examination was carried out with hematoxylin- and eosin-stained tissue preparations. One hundred paraffinized blocks of gall bladder tissue samples of histologically proven Chronic Cholecystitis and Cholelithiasis were taken into the study. Among them, thirty-six samples were of Chronic Cholecystitis (Male-6, Female- 30) and sixty-four were of Cholecystitis with Cholelithiasis (Male-9, Female-55). p53 gene expression for these was performed at the Department of Pathology, MCH, Kolkata [20- 23]. Formalin-fixed, paraffin-embedded tissue specimens were used for immunohistochemical staining. Sections of 5 μm thick from paraffin-embedded blocks were deparaffinized in xylene and rehydrated in a graded series of ethanol [24]. The sections were pretreated with autoclaving at 121°C for 15 min in 0.01 mol/L citrate-buffered saline (pH 6.0) for antigen retrieval. Endogenous peroxidase activity was blocked by incubation with 3% H2O2 for 30 min at room temperature. The sections were incubated with 10% normal goat serum for 1 h to block nonspecific binding of

the immunological reagents. After incubation with mouse monoclonal antibodies against p53 (Clone DO-7; DakoCytomation, Glostrup, Denmark) at 4°C overnight, streptavidin-biotin complex and horseradish peroxidase was applied, and reaction products were visualized using the Histofine SAB-PO (M) immunohistochemical staining kit (Nichirei, Tokyo, Japan), according to the manufacturer's instructions. The peroxidase labeling was developed by incubation of the sections in diaminobenzidine tetrahydrochloride for 1 minute. Finally, nuclear counterstaining was done using Mayer's hematoxylin solution. Two blinded observers (K. A. and Y. Z.) independently examined the immunostained sections.

Percentage of positivity of p53 staining	**Result considered Positive/Negative with Grade**
<_10 %	Negative
11-33%	Weakly Positive
34-66%	Moderately Positive
>66%	Strongly Positive

DNA EXTRACTION:

For S.Typhi DNA PCR, normal-sized tissues a single 5,m section (average collected volume 1-44 mm3), and biopsy specimen sized tissues a single 10, um section (average collected volume 0,034 mm3) were cut and processed according to the following method: After dewaxing and rehydration, the tissue section was resuspended in 50micL of digestion buffer (50 mM KCl, 1-5 mM MgCl, 10 mM TRIS-HCl, 0-5% TWEEN 20, pH 9 at 25°C) containing 200, ug/ml proteinase K (Merck, Darmstadt, Germany) and was incubated at 55°C for 3 hours.'9 The proteinase K was inactivated by boiling for eight minutes. Then we added an equal volume of buffer saturated phenol (Fisher, BP1750I-400) and mixed it by inversion. Spun for at least 5 minutes at 14,000 rpm in a microcentrifuge, transferred the aqueous layer to a new tube. Noted the interphase: if they're a lot of white material or not. Repeated steps 1 and 2 on the aqueous fraction until the interphase was clear (typically 3 or more times). Performed back extractions* when the interphase is fuzzy to increase final yield. Once the interphase is clear, added an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) (Fisher, BP1752I-400) to the extracted aqueous fraction of my sample. Mixed for 5 minutes and then spun for 5 minutes at 14,000 rpm. This reduced residual phenol and further sharpened the interphase, facilitating the extraction of the aqueous layer. Removed the aqueous layer to a new tube and treated with RNase A at 100 µg/ml for 1 hour at 37°C. Repeated steps 1 to 4 to remove any remaining RNase A and collected the aqueous fraction. One should only need 1 or 2 buffer saturated phenol steps as there should be much less protein to remove than in the initial lysate. To perform back extractions added 50-100 µl of dH20 to the sample tube containing the interphase and organic portion. Inverted the tube to mix, and spin the sample at 14,000 rpm in a microcentrifuge for 5 minutes. Collected the aqueous phase and added it to the previously acquired aqueous extraction. Continued back extractions until the interphase was clear. Then we estimated the volume of my collected aqueous layer. We added 1/10 the volume of 3 M sodium acetate pH 5.2 and 1 volume of 100% isopropanol (v/v) molecular biology grade (or 2.5 volumes of 100% ethanol)mixed well and put on ice or in a -20°C freezer for 30 minutes. Spun at maximum speed (14,000 rpm) at 4°C in a microcentrifuge for 10 minutes, discarded the supernatant, washed the pellet with 70% ice-cold ethanol to remove unwanted salts, resuspended the pellet in the buffer of choice, dH2O. All DNA samples were quantified by fluorometry using Qubit ™ dsDNA HS Assay Kit (Life Technologies, Carlsbad, California, US) on Qubit™ 3 Fluorometer (Invitrogen, Life Technologies)as per the manufacturer's instructions, and assessed for purity by NanoDrop 8000 Spectrophotometer (Thermo Scientific) 260/280 absorbance ratio measurements, in triplicate. Apart from the Qubit assay, for the quality control analysis of DNA, Infinium HD FFPE QC Assay (Illumina, Inc.) was used by performing a quantitative PCR of FFPE DNA on the CFX96™ Real-Time PCR Detection System (BioRad). Subsequent data analysis was performed as per the Manufacturer's instructions. The ΔCq was calculated to evaluate the quality of isolated DNA, since values ΔCq > 5 are not suitable for further downstream processing for Infinium HD FFPE Restore Protocol (Illumina,

Inc.) and Infinium MethylationEPIC array (Illumina, Inc.). A value ΔCq < 5 ensures the better quality of isolated DNA that is suitable for various targeted and genome-wide analyses.

PCR detection of Salmonella spp. Were done using primers. Salmonella typhi strain was retrieved from frozen stock culture and grown in 3 mL Luria–Bertani (LB) broth overnight at 37 1C (aerobically). DNA extraction from pure cultures was achieved by boiling bacterial cells, which were resuspended in 1% (v/v) Triton X-100, as described before (Wang et al., 1996). Without isolation of the DNA, 2 mL of the samples were directly added to the PCR mixture. The set of primers was designed from the sdiA gene sequence available from the GenBank database. The multiple sequence alignment was processed using CLUSTAL W software (Thompson et al., 1994). We focused on the Salmonella typhi strain. PCR amplification was carried out in a 25 mL reaction mixture containing 2.0 mM of each deoxynucleoside triphosphate (dNTPs), 1 PCR buffer [67 mM Tris-HCl, pH 8.0, 16.6 mM, (NH4)2SO4, 0.05% Tween 20], 3 mM MgCl2, 25 pmol of each primer (SdiA1, SdiA2), 1 U Taq Polymerase (HyTest Biotechnology Ltd, Cambridge, UK) and 2 mL aliquot of the sample DNA. 1.0 mg mL1 of nonacetylated bovine serum albumin (BSA, Ambion) was added in the reaction mixture. The cycling program consisted of denaturation at 94.1C for 5 min, followed by 30 cycles of 94.1C for 30 s, 52.1C for 40 s, 72.1C for 30 s. A final extension was performed at 72 .1C for 7 min. As IAC a 566 bp fragment of the 16S rRNA gene was used and amplified with universal (907R) and bacterial (341F) primers (Muyzer et al., 1997). The volume of the reaction mixture was 50 mL containing 4.0 mM of each dNTP, 2 U Taq Polymerase, 4 mM MgCl2, primers SdiA1, SdiA2, 341F, 907R (25 pmol each), 1 PCR buffer, 1.0 mg mL1 of BSA, and template DNA (4 mL). The cycling program consisted of denaturation at 94 1C for 5 min, followed by 30 cycles of 94.1C for the 30s, 52.1C for 40 s, 72.1C for 90 s. A final extension was performed at 72.1C for 10 minutes PCR amplification was conducted in a primus 96 plus Thermal Cycler (MWG Biotech AG, Ebersberg, Germany). PCR products were resolved by electrophoresis in 1.5–2% (w/v) agarose gels and visualized under UV light after ethidium bromide staining. The DNA sequence of PCR fragment was obtained from a pure culture of Salmonella typhi and was determined by the dideoxy method (Sanger et al., 1977). Sequencing was carried out by Macrogen Inc. (Korea). To confirm that the sequence is a part of the sdiA gene, a search of the GenBank DNA database was conducted by using the BLAST algorithm (Altschul et al., 1990). The similarity of over 99% to the sdiA gene of Salmonella enterica strains showed that the set of primers described here amplifies exclusively the 274 bp fragment of the target gene.

Outcome parameters- Outcome parameters were based upon Positivity of S.typhi carrier state in chronic cholecystitis, Cholelithiasis and altered expression of p53 & in Chronic cholecystitis, Cholelithiasis cases by IHC

RESULTS

Among 100 patients, a total of 14 patients were male and eighty-five patients were female. The majority of the histology was Cholecystitis with Cholelithiasis, sixty-four in number. And thirty-six patients were of Chronic cholecystitis.

Table 1: Gender and Histology Association

Gender	Histology		Total
	Cholecystitis with Cholelithiasis	Chronic Cholecystitis	
Male	9	6	14
Female	55	30	85
Total	64	36	100

Among the 100 samples, 10% samples were found positive of IHC for p53 gene expression, nine samples were weakly positive and one was moderately positive. (Table-1)

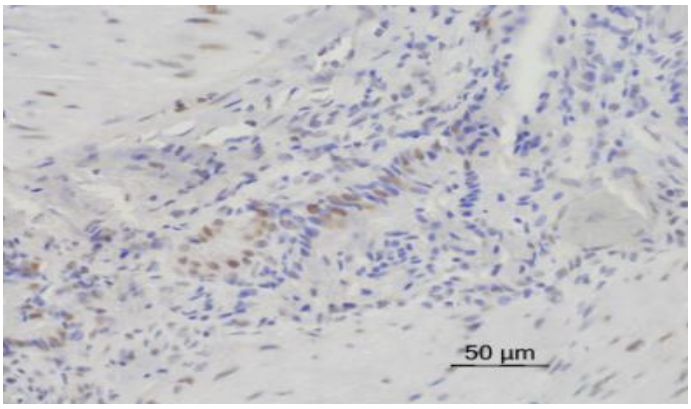

Figure 1- IHC for p53 Gene (Weakly Positive)

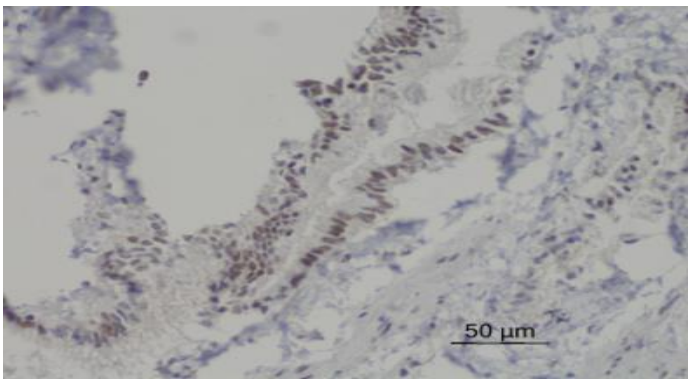

Figure 2- IHC for p53 Gene (Moderately Positive)

Grade of p53 positivity among IHC positive subjects	Frequency	Percent
Weakly positive	9	90.0
Moderately positive	1	10.0
Total	10	100.0

Table 2 Seven samples (7%) were positive for S. Typhi chronic carrier state (Six in Cholecystitis with Cholelythiasis samples, one in Chronic Cholecystitis sample); which is much higher than the overall prevalence of S. Typhi carrier state which is only 2.5% as per present epidemiological data states.

PCR	Association between PCR and P53 status	Positive	Negative	Total number & Percentage
Positive	Count % within PCR	5 71.4%	2 28.6%	7 100.0%
Negative	Count % within PCR	5 5.4%	88 94.6%	93 100.0%
Total	Count % within PCR	10 10.0%	90 90.0%	100 100.0%

Salmonella Typhi carrier state was detected in seven (7) samples demonstrated by PCR. Among the Salmonella typhi carrier state, five (5) cases were p53 positive by Immunohistochemistry.

DISCUSSION:

Seven samples were positive for S. Typhi chronic carrier state (Six in Cholecystitis with Cholelythiasis samples, one in Chronic Cholecystitis sample); in percentage, it is seven percent (7%), which is much higher than the overall prevalence of S. Typhi carrier state which is only ~2.5% in the overall population as per present epidemiological data states [18]. From the above findings, we find a strong relation between p53 gene mutation and Cholecystitis & Cholelithiasis. Among the one hundred(100) samples, ten (10%) samples were found positive for IHC for p53 gene expression. Through IHC study, grades of p53 positivity were found. (nine samples were weakly positive and one was moderately positive). Gall-bladder cancer is usually associated with chronic cholecystitis and gallstone disease. P53 gene mutation is observed in a high proportion of gallbladder cancers as it can be accurately detected with conventional immunohistochemical techniques[19, 20]. IHC data also suggest that approximately one-third of the proximal tumors were found to express weak p53immunopositivity, whereas moderate immunopositivity and a higher rate were observed in tumors of the lower mid-region. Finally, moderate and marked p53 immunopositivity was observed in tumors of the ampulla and

gallbladder. In tumors of the lower mid-region of the ampulla and the gallbladder, a significantly higher p53positivity was noted [21]. Also through the PCR analysis, it is clear that Salmonella typhi carrier state was detected in seven (7) samples demonstrated by PCR. Among the Salmonella typhi carrier state, five (5) cases were positive for p53 positive by Immunohistochemistry. And in percentage within the PCR is 71.4% which is moderately high. Previous data support that the alteration of p53 gene expression and S.Typhi carrier state lead to Carcinoma Gall Bladder [22]. Alteration of IHC expression of p53 gene in S.Typhi carrier state in Gall Bladder of Cholelithiasis & Chronic Cholecystitis patients are much high and there is a statistically significant correlation we established.

CONCLUSION

Gall bladder carcinoma/cancer is usually associated with chronic cholecystitis and cholelithiasis. P53 plays an integral part in cholecystitis and cholelithiasis. P53 gene mutation and change in codon play an important role in the above-mentioned diseases. After the study performed we got the data that p53 showed altered expression in S. typhi carrier state in chronic cholecystitis and cholelithiasis patients which is much higher than chronic cholecystitis and cholelithiasis patients who are not S. typhi carrier, and its statistically significant.

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Conflicts of Interest: Nil Source of Funding: Nil

Citation: Soumik Ghosh, Kalidas Biswas, Atrayee Chatterjee. Prevalence of Salmonella Typhi Carrier State and Altered P53 Gene Expression in Chronic Cholecystitis & Cholelithiasis Patients in Eastern India. National Journal of Medical and Allied Sciences 2022; 11(1): Online first

Date of Acceptance: 21.03.2022
Date of Submission: 27.02.2022