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Research Article

DIAGNOSTIC UTILITY OF *DAPK*-GENE PROMOTER HYPERMETHYLATION IN GASTRIC CANCER

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ABSTRACT

Background: Gastric cancer (GC) is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide, with a wide variation in incidence rates across different geographical areas. The current view is that a malignancy arises from a transformation of the genetic material of a normal cell followed by successive mutations and other chain of alterations in genes such as DNA repair genes, oncogenes, tumor suppressor genes e,g DAP-K and others. The aim of present study was to find out the role of Promoter Hypermethylation of DAP-K gene in gastric cancer with emphasis on clinicopathological parameters.

Material and Methods: Gastric cancer samples along with normal gastric samples were collected and the DNA was extracted from all which was followed by bisulphate modification. Methylation-specific polymerase chain reaction was used for the analysis of the promoter hypermethylation status of DAP-K gene.

Results: The epigenetic analysis revealed that Kashmiri population has a different promoter hypermethylation profile of DAPK gene which is unlike to other regions. As depicted by methylation-specific PCR, 60% of the gastric cancer tissues harbored methylated DAP-K promoter hypermethylation. In comparison, 79% of the histopathologically confirmed normal tissues exhibited unmethylated DAPK

promoter. The association of *DAPK* promoter hypermethylation with gastric cancer was evaluated by $\chi 2$ (Chi square) test and was found to be statistically significant (P=0.0007). Pertinently *DAPK* promoter methylation was found to be insignificantly higher in Stage III/IV compared to Stage I/ II (P =0.0820). Taking separate gender into consideration *DAPK* gene promoter hypermethylation was found to be significantly associated with Gastric cancer both in male and female categories depicting also insignificant higher frequency of hypermethylation in male cases than female cases. As far as histological grades of the disease, *DAPK* promoter methylation was found to be significantly higher in Poorly differentiated adenocarcinomas, compared to well differentiated adenocarcinomas and moderately differentiated adenocarcinomas (P =0.0479).**Conclusion:** Our study has supplemented the steadily growing list of genes inactivated by promoter hypermethylation in gastric carcinoma and has provided new insights into the molecular basis of the disease for the development of molecular marker which may contribute to the improvement of diagnosis and prognosis of Gastric cancer.

Keywords: Gastric Cancer, DAPK-gene, epigenetic alterations, promoter hypermethylation

INTRODUCTION

Kashmir has a high incidence of cancers and GC constitutes about 30%-40% of all malignancies reported in Kashmir. Till date GC has remained a major clinical challenge due to its poor prognosis, limited treatment options, relatively resistance to chemotherapy / radiotherapy and late diagnosis of the disease **(1, 2).** Gastric cancer also called stomach cancer is a malignant tumor arising from the lining of the stomach. Stomach cancer usually begins in cells in the inner layer of the stomach. Over time, the cancer may invade more deeply into the stomach wall. Stomach cancer cells can spread by breaking away from the original tumor. They enter blood vessels or lymph vessels, which branch into all the tissues of the body. Stomach cancers tend to develop slowly over many years **(3).** Before a true cancer develops, pre-cancerous changes often occur in the inner lining i.e. mucosa of the stomach. These early changes rarely cause symptoms and therefore often go undetected. Epigenetic changes such as DNA methylation is one of the most common epigenetic event that involves covalent addition of the methyl group to DNA, which plays an important role in driving tumorgenesis **(4-6).** The DNA methylation usually occurs in the CpG islands located in or near the promoter of over 70% of all genes **(7)**. DNA methylation is an important regulator of gene transcription, and its role in carcinogenesis has been a topic of considerable interest in the last few years. Alterations in DNA methylation are common in a variety of tumors as well as in cancer development **(8, 6).** As methylation occurs early and can be detected in body fluids, it may be of potential use in early detection of tumors and for determining the prognosis **(9).** Methylation of DAP-K has been reported in many cancers **(10-12)** with inclusive results. Therefore our study was focused on studying this alteration in GC patients of Kashmir Valley so as to get conclusive finding that may help in analyzing this dreadful disease and look for alternative approaches of medicine for the same. Also various clinical and pathological parameters were studied in relation to DAPK hypermethylation.

MATERIALS AND METHOD

Sample collection:

400 surgically obtained gastric tissue samples were collected among which 200 were gastric cancer cases and 200 were normal controls. Samples were obtained from the Department of Surgery, Shri Maharaja Hari Singh (S.M.H.S) hospital Srinagar in sterilized plastic vials containing 10% of normal saline and transported from the theatres to the laboratory on ice and stored at -80°C till further process. The information regarding gender and stage was collected from the record file of patients present in the hospital and histological grading of each sample was obtained from the histopathological reports. The DNA was extracted by phenol chloroform method (Sam brook & Russell).

Qualitative and quantitative analysis of genomic DNA: The integrity of the genomic DNA was examined by gel electrophoresis using 3 % agarose gel. DNA in presence of ethedium bromide was visualized with the help of gel doc system (Biorad). The quantity of DNA was determined by U.V. spectrophotometric method.

Bisulpite treatment: Modification by Sodium bisulfite converted unmethylated cytosines to uracil and hence enabled to distinguish between the hypermethylated and non hypermethylated cytosine residues. DNA was modified by kit based method (EZ DNA Methylation™ Kit) supplied by ZYMO RESEARCH. The modified DNA was stored at -20^{0}C for further use.

Methylation Analysis:

Methyl specific polymerase chain reaction (MSP): Amplification of the promoter region of the gene was carried out in Gradient Thermalcycler (Eppendorf) in a 25µl reaction mixture as shown in table I. Predesigned primers **(13)** were used and information about same is given in table II.

Reagent	**Concentration**	**Volume**
PCR MM (master mix)		12.5 µl
Forward primer	10 pmol/µl	1µl
Reverse primer	10 pmol/µl	1 µl
DNA sample	250 ng/µl	1µl
Deionised water		9.5 µl
Total volume		25 µl

Table 1: Volume and concentrations of different reagents used in PCR

Nature of Primer	Primer sequence	
unmethylated Prime (*DAPK*)	Forward primer	5ggaggatagttggattgagttaatgtt3
	Reverse primer	5caaatccctcccaaacaccaa3
methylated primer (*DAPK*)	Forward primer	5ggatagtcggatcgagttaacgtc3
	Reverse primer	5ccctcccaaacgccga3

Table 2: Primer information used for MSP (Methylation Specific PCR)

The thermal cycler was programmed as under, to carry out the PCR amplification.

Steps	Temperature ^{0}C	Time	Number of cycles
1. Initial Denaturation	95	5 min	1
2. Denaturation	95	30 sec	40
3. Annealing	58	30 sec	
4. Extension	72	30 sec	
5. Final extension	72	5 min	1
6. Hold	42	5 min	

Table 3: Thermal cycling conditions

After completion of PCR, the reaction products were run on 2% agarose gel, in presence of 100 bp ladder as marker. Methylated and unmethylated bands were noted down in all samples. The data obtained was statistically assessed by using SPSS Software.

RESULTS

Analysis of DAPK 1 promoter hypermethylation:

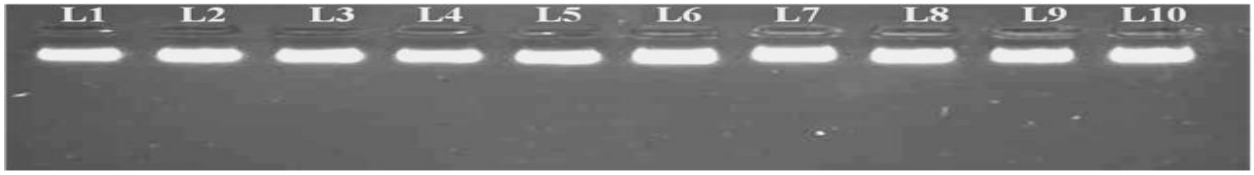


Figure 1: Lane (L) 1-10 showing the isolated DNA of samples, run on 3% agarose

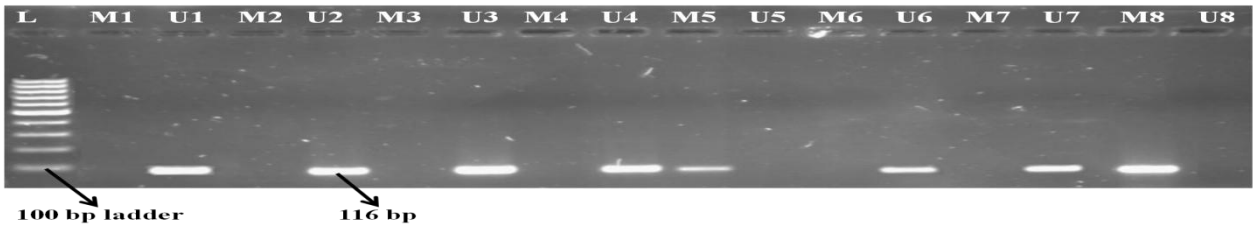


Figure 2: MSP (Methylation specific PCR) of Histopathologically Confirmed Normal gastric DNA samples run on 3% Agarose Gel

L– 100bp Ladder

M - Control amplified with methylated primer, product size was 114bp.

U - Control cases amplified with unmethylated primer, product size was 116bp.

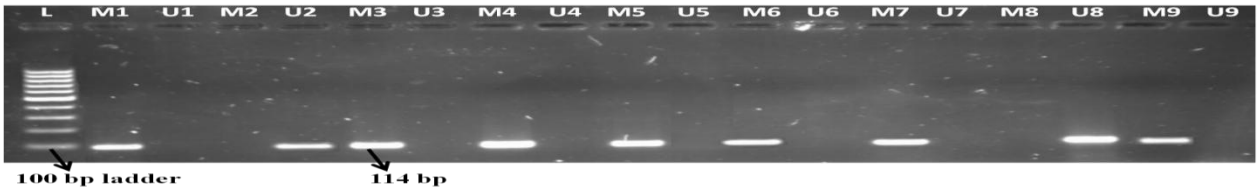


Figure 3: MSP (Methylation specific PCR) of Gastric Cancer DNA Samples Run on 3% Agarose Gel.

M – 100bp ladder

M - Cases amplified with methylated primer, product size was 114bp.

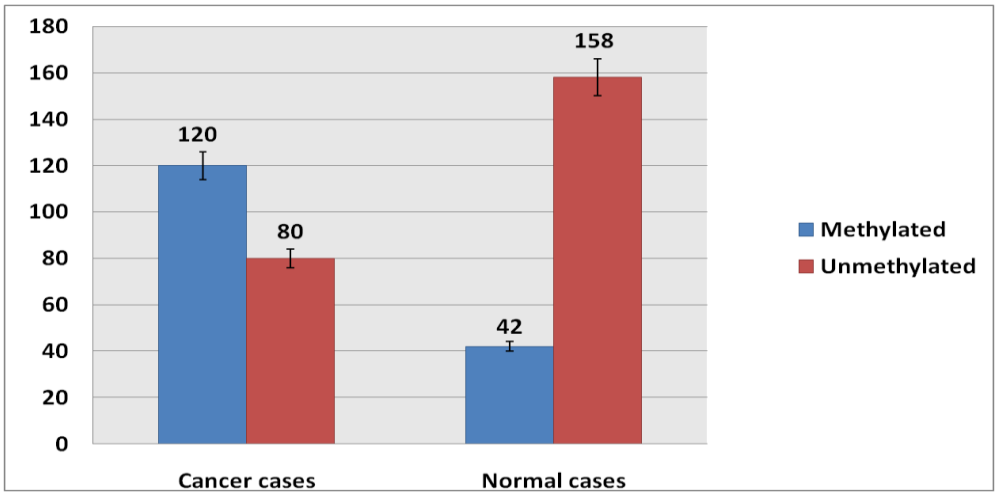
U - Case amplified with unmethylated primer, Product size was 116bp.

Relationship of Promoter hypermethylation of *DAPK* gene with gastric cancer in histopathologically confirmed cancer and normal cases

Methylation-specific PCR was performed to examine the methylation status of the promoter region of *DAPK* gene. It was observed that 60% (120/200) of the gastric cancer tissue samples harbored methylated *DAPK* promoter and 40% (80/200) depicted unmethylated *DAPK* promoter. In histopathologically confirmed normal tissue samples 79% (158/200) exhibited unmethylated *DAPK* promoter and only in 21% (24/200) normal samples, *DAPK* promoter was found to be methylated. The association of promoter methylation with gastric cancer was evaluated by χ^2 (Chi square) test, using SPSS software and was found to be significant (P=0.0007).

Cancer cases (200)		**Frequency**
Methylated	120	60 % (120/200)
Unmethylated	80	40% (80/200)
Normal samples (200)		**Frequency**
Methylated	42	21%(42/200)
Unmethylated	158	79%(158/200)

Table 4: Number & Frequency of Methylated/Unmethylated Cancer & Normal Cases

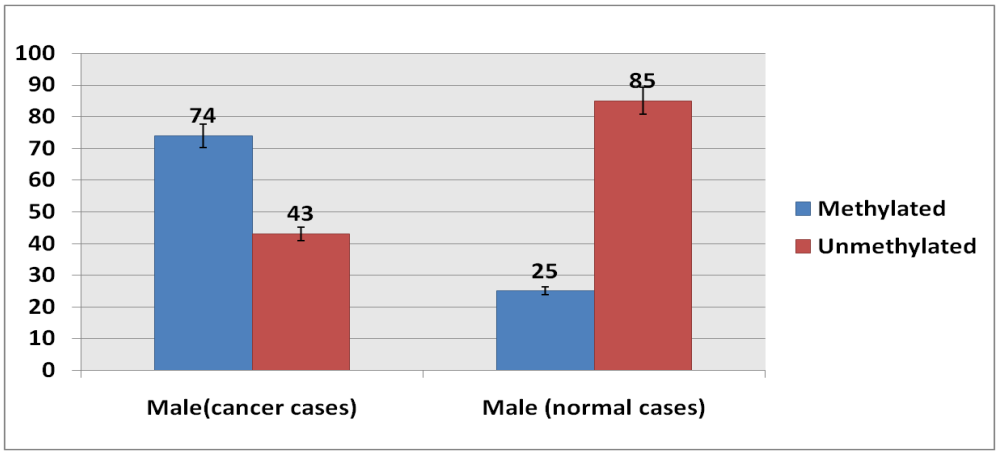


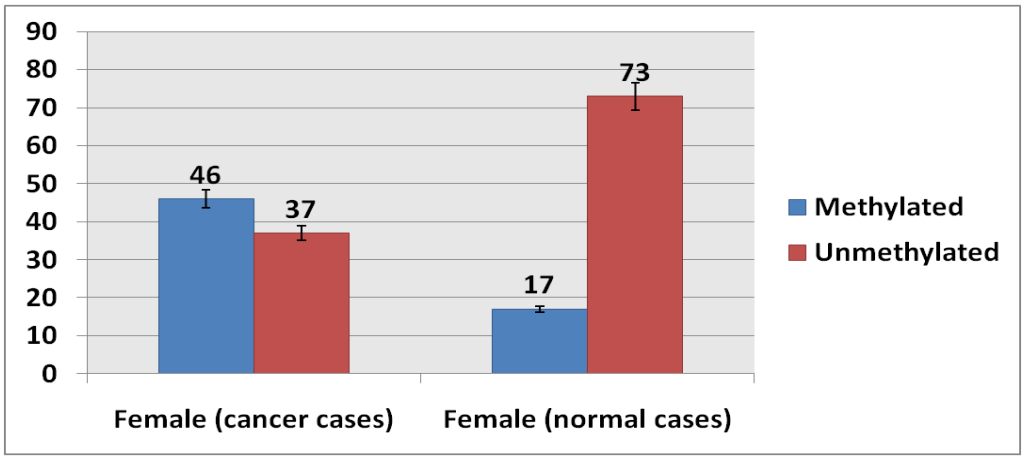
Relationship of promoter methylation within male and female groups:

Looking gender wise, among 117 male cancer cases, 74 cases were methylated and 43 cases were unmethylated and among 110 male normal controls 25 were methylated and 85 were unrmethylated. The association of promoter methylation within male gender was found significant (P =0.0055). Comparatively, among 83 female cases, 46 cases were found to be methylated and 37 were unmethylated and among 90 female normal controls 17 were methylated and 73 were unrmethylated. The association of promoter methylation within female gender was again found to be significant (P =0.0043). However, on comparing the male cases with female cases, occurrence of *DAPK promoter* methylation was found to be unequally distributed in males and females with more frequency in males than in female but the difference was not statistically significant (*P* =0.8435).

TOTAL CANCER CASES (200).		**FREQUENCY**
MALES – 117		
Methylated	74	63.2% (74/117)
Unmethylated	43	36.7 % (43/117)
FEMALES – 83		FREQUENCY
Methylated	46	55.4 % (46/83)
Unmethylated	37	44.5 % (37/83)
TOTAL NORMAL CASES (200).		
Males 110		
Methylated	25	22.7 % (25/110)
Unmethylated	85	77.2 % (85/110)
Female 90		
Methylated	17	18.6% (17/90)
Unmethylated	73	81.1% (73/90)

Table 7: Number of Cases Showing Promoter Methylation & Unmethylation sex wise





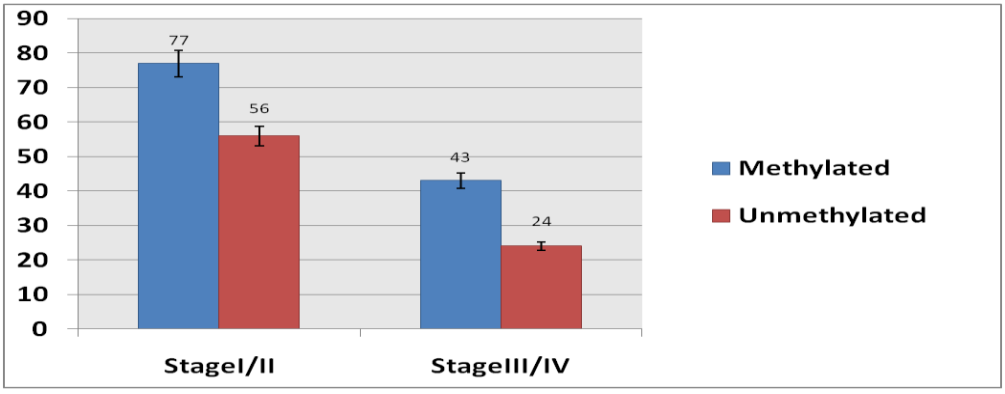
Relationship of promoter methylation of *DAPK* gene in stage I/ II and stage III/IV: Table 5 and table 6 gives detailed pattern of promoter hypermethylation among various stages of the disease. There were 133 gastric cancer cases that were in Stage I and Stage II of the disease. Among these cases 77 cases were methylated and 56 cases were unmethylated. However, among 67 cases that were in Stage III and Stage IV of the disease, 43 cases were methylated and 24 cases were unmethylated. When the frequency of *DAPK* promoter methylation was compared with clinical staging of the disease, *DAPK* promoter methylation was found to be certainly higher in Stage III/IV (64.1 %) compared to Stage I/ II (57.8%) but the difference was not statistically significant (P =0.0820).

Parameter	**Total number of cases (200)**			
	Stage I (64 cases)	**Stage II (69 cases)**	**Stage III (34 cases)**	**Stage IV (33 cases)**
Methylated	34	43	20	23
Unmethylated	30	26	14	10

Table 5: Number of Cases Showing Promoter Methylation & Unmethylation within different stages

Total number of cases (200)		
Stage I/II (133 cases)		
Parameter	**Cases**	**Frequency**
Methylated	77	57.8 % (77/133)
Unmethylated	56	42.1 % (56/133)
Stage III/IV (67 cases)		
Parameter	**Cases**	**Frequency**
Methylated	43	64.1 % (43/67)
Unmethylated	24	35.8% (24/67)

Table 6: Number of Cases Showing Promoter Methylation & Unmethylation in Stage I/II & Stage III/IV



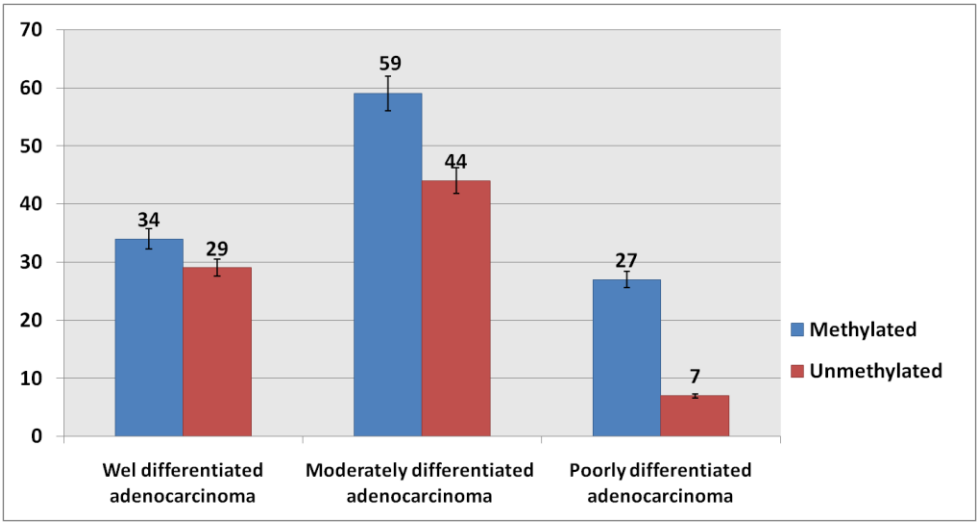
Relationship of promoter methylation of *DAPK* gene within different histological grades:

There were 63 GC cases of well differentiated adenocarcinoma and among these, 34 (34/63) were methylated and 29 (29/63) were Unmethylated. 103 GC cases belong to moderately differentiated adenocarcinomas grade and among these 59 (59/103) were methylated and 44 (44/103) were Unmethylated. Lastly 34 GC cases belonged to poorly differentiated adenocarcinomas grade and among these 27 (27/34) were methylated and 7 (7/34) were unmethylated. When the frequency of *DAPK* promoter methylation was compared within histological grades of the disease, *DAPK* promoter methylation was found to be certainly higher in Poorly differentiated adenocarcinomas 79.4%, compared to well differentiated adenocarcinomas 53.9% and moderately differentiated adenocarcinomas 57.2% and the difference was

statistically significant (P =0.0479).

Well differentiated adenocarcinomas (63)		**Frequency**
Methylated	34	53.9% (34/63)
Un methylated	29	46.0% (29/63)
Moderately differentiated adenocarcinomas (103)		
Methylated	59	57.2% (59/103)
Un methylated	44	42.7% (44/103)
Poorly differentiated adenocarcinomas (34)		
Methylated	27	79.4% (27/34)
Un methylated	7	20.5% (7/34)

Table 8: Methylation Pattern During Different histological grade



DISCUSSION

Epigenetic mechanisms, such as hypermethylation of CpG Islands of promoter regions, have been proposed as a mechanism of gene inactivation in tumour cells **(14)**. our study depicted significant association of promoter methylation of *DAPK* gene in GC which is also supported by the findings of Esteller et al., 2008; Rice et al., 2000, Wenzhuo et al.,2016, Tehseen et al., 2017 **(15, 16, 3, 5)**. However our results contradict with the findings of Aurelie et al., 2002, Knudson et al., 1985 **(17, 18)** as these studies have proposed in their

results that *DAPK* promoter hypermethylation is not involved in the cancer. Our study also supports the findings of Hanahan D. et al., 2000 **(19)** proposing that tumor tissue of GC patients harbored promoter hypermethylation of different genes. These results were in accordance to our findings, that in *DAPK* gene of GC patients, there is an alteration of methylation pattern, which may the causative agent for the gastric cancer and is also in support of the findings of Jing et al., 2014; Qu et al., 2013, Nakamura et al., 2014, predicting that alterations in *DAPK* gene are estimated to be responsible for about 50% of familial gastric cancer **(20-22)**.

In our study we found 60% mean frequency of *DAPK* gene promoter hypermethylation in cases and in comparison only 21% of the controls had the same epigenetic alteration. These findings were found to be higher than the results speculated in various previous studies carried out by Wenzhuo etal., 2016 and Tomohiro et al. (2004) **(3, 23).**

As for as clinicopathological parameters are concerned, our study depicted insignificant higher frequency of *DAPK* gene promoter hypermethylation in stage III/IV (P =0.0820). This data strongly support the findings of Wenzhuo et al., 2016, Hirofumi et al. 2011, Hora Loghmani et al. (2014). In these studies there was no significant difference of *DAPK* gene promoter hypermethylation among various age groups, stages and tumor grades (**3, 24**).

Our study confirms that *DAPK* gene promoter hypermethylation is specific characteristic of gastric cancer patients in ethnic population of Kashmir valley, but more additional and large population based studies are needed to understand the nature of association of *DAPK* promoter hypermethylation in gastric cancer and whether this epigenetic event can be used as early diagnostic and prognostic tool in this dread full disease or not.

CONCLUSION

This study revealed that promoter region hypermethylation status of tumor suppressor gene *DAPK* showed a significant increase in gastric cancer patients of Kashmiri origin as compared to controls. Findings were more apparent when the data for hypermethylation was interpreted taking Clinical stage into consideration and it was seen here that stage III/IV shows higher frequency of promoter region hypermethylation compared to stage I/II which was earlier reported in literature. Further it has been seen that, the frequency of *DAPK* gene promoter hypermethylation was insignificantly increased in male cancer cases. It is clear that promoter hypermethylation of *DAPK* gene is important in developing GC which is a multistep process involving genetic and epigenetic changes. Our study has supplemented the steadily growing list of genes inactivated by promoter hypermethylation in gastric carcinoma and has provided not only new insights into the molecular basis of the diseases but also list of interesting candidate genes for the development of molecular markers which may contribute to the improvement of diagnosis and prognosis of Gastric cancer.

Conflict of interest: Authors declare that they have no conflict of interest.

Author's contribution: All the authors have contributed equally. All authors read and approved the final manuscript.

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