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RESEARCH ARTICLE

STUDY OF DNA METHYLATION IN THE PROMOTER REGION OF P15 AND E-CADHERIN GENES FROM HEAD & NECK TUMOURS

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ABSTRACT

Background: Hypermethylation of promoter regions of tumour suppressor genes is known to cause downregulation of tumour suppressor genes. We evaluated DNA methylation of two tumour suppressor genes p15 & E cadherin on histopathologically proven cases of head and neck cancers. ***Objectives:*** To study Methylation in the promoter region of p15 and E Cadherin genes from tissue samples of head & neck cancer and compare it with peripheral blood. ***Methodology:*** Forty three cases of head & neck cancer were taken as study subjects. DNA was extracted from HPE proven head & neck cancers. This DNA was bisulphite modified. Methylation Specific PCR was done on the modified DNA to detect methylation pattern. DNA was extracted from peripheral blood of the same patients and the same procedure was followed on the modified DNA to compare the methylation patterns in head & neck cancer tissue as well as in the blood. ***Results:*** DNA methylation was detected in 49% tissue samples and 40% peripheral blood samples in p15 gene & 63% tissue samples and 44% peripheral blood samples in E cadherin gene. Kappa coefficient is 0.346 for p15 gene and 0.277 for E cadherin. ***Conclusion:*** DNA methylation of promoter region of E cadherin & p15 tumour suppressor genes which causes them to become downregulated may be one of the mechanisms of development of head & neck cancer. This is a reversible mechanism. Demethylating agents like Azacytidine and Decitabine can be exhibited to patients of Head & neck carcinoma who show evidence of DNA methylation.

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INTRODUCTION

In India and worldwide head and neck cancers accounts for one of the highest morbidity and among them oral cancers predominate.[1] In the future with the combination of all cytotoxic drugs, surgery and radiation it is indeed impossible to predict a drastic improvement in cure rate. Thus we should aim our research work to identify newer molecular markers which can be taken as targets for therapy or for prognosis.[1] In the United States in the year 2010, estimated new cases for head & neck cancer were 47,560 and deaths were 11,480[2] and among them squamous carcinoma were in majority.[3] The primary risk factors which are known worldwide are cigarette smoking and alcohol and together they are 75% of all the cases.[4] Other risk factors for the disease include dietary factors,[5,6] environmental and occupational exposures,[7] gastro-esophageal reflux[8] and inherited cancer syndromes, such as Bloom, Li Fraumeni, ataxia telangiectasia or xeroderma pigmentosum,[9] although these account for a much smaller risk factors of HNSCC relative to tobacco, alcohol and HPV16. In cancer research epigenetics is new achievement but with many gray areas. With research it has been made clear that the genome has information as genetics and epigenetics. Epigenetics means that the changes in gene expression is regulated by a different mechanism that donot involve changes in DNA sequence. The changes in epigenetics are chromatin structure modulation, transcriptional repression, X-chromosome inactivation, genomic imprinting, and the suppression of the detrimental effects of repetitive and parasitic DNA sequences on genome integrity. The most significant epigenetic information that is present in mammalian cell is methylation, or methyl group covalently attached to the 5th position of cytosine present in CpG dinucleotide which is abundantly present in the promoter region of a gene.It has been recently proven that methylation is also involved in many other processes like DNA repair, genome instability, and regulation of chromatin structure.DNA methylation causes binding of

DNA to histone more firmly due to opposite charges and has profound impact on gene expression.[10] It has been recently identified that hypermethylation of CpG islands present in promoter region is one of the etiology for pathogenesis of HNSCC.[11] The cell cycle is regulated by different genes and gene products and among them the most essential role is played by cyclin-dependent kinases along with their binding partners and also the natural inhibitory molecules such as p16,Rb (retinoblastoma) and p15 gene. Among these genes p15 is most widely studied in oral squamous cell cancer. Abnormal methyaltion of p15 gene occurs early in tumour cell population in both *in situ* and invasive tumours with a frequency of 23% to 50%.[11-14] Due to the known fact that Rb and p15 gene are inactivated by methylation in other tumours, still there is hardly any work done on head & neck tumours.[11] In head and neck cancers the methylation in promoter region of E-cadherin gene has been studied.

The frequency of methylation in promoter region of E-cadherin in oral squmous cell cancer varies from 35% to 50%.[15] Detection of epigenetic changes in HNSCC can serve as a new molecular marker for early detection as well as prognosis of the disease. Epigenetic studies can help in implementing new therapeutic strategies for head and neck cancer patients in the form of demethylating agents like 5 azacytidine and decitabine. Drugs like hydralazine & procainamide has been recently identified to show some demethylating properties thus know to cause reactivation of tumour suppressor genes which has been methylated.[16] This study was taken up to assess the methylation pattern in a panel of two genes in head and neck cancer tissue and in peripheral blood of the patients of HNSCC by using methylation specific PCR.

MATERIAL AND METHODS

The present study was conducted in the department of Biochemistry and department of Otorhinolaryngology of an urban tertiary care teaching hospital. Ethical clearance was obtained from the college ethical committee prior to starting the study. Informed consent was taken from all the patients.
Forty three (43) cases of head & neck cancer were taken as study subjects. The tumour sites of these 43 HNSCC included oral cavity (n=16), oropharynx (n=5), hypopharynx (n=13) and larynx (n=9). DNA was extracted from these cases of histopathologically proven head & neck cancers tissue.

The DNA was bisulphite modified. Methylation Specific polymerase chain reaction (MSP) was done on the modified DNA to detect methylation pattern. DNA was also extracted from peripheral blood of the same patients and the same procedure was followed on the modified DNA to compare the methylation patterns in head & neck cancer tissue as well as in the blood. All the above mentioned parameters were evaluated by MSP in head & neck cancer patients.

Sample collection:

- Forty three fresh (non formalin fixed) histopathologically proven cases of head and neck cancer tissues from primary site were collected at the time of surgical resection.
- The tissues was immediately stored in allprotect tissue reagent (Qiagen) and stored at -70°Cfor preservation till an analysis was done.
- The blood samples from the same patient were collected in EDTA tube and stored at -70°Cfor preservation till analyses was done.

Exclusion criteria: Benign lesions, inflammatory lesions and secondaries in cervical lymph nodes were excluded

DNA extraction from tissue and peripheral blood sample and bisulphite conversion protocol: DNA was extracted by using PureLink Genomic DNA kits by Invitrogen. The extracted DNA was quantified and bisulfite modification was performed using methly code bisulphite conversion kit by Invitrogen. The optimal concentration of DNA was kept between 200-500ng/µl for bisulfate modification.

Methylation Specific Polymerase chain reaction (MSP) Protocol: Hot start Methylation specific PCR was performed by using kits from chemicon international in a 25 µl reaction volume which comprised of water 19 µl, Primers U/M 1 µl, Axygen Qarta buffer mix 2.5 µl and DNA template 2.5 µl. Universal methylated & un-methylated DNA controls from Zymogen were bisulfite modified and run with each batch. Universal methylated & un-methylated commercially bisulfite modified DNA controls from Qiagen were also run with each batch. Thermal cycler settings for both the genes were as follows: Initial denaturation for hot start Taq activation was 95^0C for 14 minutes, Denaturation at 94^0C for 60 seconds, Annealing at 60^0C for 60 seconds, extension at 72^0C for 60 seconds for 35 cycles and final extension at 72^0C for 07 minutes. 1.5% agarose gel electrophoresis with Ethidium bromide was performed for detection of PCR products. The gel was visualized on the UV - transilluminator to read the final results.

Statistical analysis: Data was analyzed by using MedCal statistical software. Kappa coefficient was calculated with confidence interval of 95% to see the agreement between the methylation status in HNSCC tissue and peripheral blood specimens from cases.

RESULTS

HNSCC patients: ***(Figure 1):*** A total of 43 HNSCC patients (histologically proven squamous cell carcinoma) with mean age of 60.20 years (39 to 76 years) were included in the study. Out of 43 HNSCC patients 04 were female (9.3%) and 39 were male (90.7%).

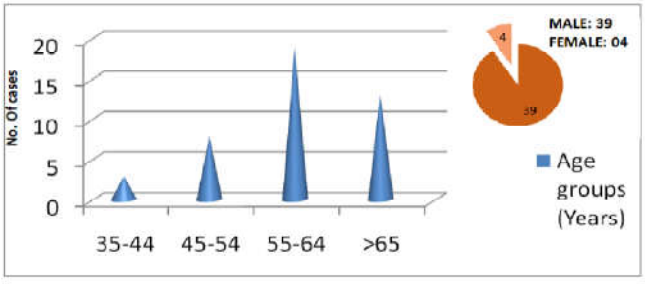


Figure 1: Age and gender wise distribution of cases

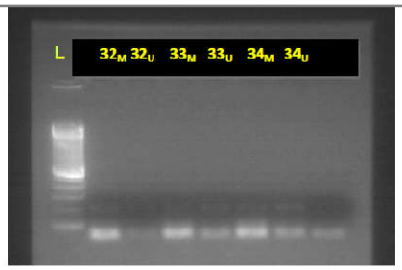
Amplicon size of PCR products:

For p15 gene: Amplicon size of M primer is 154 base pair (bp) and for U primer it is 162 bp.

For E cadhrin gene: Amplicon size of M primer is 206 base pair (bp) and for U primer it is 212 bp.

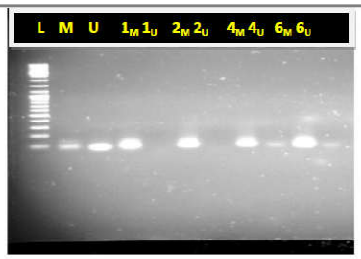
Promoter methylation in HNSCCs: Methylation specific polymerase chain reaction(MSP) was used to analyze the promoter methylation status of two genes (p15 and E-cadherin) from cancerous tissue and peripheral blood in 43 patients of HNSCC.

MSP results: PCR for the above mentioned two genes (p15 and E-cadherin) was carried out after extraction of DNA from tissue and peripheral blood. The results of the same are given in the Table 1 (Figure 2, 3, & 4). Samples showing amplification with 'M' primer were taken as 'methylated', including those showing amplification with both "M' and 'U' primers. Samples showing amplification with only 'U' primer was taken as 'unmethylated'.



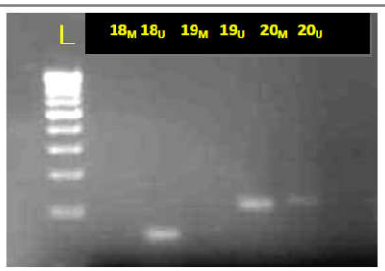
(L – 100 bp Ladder, M – M primer product, U – U primer product, 32 to 34 – tissue samples)

Figure 2: Gel Picture of tissue samples with p15 primers



(L- Ladder, M- Methylated control, U – unmethylated control, 1_M, 2_M, 4_M & 6_M peripheral blood samples with M primer, 1_U 2_U 4_U & 6_U – peripheral blood samples with U primer)

Figure 3: Gel Picture of peripheral blood samples with p15 primers



(L – DNA Ladder, 18 to 20 – tissue samples, M – M primer product, U – U primer product)

Figure 4: Gel Picture of tissue samples with E – Cadherin primers

p15 gene: 49% (21/43) tissue samples had shown amplification with M primers. 40% (17/43) peripheral blood samples had shown amplification with M primers. 12 cases out of 43 (28%) HNSCC showed methylation of p15 gene in both tissue and peripheral blood samples. 17 cases out of 43 (40%) HNSCC showed un-methylation in tissue and peripheral blood samples. Kappa coefficient calculated was 0.346 (95% confidence interval, 0.0695 -0.622) between methylation status in tissue specimens (21/43) and peripheral blood specimens (17/43), which showed fair correlation between them.

E – Cadherin gene: 63% (27/43) tissue samples had shown amplification with M primers. 44% (19/43) peripheral blood samples had shown amplification with M primers. 15 cases out of 43 (35%) HNSCC showed methylation of E-cadherin gene in both tissue and peripheral blood samples. 12 cases out of 43 (28%) HNSCC showed un-methylation in tissue and peripheral blood samples. Kappa coefficient calculated was 0.277 (95% confidence interval, 0.0129 -0.542) between methylation status in tissue specimens (27/43) and peripheral blood specimens (19/43), which showed fair correlation between them.

Methylation pattern in panel of genes: The results of the two genes were analyzed together to assess the utility of the panel of markers. The combined methylation pattern of the two genes analyzed together is shown in Table 2. 30% (13/43) tissue samples had shown amplification with M primers of both the genes. 51% (22/43) tumor tissues samples showed methylation of either p15 or E-cadherin. 19% (8/43) blood samples had shown amplification with M primers of both the genes. 47% (20/43) peripheral blood samples showed methylation of either p15 or E-cadherin.

Methylation pattern of genes (p15 and E-cadherin) in relation to site of tumors:

Methylation pattern of p15 gene: (Table 3)

69% (11/16) tissue samples taken from oral cavity showed amplification with M primer whereas 44% (07/16) peripheral blood samples from these patients showed amplification with M primer. 20% (01/05) tissue samples taken from oropharynx showed amplification with M primer whereas 60% (03/05) peripheral blood samples from these patients showed amplification with M primer. 54% (07/13) tissue samples taken from hypopharynx showed amplification with M primer whereas 38% (05/13) peripheral blood samples from these patients showed amplification with M primer. 22% (02/09) tissue samples taken from larynx showed amplification with M primer whereas 22% (02/09) peripheral blood samples from these patients showed amplification with M primer.

Methylation pattern of E-cadherin gene: ***(Table 4)***

56% (09/16) tissue samples taken from oral cavity showed amplification with M primer whereas 25% (04/16) peripheral blood samples from these patients showed amplification with M primer. 60% (03/05) tissue samples taken from oropharynx showed amplification with M primer whereas 60% (03/05) peripheral blood samples from these patients showed amplification with M primer. 69% (09/13) tissue samples taken from hypopharynx showed amplification with M primer whereas 62% (08/13) peripheral blood samples from these patients showed amplification with M primer. 67% (06/09) tissue samples taken from larynx showed amplification with M primer whereas 44% (04/09) peripheral blood samples from these patients showed amplification with M primer.

Amplification of both M and U primers in the samples:

p15 gene: Out of the 21 tissue samples which had shown amplification with M primer, 19 tissue samples also showed amplification with U primer. Out of the 17 peripheral blood samples which had shown amplification with M primer, 13 peripheral blood samples also showed amplification with U primer

E-cadherin gene: Out of the 27 tissue samples which had shown amplification with M primer, 20 tissue samples also showed amplification with U primer. Out of the 19 peripheral blood samples which had shown amplification with M primer, 11 peripheral blood samples also showed amplification with U primer.

peripheral blood specimen. In our study, we analyze methylation pattern of promoter region of two genes by using MSP and we observed promoter hypermethylation in both the genes (p15 and E-cadherin). Both the genes (p15 and E-cadherin) were methylated in head and neck cancerous tissues and peripheral blood specimens.

Table 1. p15 & E-cadherin MSP PCR results of HNSCC tissue & peripheral blood

		p15 in tissue (n=43)					E-cadherin in tissue (n=43)		
		M	U				M	U	
p15 in blood (n=43)	M	12 (28%)	5	17 (40%)	E-cadherin in blood (n=43)	M	15 (35%)	4	19 (44%)
	U	9	17 (40%)	26		U	12	12 (28%)	24
		21 (49%)	22	43			27 (63%)	16	43

Table 2. Panel of E-Cadherin & p15 genes results in HNSCC tissue & peripheral blood samples

Type of samples (n=43)	Number of cases showing amplification with 'M' primer in both genes	Number of cases NOT showing amplification with 'M' primer in both genes
Tissue	13	30
Peripheral blood	08	35

Table 3. Methylation pattern of p15 gene according to tumor sites

Tumor sites	HNSCC cases (n=43)	
	Tissue specimen showing methylation of p15 gene (n=43)	Peripheral blood specimen showing methylation of p15 gene (n=43)
Oral cavity (n=16)	11 (69%)	07 (44%)
Oropharynx (n=05)	01 (20%)	03 (60%)
Hypopharynx (n=13)	07 (54%)	05 (38%)
Larynx (n=09)	02 (22%)	02 (22%)

Table 4. Methylation pattern of E-cadherin gene according to tumor sites

Tumor sites	HNSCC cases (n=43)	
	Tissue specimen showing methylation of E-cadherin gene (n=43)	Peripheral blood specimen showing methylation of E-cadherin gene (n=43)
Oral cavity (n=16)	09 (56%)	04 (25%)
Oropharynx (n=05)	03 (60%)	03 (60%)
Hypopharynx (n=13)	09 (69%)	08 (62%)
Larynx (n=09)	06 (67%)	04 (44%)

DISCUSSION

Head and neck cancer is a highly diverse group of malignant disease and in developing countries it is sixth most commonly occurring cancer.[17,18] Head and neck cancer is a complicated disease with varied anatomical sites and different types of tumours that show diverse etiology and characteristics. It has been seen that both genetic and environmental factor has a role in etiology of the disease but the main pathogenesis is still unclear.[19] One of the major epigenetic changes in humans is methylation and it play a very important role in development of tumour.[20,21] Hypermethylation is commonly seen in CpG islands of promoter region or 5' end of genes.[21] Aberrant methylation of CpG islands in promoter region of a gene can lead to inhibition of transcription of the same gene. Increasing number of studies determining methylation status of the host gene in head and neck cancer tissue has come forward in the recent years. Recent advances in technology make it possible to analyze DNA methylation by highly sensitive and quantitative methods.[19] The detection of methylated genes from head and neck cancerous tissue specimen is technically feasible and represents a source for detecting potential biomarkers of relevance to head and neck carcinogenesis. This study was taken up to identify the utility of two genes singly and as a panel of genes, as molecular markers of epigenetic changes in early HNSCC. We used two genes p15 and E – Cadherin; and detected the methylation pattern of the promoter regions of these genes in head and neck cancerous tissue and

Methylation found in p15 gene:

Methylation in head and neck cancerous tissue specimens: Our study showed 49% (21/43) methylation in p15 gene in head and neck cancerous tissue specimens. In comparison to our study, other study done by Viswanathan M et al[11] observed less incidence/frequency of promoter methylation in p15 gene in HNSCC tissue specimens, which was 23% (12/51). In contrast to this Wong T. S. et al[22] showed higher incidence of hypermethylation in p15 gene in HNSCC tissue specimens, which was 60% (44/73). The discrepancy of p15 methylation may be due to sample selection random bias of small sample size. Other explanation could be that since methylation patterns of CpGs have been observed to be heterogeneous in their occurrence; *i.e.,* CpG sites methylated in one patient need not be the same as in another patient.[23] A true methylation profile could be generated by bisulfite sequencing, but this would be a cumbersome technique for studying a panel of genes in a large number of samples.[11]

Methylation in peripheral blood specimens: Kwong J et al[24], Wong T. S. et al[22] and Chang H. W. et al[25] observed p15 methylation in peripheral blood, which were 17%, 60% (12/20) and 20% (6/30), respectively. In comparison with their results we have observed 40% (17/43) p15 gene methylation in peripheral blood specimens, which was compatible with their results. 12/43 (28%) HNSCC cases showed methylation of p15 gene in head and neck cancerous tissue & peripheral blood specimens and the overall agreement by kappa coefficient was

0.346 (95% CI 0.0695 to 0.622), which showed fair correlation in methylation status of the two samples.

Methylation found in E-cadherin gene:

Methylation in head and neck cancerous tissue specimens: Viswanathan M et al[11], Chang H. W. et al[25], Nakayama S et al[26], and Saito H et al[27] observed E-cadherin gene methylation in head and neck carcinoma tissue, which were 35% (35/99), 53% (16/30), 94.4% (17/18) and 17% (9/52), respectively. In comparison with their results we have observed 63% (27/43) E-cadherin gene methylation in head and neck carcinoma tissue specimens, which was compatible with their results. The high frequency of methylation may be related to the advance grades and stages of HNSCC and may be due to different methods used for the detection of methylation in a particular gene.

Methylation in peripheral blood specimens: Chang H. W. et al[25] and Yonggang et al[28] observed E-cadherin gene hypermethylation in peripheral blood, which were 20% (6/30) and 45% (9/20), respectively. In comparison with their results we have observed 44% (19/43) E-cadherin gene hypermethylation in peripheral blood specimens, which was compatible with their results. 15/43 (35%) HNSCC cases showed methylation of E-cadherin gene in head and neck cancerous tissue & peripheral blood specimens and the overall agreement by kappa coefficient was 0.277 (95% CI 0.0129 to 0.542), which showed fair correlation in methylation status of the two samples.

Methylation pattern in panel of genes: Viswanathan M et al[11] studied 51 OSCC samples in which methylation status of all 5 genes (p16, p15, hMLH, MGMT and, E-cdherin) was analysed. 74.5% cases showed methylation for at least one of the gene, 40% for two of the genes and 16% for 3 of the genes. Only one sample showed methylation in 4 genes. Wong T. S. et al[22] studied 73 HNSCC samples in which methylation status of two genes (p16 and p15) was analysed. Methylation of both the genes was found in 24/73 (33%) of the tumour samples. 56/73 (77%) tumours showed methylation of either p15 or p16. We studied two genes (p15 & E-cadherin) as a panel in 43 HNSCC cases. 13/43 (30%) HNSCC tissue samples showed methylation in both the genes. 22/43 (51%) HNSCC tissue samples showed methylation in either of the gene. 8/43 (19%) peripheral blood samples from HNSCC cases had shown methylation of both the genes. 20/43 (47%) peripheral blood samples from HNSCC cases had shown methylation of either of the gene.

Methylation pattern of genes (p15 and E-cadherin) in relation to site of tumors: In our study, we found that irrespective of HNSCC tumour sites, tissue samples showed higher percentage of methylation in both the genes compared to peripheral blood specimens. Exception was seen in tissue samples taken from oropharynx (n=05), where methylation of p15 gene in tissue specimen was less, which was 20% (01/05) as compared to methylation in peripheral blood samples, which was 60% (n=03/05). This could be due to less number of samples taken from oropharynx in our study. Our results were comparable with the other studies done by Wong T. S. et al[22] and Viswanathan M et al[11].

Amplification of both 'M' and 'U' primers in the samples: In this study there were amplifications by 'U' primers along with 'M' primers in the methylated cases. 19/21 HNSCC tissue samples had shown amplification with both M and U primers for p15 gene promoter region. 13/17 peripheral blood samples from HNSCC cases had shown amplification with both M and U primers for p15 gene promoter region. 20/27 HNSCC tissue samples had shown amplification with both M and U primers for E-cadherin gene promoter region. 11/19 peripheral blood samples from HNSCC cases had shown amplification with both M and U primers for E-cadherin gene promoter region. Since methylation patterns are heterogenous by nature therefore they cannot be presumed to be either methylated or unmethylated at any one particular site. Therefore it is always beneficial to perform standard bisulphite sequencing on a target region before proceeding to MSP, which will ensure that the target CpG sites are fully methylated, and do not show any polymorphisms.

Conclusion

Identifying abnormal methylation events in malignancy is a promising avenue of research for developing tests which can be used for diagnosis, prognostication or treatment. This study was an attempt to assess the utility of epigenetics in the form of 'Methylation status of promoter regions of two genes (p15 & E-cadherin) in HNSCC cases and also to compare the methylation status of the two genes in cancerous tissue and peripheral blood of the same patients. So that non invasive specimen collection like peripheral blood can also be used for screening of HNSCC patients in a tertiary care centre. Our results suggest that identification of methylation status of these tumour suppressor genes in peripheral blood might be a useful tool in the future for HNSCC screening. However, prospective collection of blood specimens from cancer patients and a large scale population screening study are necessary to clarify the potential of methylated p15 and E-cadherin as tumour markers in HNSCC. The sample size in this study was small and we used only two genes in the study, for more significant contribution to the field of head and neck malignancy, more number of genes in more number of samples should be further analyzed in future.

Conflict of interest: None

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