DNA METHYLTRANSFERASE 1 (DNMT1) GENE ACTIVITY IN HUMAN LYMPHOMAS CORRELATES WITH ABERRANT p53 GENE EXPRESSION

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Background: The DNA Methyltransferase 1 (DNMT1) gene has been implicated as a mutagen for tumor suppressor genes by causing hypermethylation and subsequent TA mutations of CpG islands located in the promoter regions of these genes. The present study was undertaken to determine if increased DNMT1 gene activity correlated with increased aberrant p53 gene expression in human lymphomas. **Methods:** The study was undertaken on randomly selected archival human lymph nodes comprising 50 normal or reactive lymph nodes and 50 lymphoma lymph nodes. These were subjected to Fluorescent In Situ Hybridization (FISH) using oligonucleotide Antisense probes for the DNMT1 and the p53 mRNA according to standard FISH protocols. Percent cells stained, mean 'dots' stained per cell and staining signal intensity were taken as the criteria for comparing control and lymphoma lymph nodes. Quantitation of probe signals was done both by manual visualization of fluorescent signals and computer based image analysis. Correlation analysis was performed by calculation of Pearson's correlation coefficient. **Results:** Data indicated significantly increased expression of the DNMT1 and the p53 mRNA in lymphoma cases as compared to controls ($p<0.001$). Moreover significant correlation was obtained for the expressions of these two genes in lymphomas ($p<0.001$), but not in control lymph nodes. **Conclusion:** Increased DNMT1 gene activity may contribute to increased p53 gene expression in human lymphomas, supporting a mutagenic role for the DNMT1 gene.

Key Words: Fluorescent In Situ Hybridization, p53, DNA Methyltransferases, lymphomas.

INTRODUCTION

There are convincing reports of altered DNA methylation patterns in the genomes of malignant or transformed cells. Generally it is agreed that there is a global hypomethylation in malignant cells[1-3] accompanied by regional hypermethylation[4-6] and in most cases also an increase of the methylation enzyme DNA methyltransferase activity.[7-9]

Studies on malignant cells have shown that the tumor suppressor genes have been hypermethylated (hence inactivated) in a large number of tumors; there are reports also of hypomethylated tumor promoter genes.[10-13]

The group of haematological malignancies is no exception to abnormal methylation patterns.[14-24]

Hypermethylation with resultant silencing of p53 and its homologous p73 (tumor suppressor) gene expression has been reported in acute lymphoblastic leukaemia, Burkitt's lymphoma, non-Hodgkin's lymphoma, acute myelogenous leukaemia and multiple myeloma.[20,22,23] It is pertinent that there were no mutations or other changes in the gene in question and hypermethylation was considered the sole explanation for the observations.

An additional basis for transformation is provided not by an epigenetic change, but by the effects of methylation of the Cytosine bases themselves. It has been established that 5-Methylcytosine (5MeC) acts as a 'hot spot' for further mutations, so that it is changed to Thymine by spontaneous deamination and the subsequent addition of a keto group.[25-27] In one study it was found that 28% of mutations at the human p53 gene locus were due to C to T transitions at Cytosine-Guanine pair (CpG) dinucleotides located at the promoter regions of these tumor suppressor genes.[28]

Since DNA methylation is largely controlled by the DNA Methyltransferase 1 (DNMT1) enzyme[29-33] it is worthwhile to study if the gene expression of this enzyme correlates with aberrant (mutated) p53 gene expression in human lymphomas. This would provide preliminary evidence of involvement of the epigenetic changes of altered DNA methylation in p53 mutation.

MATERIAL AND METHODS

The study was conducted at the Department of Pathology, Ayub Medical College Abbottabad Pakistan and the Department of Biological Sciences, Quaid-i-Azam University Islamabad Pakistan from March to November 2003. Samples were archival paraffin blocks of normal / reactive and lymphoma lymph nodes processed in the Department of Pathology at Ayub Medical College Abbottabad Pakistan. A computer based random sample of all lymph nodes processed from January 2000 to December 2002 in the department was generated and 50 normal / reactive lymph nodes and 50 lymphoma

lymph nodes were randomly selected as controls and cases respectively after checking these samples for technical soundness.

All oligonucleotide probes were purchased from GeneDetect.com Ltd, Auckland, New Zealand. Antisense DIG-labelled DNMT1 Probe was synthesized using the sequence generated in the NCBI sequence viewer for Homo sapiens DNA (cytosine-5-)-methyltransferase 1 (DNMT1), mRNA; a 5434 bp linear sequence was generated. The oligonucleotide probe synthesized was a 48mer DNA fragment with the following sequence: TCTGTCCCAGCGTACCCCAGCCAGCTTGATC AGGTCCCGCATGCAGG, complementary to nucleotides 1973-2020 of NM-001379 and with a 96% sequence homology to nucleotides 1973-2002 by BLAST analysis. The Sense DIG-labelled DNMT1 control probe was supplied as part of the test probe kit, whereas the Antisense DIG-labelled PolydT probe was purchased from the same source separately.

The p53 Antisense Human specific, 48mer DNA probe had the following sequence: TCATGGTGGGGGCAGCGCCTCACAACCTCCG TCATGTGCTGTGACTGC. The probe was synthesized to hybridize to nucleotides 743-790 located within the coding sequence of the human (mutant) p53 mRNA. BLAST analysis revealed 96% homology with the Sense Strand of the mutant human p53 gene. The corresponding Sense Strand was supplied by the manufacturer as part of the test probe kit.

TSA™ Plus was purchased from Perkin Elmer Life Sciences, while other reagents and chemicals were purchased from DAKO Corporation and Sigma-Aldrich, Inc. VectaShield™ Mounting Medium was supplied by Vector Labs, Inc.

The laboratory protocol used for FISH was as described previously.[34] All areas of the sections were examined and mean percentages of cells stained positive over 10 random high power fields (x200 and x400) for each slide was recorded.

The number of stained 'dots' (speckled cytoplasmic staining) per cell was also taken as an index of staining. From 300-350 cells counted per slide, the mean spots per cell were calculated for each slide. The mean (± S.D.) spots per cell for control and lymphoma groups were also calculated for each probe.

The intensity of staining was recorded visually in 10 random high power fields (x400, x1000 oil). Staining intensity was categorized as low, medium and high.

For computer based image analysis, images were captured and analyzed by computer software Adobe Photoshop 7.0 as described previously.[34, 35]

The computer software SPSS version 8 was used for analysis. Differences were tested for by the Chi Square Test for qualitative variables and the Student's T test for quantitative variables. A p value ≤ 0.05 was considered significant.

RESULTS

Examination of the 50 control lymph nodes showed 44 (88%) with reactive changes while the remaining 6 (12%) were of normal architecture. Of the 50 lymphoma cases, 33 (66%) were of non Hodgkin's Lymphoma and 17 (34%) had Hodgkin's Lymphoma.

Figure 1 shows an example of a positively stained lymphoma lymph node, utilizing the p53 Antisense probe followed by TSA™ Plus signal amplification. Positive staining is seen as speckled 'dots' in the cytoplasm of lymphoma cells.

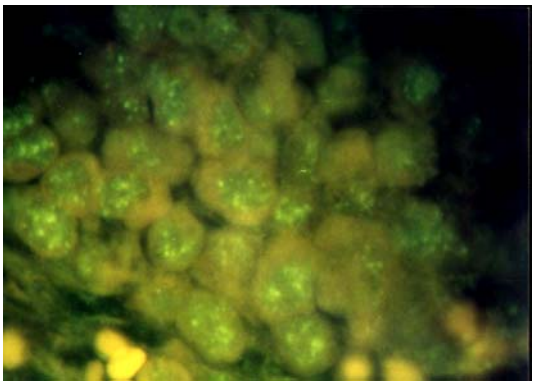

Fig-1: Lymphoma lymph node showing positively stained fluorescent speckles or 'dots' in cytoplasm of lymphoma cells (p53 Antisense probe, TSA™ Plus amplification, x1000, oil).

The mean percentages of cells stained for control and lymphoma lymph nodes are provided in Table 1.

Table-1: Mean percentages of cells stained for the control and lymphoma groups (n = 50 each)

Probes (mRNA)	Control Lymph Nodes Mean ± S.D.	Lymphoma Lymph Nodes Mean ± S.D.
Antisense PolydT	62.10 ± 7.01	66.30 ± 6.98*
Antisense DNMT1	14.20 ± 4.88	36.10 ± 15.53**
Sense DNMT1	4.70 ± 1.02	4.88 ± 0.72
Antisense p53	7.74 ± 2.39	33.70 ± 12.11**
Sense p53	6.16 ± 2.00	6.68 ± 2.23

*p=0.003 as compared to the control group value; p<0.001 as compared to the control and lymphoma Sense DNMT1 probe values.

**p<0.001 as compared to the control group value and the Sense DNMT1 and Sense p53 probe values.

There was an overall increased mRNA

expression in lymphoma cells compared to normal lymph node cells; similarly gene expressions of the DNMT1 gene and the aberrant p53 gene were significantly increased over their control counterparts.

Results for the mean 'dots' per stained cells are provided in Table 2. Significant differences are noted between the control and lymphoma cells for expressions of the PolydT, DNMT1 and p53 genes.

Table 2: Mean 'dots' (speckled cytoplasmic staining) per cells stained for the control and lymphoma groups (n = 50 each)

Probes (mRNA)	Control Lymph Nodes Mean ± S.D.	Lymphoma Lymph Nodes Mean ± S.D.
Antisense PolydT	8.84 ± 2.35	11.34 ± 3.80*
Antisense DNMT1	8.76 ± 2.51	11.30 ± 3.15*
Sense DNMT1	3.20 ± 0.20	3.38 ± 0.81
Antisense p53	8.90 ± 2.61	11.46 ± 2.77*
Sense p53	3.20 ± 0.73	3.26 ± 0.78

*p<0.001 as compared to corresponding control probe values, Sense DNMT1 and Sense p53probe values

Signal intensity was analyzed by computer based software with the results as shown in Table 3. The mean signal intensity histograms showed significant differences between the control and lymphoma lymph nodes for the PolydT, DNMT1 and p53 genes.

Correlation analysis was performed between the expressions of the genes of interest (DNMT1 and p53). It can be seen in Figure 2 that a highly significant linear correlation was present between the percent areas stained for the DNMT1 and the aberrant p53 genes in lymphoma lymph nodes.

Table 3: Distribution of computer based mean intensity histograms of cells stained for the control and lymphoma groups (n = 50 each)

Probes used	Control lymph nodes Mean ± S.D.	Lymphoma lymph nodes Mean ± S.D.
Antisense:		
PolydT	92.93 ± 24.26	104.40 ± 31.73**
DNMT1	89.75 ± 28.47	101.85 ± 28.17*
Sense DNMT1	77.01 ± 18.91	78.33 ± 22.57
Antisense p53	89.17 ± 24.97	105.10 ± 26.65†
Sense p53	81.15 ± 19.33	81.40 ± 23.88

*p=0.035 as compared to corresponding control value and p<0.001 as compared to Sense probe values

**p=0.045 as compared to corresponding control value and p<0.001 as compared to Sense probe values

†p=0.003 as compared to corresponding probe values and p<0.001 as compared to Sense probe values

Correlation graph of the percent areas stained with the DNMT1 and the p53 Antisense probes for control lymph nodes showed a positive linear correlation, which was not significant, with *r* being 0.236 and p=0.10.

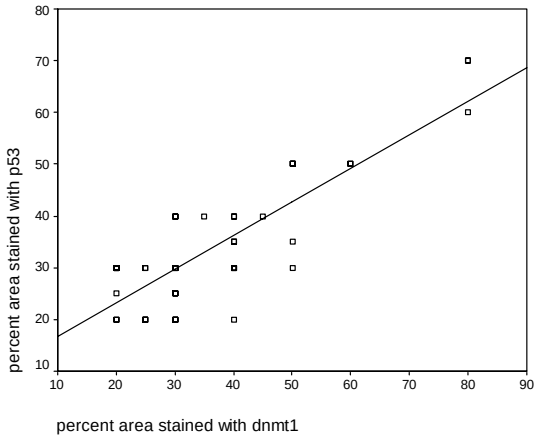

Fig-2: Correlation graph of the percent areas stained with the DNMT1 and the p53 Antisense probes for lymphoma lymph nodes (n = 50). A positive linear correlation is seen, which is highly significant with *r* being 0.832 and *p*<0.001.

DISCUSSION

Significant increases in expressions of the DNMT1 gene and the aberrant p53 gene were observed in lymphoma lymph node cells as compared to control lymph node cells, as judged by three parameters: mean percentage areas of cells stained (Table 1), mean 'dots' per stained cells (Table 2) and computer based intensity histograms of stained cells (Table 3).

It could be argued that increased expressions of the DNMT1 and p53 genes in lymphoma cells merely reflects an overall increased mRNA expression in lymphoma cells as judged by significantly increased PolydT expression (Table 1). If this were so, then for a ratio of 1:1.07 between control and lymphoma PolydT values, the ratios for control and lymphoma DNMT1 gene expression would not be 1:2.46, or about 2.5 times that expected if the increased DNMT1 followed the increased expression of PolydT in lymphoma cells. Moreover, DNMT1 gene expression forms 22.86% of the control lymph nodes' PolydT, while the corresponding figure for lymphoma nodes is 54.45% - an increase of 2.38 times (Table 1). For the p53 gene, the increase in ratio is 1:4.35 or an increase of over 4 times compared to the control lymph node cells; p53 gene expression forms about 12.5% of the control lymph node PolydT values and about 51% of the lymphoma lymph node PolydT values,

representing an increase of over 4 times in the lymphoma group.

Correlation analysis revealed a highly significant linear positive association between the gene expressions of the DNMT1 and the aberrant p53 genes in lymphoma cells (Figure 2); however there was no correlation between the expressions of these genes in control lymph node cells.

Taken together, these findings could be interpreted as implying an association between increased expressions of the DNMT1 and the aberrant p53 genes in lymphoma cells.

It cannot be said as to which was the primary event in our series of cases; however other evidence[7-9] points out that changes in DNA methylation and increased DNMT1 activity are initial events in carcinogenesis and bring about the early phenotypic changes that characterize transformed cells (initiation?). Persistent hypermethylation brings about further changes such as base mutations in tumor suppressor genes such as p53 (promotion?.)[25-27] It is plausible that the results of our series are an indicator of this sequence of events.

From the viewpoint of carcinogenesis, these findings imply two potentially oncogenic events occurring in lymphoma cells. It has been shown that hypermethylation resulting from increased DNMT1 gene expression is an independent event found in a number of haematological malignancies;[20-22] the presumed oncogenic mechanism is silencing of the tumor suppressor genes such as p53 or p73.

Paradoxically, increased DNMT1 activity has been related to DNA hypomethylation and indeed carcinogenesis, a fact that is explained by decreased levels of the methyl donor S-Methyl Adenosine (SAM) in some tumor cell types;[13] the presumed pathway is increased hypomethylation of promoter regions of tumor promoter genes in excess of the presumed hypomethylation of tumor suppressor genes. It appears then that DNMT1 controls methylation in both suppressor and promoter genes and other circumstances may well be determining factors for or against carcinogenesis, at least in some tumor types.

Aberrant p53 (and its homologues) is a hallmark of about 50-70% of malignancies in general.[36, 37] The role of p53 has also been studied extensively in human leukaemias and lymphomas. Despite some conflicting studies, the evidence points to a role for p53 mutations in the early stages of cell transformation in these malignancies. High levels of Tumor Promoter (TP)53 expression have been found in non Hodgkin's[38-43] and Hodgkin's Lymphomas[44, 45] as well as in Multiple Myelomas[46-48] with implications not only for tumorigenesis but also for disease prognosis[49, 50]

It would be worthwhile to pursue this line of evidence with further research studies exploring the quantitative changes in the DNMT1 gene expression, so that a differentiation profile of transformed and malignant cells from normal cells could be made; this difference could be useful in identification of early transformed cells (initiated cells?) and put to therapeutic advantage with tumor-cell directed selective targeting by drugs or other antineoplastic agents.[51-53] A similar quantitative profiling for aberrant p53 gene expression in body cells could also be used to identify and target cells 'committed' to malignant proliferation ('promoted cells').

A role for increased expression of DNMT1 gene as a mutagenic agent for p53 is plausible in light of earlier studies[28] as well as supported by the present study. It would also be worthwhile to study its role in causing mutations in other tumor suppressor or tumor promoter genes implicated in a variety of tumors. Thus not only would hypermethylation act as an initial event for transformation but persistent (faulty) hypermethylated states cause mutations in selective genes to promote final oncogenesis.

ACKNOWLEDGEMENTS

We gratefully acknowledge the help extended by Dr. Steve Marshall and Dr. Tasha Alexei, scientists at GeneDetect.com Ltd, Auckland, New Zealand, for procurement of essential reagents and chemicals needed for the laboratory work.

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