# INCREASED DNA METHYLTRANSFERASE 1 (DNMT1) GENE EXPRESSION IN HUMAN LYMPHOMAS BY FLUORESCENT IN SITU HYBRIDIZATION

**Iftikhar Qayum, Muhammad Ashraf***

**Department of Pathology, Ayub Medical College Abbottabad, *Department of Genetics, Quaid-i-Azam University Islamabad Pakistan.**

**Background:** The DNA Methyltransferase 1 (DNMT1) gene is among the better known 'epigenetic' systems that can regulate normal and abnormal gene expression as well as create 'hot spots' for DNA mutations. Its role has been studied in a number of malignancies with important implications for involvement in early events of malignant transformation. The present study describes the findings with respect to expression of this gene in human lymphomas studied by Fluorescent In Situ Hybridization (FISH). **Method:** The study was undertaken on randomly selected archival human lymph nodes comprising 50 specimens of normal or reactive lymph nodes and 50 specimens of lymphoma lymph nodes. These were subjected to FISH using oligonucleotide Antisense probes for the DNMT1 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 visualisation of the fluorescent signals and computer based image analysis. **Results:**Data indicated a significantly increased expression of the DNMT1 mRNA in lymphoma cases as compared to controls ($p<0.001$). **Conclusion:** This implies a possible role of the DNMT1 gene in transformation / oncogenesis in human lymphomas.

**Key Word**s: DNA methyltransferases, FISH, lymphomas.

## INTRODUCTION

Epigenetic mechanisms involving abnormal patterns of DNA methylation of both the tumor promoter and tumor suppressor genes are brought about by the primary DNA methylation enzymes, the DNA Methyltransferases (DNA-MTases).[1-4] The principal enzyme involved in major methylation events is DNA Methyltransferase 1 (DNMT1) which is involved primarily in maintenance methylation, while DNMT3a&b are primarily involved in de novo methylation; DNMT2 is currently not known to have any methylation activity though it has structural homology with the other DNA-Mtases.[5-8] DNA methylation involves the addition of methyl groups to the base Cytosine, preferably at Cytosine-Guanine pair sites or CpG islands.[1-5]

Many studies have implicated abnormal DNA methylation patterns in human malignancies, including human leukaemias and lymphomas.[9-12] The main findings seem to be hypermethylated tumor suppressor genes such as p53, p73, p15, p16, etc. along with global hypomethylation of DNA including the tumor promoter genes such as c-myc, c-ras, c-fos, c-jun, etc.

Increased DNMT1 activity has been reported from a number of human lymphomas and other malignancies as well.[2,13,14] It has been proposed that this increased DNMT1 activity has a role to play in early transformation and oncogenesis, as increased enzyme activity has been linked to possible hypermethylated status of tumor suppressor genes thus promoting oncogenesis.

Further evidence links methylated cytosines (5-MeC) to the later step of mutation. Methylcytosine is unstable and undergoes spontaneous deamination, after which a keto group is added to it and it is converted to Thymine.[2,6,15,16] Proofreading enzymes recognize this base change and the complementary base is changed from G to A. Thus a TA mutation occurs. In this way, the presence of hypermethylated CpG islands may act as a means of inducing DNA 'hot spots' for mutations as a further or final step in carcinogenesis.

Recent improvements in in situ hybridization protocols allow for quantitative assessment of gene expression by computer based image analysis after generating a signal by use of amplification systems, so that results comparable to the PCR based results can be obtained.[17, 18] This has facilitated use of in situ hybridization techniques [19, 20] which are often easier to perform than the PCR based ones in addition to providing additional data on cellular and tissue morphology not possible with the PCR based protocols.

## MATERIAL AND METHODS

The study was performed at the Department of Pathology Ayub Medical College Abbottabad Pakistan and the Department of Genetics 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 48 bp DNA fragment with the following sequence: TCTGTCCCAGCGTACCCCAGCCAGCTTGATCAGGTCCCGCATGCAGG. It was complementary to nucleotides 1973-2020 of NM-001379 and had 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. TSA Plus™ was purchased from PerkinElmer 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 derived from the manual supplied by the manufacturer of the probes and fluorescent staining systems. Briefly, 5-7µ sections were cut and prepared for FISH. Sections were then passed through steps of postfixation, blocking of endogenous peroxidase, acetylation, permeabilization, prehybridization with ISH buffer, followed by hybridization with probes and controls (PolydT and Sense probes); hybridization was done for 18-20 hours at $37^0$C in a moist chamber. Posthybridization steps included stringent washes, incubation with blocking reagent, incubation with anti-digoxigenin-HRP antibody, washing and incubation with TSA Plus™ reagent for signal generation and amplification. Slides were finally mounted for examination.

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 by a Hitachi Micro Color Camera or a Mercury 2.1 megapixel digital camera from 5-10 random high power fields (x1000 oil) and transferred to a computer hard disk for storage and analysis. Similarly photomicrographs were taken using an Olympus Microphotography Camera attached to a PM10-AD automatic exposure outfit; these were developed and scanned for feeding into the computer database.

The computer software Adobe Photoshop version 7.0 was used to develop histograms of signal intensity in order to quantify signal intensity as a correlate of the quantity of mRNA stained by probes and visualised by the TSA fluorescent system. A histogram of the intensity was generated which provided the mean intensity and the S.D. in arbitrary units ranging from 0 (dark) to 255 (full light). [17] The mean histogram value was calculated for all the images per slide and recorded.

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 $\leq 0.05$ was considered significant.

## RESULTS

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

Figure 1 shows positive staining of a diffuse small cell lymphoma lymph node cells with the Antisense DNMT1 mRNA probe. A characteristic speckled cytoplasmic staining is seen depicting sites of probe hybridization with cytoplasmic mRNA.

**Figure 1: A diffuse small cell lymphoma lymph node, stained positive with Antisense DNMT1 mRNA oligonucleotide probe, followed by TSA™ Plus signal amplification, showing speckled dots in the cytoplasm of lymphoma cells, 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 DNMT1 | 14.20 ± 4.88 | 36.10 ± 15.53* |
| Sense DNMT1 | 4.70 ± 1.02 | 4.88 ± 0.72 |
| Antisense PolydT | 62.10 ± 7.01 | 66.30 ± 6.98** |

*p<0.001 as compared to the control group value and the Sense DNMT1 probe values.
**p=0.04 as compared to the control group value; p<0.001 as compared to the control and lymphoma Sense DNMT1 probe values.

For Antisense DNMT1 probe in control lymph nodes, the mean percentage of cells stained was 14.20 ± 4.88. For lymphoma lymph nodes, the mean percentage of cells stained was 36.10 ± 15.53 (p<0.001). The differences between the control group Antisense DNMT1 values and the Sense DNMT1 values were also statistically highly significant (p<0.001); the difference of values between the lymphoma Antisense DNMT1 and the lymphoma Antisense PolydT probes were also highly significant (p<0.001). The difference of values for the control and lymphoma Antisense PolydT probes from their corresponding Sense DNMT1 probe values were highly significant p<0.001). Values for the Antisense PolydT probes between control and lymphoma groups were also significantly different (p=0.03)

Table 2 shows the distribution of mean 'dots' per cell for control and lymphoma groups. For the control group, the mean for the Antisense DNMT1 probe was 8.76 ± 2.51 dots per cell, while the value for the lymphoma group was 11.30 ± 3.15 dots per cell (p<0.001). Similarly the difference of the control and lymphoma Antisense DNMT1 probes from the corresponding Sense DNMT1 probes was highly significant (control group 8.76 ± 2.51 and 3.20 ± 0.70; lymphoma group 11.30 ± 3.15 and 3.38 ± 0.81) with a p<0.001.

**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 DNMT1 | 8.76 ± 2.51 | 11.30 ± 3.15* |
| Sense DNMT1 | 3.20 ± 0.20 | 3.38 ± 0.81 |
| Antisense PolydT | 8.84 ± 2.35 | 11.34 ± 3.80* |

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

The distribution of the intensity of staining as judged visually by fluorescence microscopy is depicted in Table 3. The differences between the two groups are not significant.

**Table 3: Distribution of visual intensity of cells stained for the control and lymphoma groups (n = 50 each)**

| Probes used | Control lymph nodes | | | Lymphoma lymph nodes | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| AntisenseDNMT1 | 30 | 17 | 03 | 30 | 18 | 02 |
| Sense DNMT1 | 35 | 15 | - | 35 | 13 | 02 |
| AntisensePolydT | 26 | 22 | 02 | 23 | 22 | 05 |

1 = low intensity, 2 = moderate intensity, 3 = high intensity

Results of the computer based image analysis histograms are shown in Table 4. For the control group, mean histogram values of the Antisense DNMT1 probe were 89.75 ± 28.47, whereas for the lymphoma group the corresponding value was 101.85 ± 28.17, giving a significant difference (p<0.035). The differences between the control and lymphoma Antisense DNMT1 probe values and the corresponding Sense DNMT1 probe values were highly significant (p<0.001).

**Table 4: Distribution of computer based mean intensity histograms of cells stained for the control and lymphoma groups (n = 50 each)**

| Probes used | Control lymph nodes<br>Mean ± S.D. | Lymphoma lymph nodes<br>Mean ± S.D. |
|---|---|---|
| AntisenseDNMT1 | 89.75 ± 28.47 | 101.85 ± 28.17* |
| Sense DNMT1 | 77.01 ± 18.91 | 78.33 ± 22.57 |
| AntisensePolydT | 92.93 ± 24.26 | 104.40 ± 31.73** |

*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

## DISCUSSION

The results support the observation that DNMT1 activity is significantly increased in lymphoma lymph nodes as compared to normal or reactive lymph nodes. This was evident from three main outcomes – the first being the significant differences between control and lymphoma lymph nodes in the mean percentage of cells staining positive by the Antisense DNMT1 mRNA probe (Table 1), the second being the significant differences in the number of stained 'dots' per cell between the two groups (Table 2) and the third being the significant differences between the two groups in their computer based signal intensity histograms (Table 4). The difference between the groups for visual assessment of staining intensity was not statistically significant (Table 3).

Increased gene expression of the DNMT1 gene in lymphoma cells could be taken as merely a reflection of increased total gene expression in these cells. However this is belied by the fact that for a ratio of 1:1.07 between control and lymphoma PolydT values, the ratios for control and lymphoma DNMT1 gene expression is 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).

This implies that more cells are being recruited during neoplastic transformation for synthesizing the enzyme DNMT1 and more DNMT1 is being synthesized, so that there may well be a plausible role for the enzyme in some step of oncogenesis. As the enzyme is known to be a powerful methylator of the base Cytosine, it would go along with the earlier observations of some studies that tumor cells have increased content of 5MeC in them as compared to non tumor cells of the same histogeneticorigin.[6,9-13] This observation could be linked further to the finding of hypermethylated CpG islands in tumor DNA in general and in regulatory areas of tumor suppressor genes in particular. Of interest is the finding that hypermethylated tumorsuppressor genes are found almost exclusively in tumor cells that contain adequate or high amounts of the methyl donor S-adenosyl methionine (SAM).[2] DNMT1 activity can be brought to a virtual stop by depletion of this primary donor for DNMT1 methylation activity, and this fact has been exploited by some researchers working on methods to decrease the effects of increased DNMT1 activity on tumor suppressor genes in tumor cells.[21-23]

Paradoxically, increased DNMT1 activity has been related to DNA hypomethylation and indeed carcinogenesis, a fact that is explained by decreased levels of SAM in some tumor cell types;[2] the probable 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 sets (suppressor and promoter) of genes and other circumstances may well be determining factors for or against carcinogenesis, at least in some tumor types.

Increased DNMT1 activity in the presence of low levels of SAM also triggers the development of mutational 'hot spots' by causing deamination of 5MeC to thymine.[2,6,15,16] Levels of DNA-MTase as high as 4- to 3000- fold have been reported in many tumor cells; [24] human colorectal adenomas showed a 60- to 200- fold increase in DNA-MTase activity despite a reduced content of 5MeC. [25] The demonstration of increased DNA-MTase activity is thus an essential prerequisite to any postulate of an 'epigenetic' pathway of growth control and may well function by selective hypermethylation of tumor suppressor genes or hypomethylation of tumor promoter genes depending on the biochemical conditions prevailing in each particular cell type at the time of neoplastic transformation.

The present study thus supports the contention that significant differences in normal and lymphoma lymph node cells exist in terms of their DNMT1 activity and that this mechanism may well be operating in human lymphomas.

FISH followed by computer based image analysis for quantitation of the staining signal intensity appeared to be effective in quantitative analysis of the differences between the control and lymphoma group (Table 4). This is more significant when compared with the assessment of signal intensity by visual observation through the microscope, which produced non-significant results between the two groups (p = 0.15). Thus visual assessment cannot be relied

upon to categorize or grade signal intensity reliably, perhaps because of its subjectivity and decreased sensitivity of the human eye to subtle differences in fluorescent signal intensities. Computer based image analysis is a sensitive technique that can detect differences between groups and should be used in preference to visual assessment for semi-quantitative assessment of signal intensity, as also in the study by Lehr et al.[17]

The use of in situ hybridization techniques along with modern imaging methods may bring about a useful shift from PCR based strategies to detect differences of gene expression between normal and abnormal tissues. The technique has advantages over PCR based methods for histopathologists and cancer researchers and now that the hurdle of quantitative analysis appears to be reducing, it is expected that this technique will replace the PCR based methods to a large extent, or preferentially in early studies to detect differences by this relatively simpler method. The use of enzyme based signal generating systems coupled with computerised image analysis will further augment work in this area, as slides can be stored without deterioration for longer time periods, allowing more detailed or sequential studies to be conducted at later dates.

## CONCLUSION

Significantly increased DNMT1 expression appears to be a feature of human lymphoma cells and may have a role to play in oncogenesis in this group of tumors. Furthermore in situ hybridization followed by computer based image analysis offers a reliable and useful method to study the large number of genes involved in normal or abnormal cellular processes.

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**Address For Correspondence:**
**Dr. Iftikhar Qayum,** Department of Pathology, Ayub Medical College, Abbottabad 22040, Pakistan.
**Email:** iqayum@brain.net.pk