Evaluation of MLL2 (KMT2B) Gene Expression in Colorectal Cancer Tissue Compared with Adjacent Tumor Free Tissue

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\begin{abstract}
\textbf{Background and Aims:} An impairment of expression in MLL2 gene involved in histone methylation induces alteration in the methylation patterns at enhancers and causes several cancers including colon cancer. In this study, MLL2 expression was evaluated in North West of Iran.

\textbf{Materials and Methods:} To evaluate the MLL2 expression in patients with colon cancer, 25 tumor samples and 25 samples of tumor margins were collected and analyzed by real-time polymerase chain reaction.

\textbf{Results:} KMT2B level was not associated with age (p=0.3), gender (p=0.05) and tumor size (p=0.2) but had relation with stage of tumor (p=0.04).

\textbf{Conclusions:} According to the results obtained from investigating the MLL2 gene expression, it was found that it has a significant relationship with the stage of tumor.
\end{abstract}
Introduction

Colorectal cancer (CRC) is the fourth leading cause of cancer-related death in Iran [1]. The process of tumor formation is linked with gene mutations [2, 3], deletions or translocations [2, 4]. Apart from genetic harms which are inheritable, the epigenetic changes do play a crucial role in CRC progression [5].

Epigenetic changes are defined as inheritable alterations which occur in the genetic material due to chemical modifications [6] in a way that they do not involve changes in the primary gene nucleotide sequence [7]. These changes may affect chromatin structure and function and alter connection between DNA strands with histone proteins [5, 8]. In eukaryotes, DNA and histone proteins are established into nucleosomes, which, in turn, form the higher-ordered structure of chromatin [9]. The amino-terminal tails of histone proteins are subject to posttranslational modifications [10]. Posttranslational modifications of histone tails regulate chromatin structure and transcription. Epigenetic changes can be divided into several groups, including methylation, ubiquitination, acetylation and the like [11, 12]. Each change can increase or reduce genes expressions, and can therefore affect cellular processes and cause various diseases including cancer [6, 7]. Methylation refers to the transfer of methyl groups on the central histones of nucleosome structure and DNA (without altering the sequence) [10]. Regulation of chromatin accessibility through chromatin remodeling and histone modification (such as methylation and acetylation) is a critical step in regulating eukaryotic gene transcription [13]. Enzymes that transfer methyl group on lysine or arginine residual in the tail of histones are called histone lysine or arginine methyltransferases [10, 14]. lysine may be mono-, di-, or tri-methylated, and arginine residues may be symmetrically or asymmetrically methylated [15]. They comprise a SET domain catalyzing the addition of methyl groups to specific lysine remnants [9, 16]. The mix lineage leukemia (MLL) or KMT2 family exists in multi-protein complexes and is involved in a variety of processes such as normal development and cell growth [6]. Although some of the MLL family members have already been described to be involved in cancer, MLL2 is one of the members of the KMT2 enzyme family whose contribution to cancer is more common than other components of this enzyme family [17]. Histone methylation can result in activation or repression of genes expression. Recent studies have shown evidence that MLL2 (KMT2B) is involved in methylation of the histone H3K4 at enhancers [18], however, H3 lysine 4 methylation (H3K4me) is associated with transcriptional activation and leads to increased expression of genes involved in the transcription [19]. Hence this could be a contributing factor in various cancers including colorectal ones. Several studies conducted on MLL2 gene imply its association with other genes, especially genes involved in cellular processes [20]. KMT2B gene expression has been studied in various cancers the results of which are as follows.

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an investigation conducted at the level of protein and transcription in 2010, Natarajan and his research group found that the level of MLL2 gene increases in the cell line of breast cancer [3, 14]. According to the research carried out by Yin on the situations of this gene on patients with lung cancer, decreased expression of MLL2 gene was resulted [16].

Materials and Methods

Sample collection
Samples were collected from Imam Reza and Shahid Madani Hospitals in a period of one year. The samples were discharged in micro tubes that were first placed Diethyl pyrocarbonate (DEPC) then were transported to liquid nitrogen in the laboratory at -80 degrees fridge until extraction. Colonoscopy of tumor samples were also transported to the laboratory in the formalin to search for pathological results: For each sample, the pathologic findings, the ethical and protecting confidential patient information were collected from the pathology lab. Patients with CRC as determined by histopathological evaluation were not on any treatment program at that time.

RNA extraction from samples had to be performed in an environment free from RNAse. Therefore, special care was taken to avoid contamination of utensils and surfaces with RNase enzymes. To deactivate the RNase enzyme, DEPC-treated microtubes were used.

RNA extraction process
Samples of tumor and tumor margin were extracted by using RNA xPLus kit (sigma company), followed by five steps. Step one: putting up 1000 microliter RNA xPlus [21] and incubating for 5 min; step two: putting up 250 microliter chloroform, centrifuged with 13500 rpm for 15 min, step three: adding 500 microliter Isopropanol, and then incubating for 20 min, and centrifuging with 12000 rpm for 15 min; step four: adding alcohol 75% and centrifuging with 7500 rpm for 8 min; and step five: putting up 15 microliter DEPC water and keeping them in a -80 degrees fridge.

Electrophores gel analysis
In order to check and ensure the extraction process, agarose gel was made. Results in a column with three bands, corresponding to 28s, 18s, 5s were found on the gel. Standard curve of MLL2 with set primers to measure performance of real-time reaction was drawn. The serial dilutions were prepared from cDNA with concentration of (0.1, 0.01, 0.001, and 0.0001).

Real-time polymerase chain reaction (PCR)
PCR test was performed to determine expression of MLL2 in colorectal cancer patients. RNA was DNaseI treated in a 10 μl volume using the takara Kit and then reverse transcribed in the same tubes using combination of oligo (dT) and random hexanucleotide primers. Real-time PCR (10 μl) was performed using the SybrGreen 480 Master Mix that contained 1 μl of 1:5 dilution of complementary DNA (cDNA) and 0.3 μM of each primer. Samples were repeated three times in each well. Temperature cycling conditions were as follows: 1 cycle of 95°C for 10 min., 45 cycles of 95°C for 25 seconds (s), 61°C 30 s, and 72°C 30 s. Mix lineage leukemia (MLL2) and GAPDH (housekeeping gene) primers were respectively as follows:
(forward 5′ CGTGGATCCAAGCACCTCCT 3′,
reverse 5’ TCTTACAGCGCACACAGGCT 3’,
(product: 135 bp);
(forward 5’ TGTGAACCATGAGAAGTAT 3’,
(reverse 5’ CACGATACCAAAGTTGTC 3’,
(product 112 bp). Primer design was done
by the Gene runner software. The Ethics
Committee of Tabriz University of Medical
Sciences approved this study.

Statistical analysis
Statistical analysis of MLL2 expression was
performed by unpaired Student’s t-test and two-
way ANOVA when appropriate. The graphs
were generated using SPSS software.

Results
Real-Time analysis result
Statistical analysis indicated that MLL2
expression was not significantly related
between normal and tumor samples (Table 1).
The linear correlation of MLL2 expression
and different factors of age, gender and tumor
size (p=0.3, p=0.05 and p=0.2 respectively),
revealed no statistical significance. However
the stage of tumor showed significant result
(Table 2).

<table>
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<th>Kind of tissue</th>
<th>Mean±SD (%)</th>
<th>Fold Change</th>
<th>P-value</th>
</tr>
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<tr>
<td>Tumoral tissue</td>
<td>0.25±30.50</td>
<td>2.25</td>
<td>0.1</td>
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<tr>
<td>Normal tissue</td>
<td>0.05±31.70</td>
<td>0.1</td>
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</table>

<table>
<thead>
<tr>
<th>Stage</th>
<th>P-value</th>
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<tbody>
<tr>
<td>Stage I</td>
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<tr>
<td>Stage II</td>
<td>0.71</td>
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<tr>
<td>Stage III</td>
<td>0.04</td>
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Discussion
To obtain a global perspective on histone
methylation at Lys 4 expression patterns
in tumoral and normal cells, Real-time
PCR technology was used. Confirming and
extending prior conventional studies, it was
found that histone methylation at the MLL2
was not related to greater expression of
internalizing symptoms except for the stage of
tumor. In a research carried out by Prives
and Lowe, MLL pathways were associated
with GOF p53 oncogenic phenotypes and
therefore lead to cancer progression [22]. The
correlation of MLL2 with multiple cellular
signaling pathways suggested the potential
mechanisms underlying tumorigenesis mediated
by MLL2 alterations. These studies are
in line with our study supporting the role of
mutant MLL2 in driving tumor progression.
In contrast, no significant difference was
observed between patients with relatively high
or low MLL2 expression in tumor samples.
Ladopoulos’s observations found that the
KMT2B knock down was followed by the decrease of the active chromatin marks and progressive loss of RNA polymerase II binding [23]. Studies by Albert and Helin have shown that expression of KMT2B gene directly connects to transcription factors of specific promoters or enhancer areas [24]. Zhu and coworkers observed that when p53 suffered a missence mutation, it led to an increase in the expression of MLL2 and moz genes. However, in the normal state, it fails to reduce the putative expression of these genes [25]. The findings and approaches undertaken here can lay the basis for further understanding of the function of MLL2 and provide implications for further research on the epigenetic regulator genes that are found to be altered in cancers.

**Conclusion**

According to the results, investigating the MLL2 gene expression indicated a significant relationship with the stage of tumor.

**Conflict of Interest**

There are no conflicts of interest or financial involvement with this manuscript as confirmed by all authors.

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**References**


