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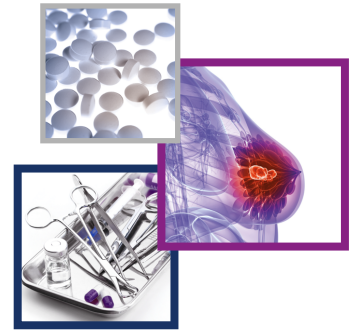
Breast Cancer Management



Study of *KMT2B* (*MLL2*) gene expression changes in patients with breast cancer

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Aim: This study aimed to demonstrate misregulation of *KMT2B* gene expression in breast cancer tissue. **Materials & methods:** Cancerous and marginal tissue samples were collected from 43 female patients. After RNA extraction and cDNA synthesis, quantitative-PCR was used to evaluate the expression level of the *KMT2B* gene. REST, Sigma plot and SPSS software were used to analyze data. **Results:** *KMT2B* gene expression was significantly decreased in tumor tissue compared with marginal tissue ($p = 0.02$). No significant correlation was found between expression levels of *KMT2B* and clinical parameters of patients ($p > 0.05$) **Conclusion:** Our study demonstrated that downregulation of *KMT2B* is associated with breast cancer and its misregulation may play an important role in tumorigenesis.

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Keywords: breast cancer • epigenetic • gene expression • *KMT2B*

Breast cancer is a heterogeneous disease which makes it resistant to clinical therapies [1]. Therefore, it is necessary to carry out extensive research to identify new molecular mechanisms in order to reach a successful therapy based on molecular markers. Until now, multiple types of genetic and molecular pathways have been identified in breast cancer. Mutation of *BRCA1* and *BRCA2* genes, which have a role in integrity of genome, is 80–90% of hereditary breast cancers but these mutations rarely occur in sporadic cases [2]. The prevalent mutant genes that are shared in primary developed and metastatic sporadic breast cancers are *TP53*, *PIK3CA*, *GATA3* and *ERBB2*. There are also metastatic specific mutant genes such as inactivation of *ARID1A*, *ARID1B* and *ARID2* (SWI/SNF chromatin remodeling complexes) that are rarely influenced in primary development of breast cancers [3]. Such information can help researchers to breakdown breast cancer in precise molecular subdivisions to help make a more reliable prognosis and determine therapeutic proceedings.

So far, most categorization of breast cancer subtypes is based on immunohistochemical analysis of estrogen receptor (ER), progesterone receptor (PR), human EGFR-1/2 (HER1/2) and cytokeratin (CK) 5/6. According to these markers, breast cancer can be subdivided into four groups: Luminal A (ER^+/PR^+, $HER2^-$), Luminal B (ER^+/PR^+, $HER2^+$), $HER2^+/ER^-$ and basal-like or triple negative (ER^-, PR^-, $HER2^-$, $HER1^+$ or CK $5/6^+$). These markers are targets of drugs such as tamoxifen and trastuzumab that are routinely used in clinical treatments [4]. However. triple negative breast cancers are defined by the lack of expression of ER, PR and HER2, and therefore are resistant to these marker targeting therapies. Instead they are often involved in upregulation of growth hormone signaling pathways which make them sensitive to treatments that target DNA-repair pathways such as platinum compounds and growth factor receptors such as anti-EGFR monoclonal antibodies or TGF-β receptor kinase inhibitors [5]. Due to the critical role of epigenetic disregulation in cancer, researchers seek epigenetic pathways that are involved in breast cancer. It has been shown that TGF-β, by inducing epithelial–mesenchymal transition, causes the increase of global H3 lysine 4 trimethylation (H3K4me3) and reduction of H3 lysine nine trimethylation (H3K9me2) histone markers which leads to metastatic behavior of epithelial cells [6]. A recent study of the genome-wide H3K4me3 and H3K27me3 in human mammary epithelial cells and three subtypes including luminal, HER2, and basal-like cells showed each of them have unique patterns of H3K4me3 and H3K27me3 in target sites and as a result lead to disregulation of subtype-specific target genes. In addition, it showed a weak level of H3K4me3 marker

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in promoters of ER, PR and HER2 at basal-like cells but a high level at HER2 in luminal and human mammary epithelial cells [7]. Because the most probably cause of disorder in such H3K4me3 patterns is due to abnormal expression or function of H3K4 methyltransferases, investigation of dysregulation of H3K methyltransferase genes in breast cancer is an important step in identifying one of bottom-most mechanisms of deregulation for many oncogenic and tumor suppressor genes.

In this study, we assayed the expression changes of *KMT2B* (*MLL2*; also known *MLL4*) as a histone methyltransferase gene in patients with breast cancer and its relationship with pathological parameters. KMT2B as a member of histone-lysine methyltransferase 2 (KMT2; also known as mixed-lineage leukemia [MLL]) family, has a methylation role on H3K4 at promoters of target genes which causes them to gain access to the relevant transcription factors [8]. Other members of this family are *KMT2A* (*MLL1*), *KMT2C* (*MLL3*), *KMT2D* (*MLL4*; also known *MLL2*), *KMT2E* (*MLL5*), *KMT2F* (*hSET1A*/*SETD1A*) and *KMT2G* (*hSET1B*/*SETD1B*) genes. Aside from KMT2F, which has non-methyltransferase function, other members of this family have the same methyltransferase role on H3K4 [9]. It is shown that members of this family alone have weak activity that needs to be reinforced, they need to make a multisubunit complexes with multiple proteins such as WDR5, RBBP5, ASH2L and DPY30 as well as other unique proteins including Menin and LEDGF for KMT2A/B, PTIP and NCOA6 for KMT2C/D and, finally, CFP1WDR82 for KMT2F/G. It is suggested that these unique proteins guide KMT2 members to specific regions [10]. *KMT2* family members are among the most mutated genes in cancer. However, few studies of *KMT2D*, *C*, *A*, *F* and *G* have been carried out in breast cancer. *KMT2C* and *D* genes act as tumor suppressor genes because they are inactivated in many cancers; it has been shown that expression of *KMT2C* is reduced in breast tumor tissues [11]. However, recent studies have also shown that KMT2C and KMT2D have important roles in progression of ER^+ breast cancer. For instance, by monomethylation of ER target loci enhancers *KMT2C* is necessary for the amplification of ER positive cancer cells and the knockdown of *KMT2C* gene leads to the suppression of estrogen-dependent genes [12]. On the other hand, KMT2D makes resistance of ER-positive breast cancer patients to PI3Ka inhibitors. Normally AKT attenuates its function in ER target sites by the phosphorylation of KMT2D. It has been demonstrated that the inhibition of PI3Ka causes inactivation of AKT and leads to overactivation of *KMT2D* in ER target loci [13]. These findings could lead to a new, promising treatment by designing drugs against KMT2C and D. The genes of *KMT2F* and *KMT2G* have been also assayed in breast cancer. The expression of *KMT2F* is increased in metastatic breast cancer cells and tumor tissues compared with normal breast tissues [14]. Also, overexpression of *KMT2F* leads to survival and metastatic behavior of ER positive cells by recruiting the ER target genes and its knocking down suppresses proliferation of tamoxifin-resistant MCF-7 cells [15]. In contrast, the cytoplasmic form of KMT2G by interacting with BOD1 leads to survival of triple-negative breast cancer cells with its knockdown resulting in the overexpression of the *AdipoR1* tumor suppresser gene and inhibition of colony formation [16]. Despite *KMT2A* rearrangements in leukemia and lymphoma, it has important roles in solid tumors as well as breast cancer. Overexpression of KMT2A activates expression of *HIF1α*, *VEGF* and *CD31* genes in tumors and leads to angiogenesis and tumor growth [17]. Gain of function mutations of *TP53*, by overexpression of *KMT2A*, *KMT2D* and *MOZ* genes, increases genome wide methylation and acetylation, resulting in breast cancer progression [18]. The research of *KMT2B* gene in breast cancer is very limited. *KMT2B* is located on chromosome 19q13.1, a location shown to be amplified in pancreatic carcinoma and glioblastoma cell lines [19]. It appears that KMT2B has an important role in cancers; however, reports investigating the dysregulation of *KMT2B* in breast cancer are still limited. Therefore, our aim was to investigate *KMT2B* gene expression changes in mammary cancer and marginal free tumor samples of patients from northwest of Iran. Also, for the first time, we analyzed the association of this gene expression changes with clinicopathological parameters including age, stage, side, grade, tumor size, number of involved lymph nodes and ER/PR/HER2 markers.

Materials & methods

Tissue specimens

In this study, 43 fresh samples of surgically removed tumorous tissues and 43 surgically marginal breast tissues were collected from female patients at the Nournejat hospital of Tabriz, Iran. The samples were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction. For recognition and characterization of tumorous tissues, pathological examination was carried out on the basis of histological and molecular traits. All of selected samples were from patients without a family history of cancer. They also did not have any underlying diseases such as diabetes or hypertension. Until their surgical removal and sampling, they had not undergone chemotherapy or radiotherapy and were drug naive.

Primer designing

Primers were designed by using Gene Runner software. *KMT2B* primers sequences were forward 5′- *CGTGGATCCAAGCACCTCCT* -3′ and reverse 5′- *TCTTACAGCGCACACAGGCT* -3′. *GAPDH* primer sequences were forward 5′- *GAGAAGTATGACAACAGCCTC* -3′ and reverse 5′- *TGAGTCCTTCCACGATACC* -3′.

RNA extraction & cDNA synthesis

All RNA was extracted by using RNX-plus as a RNA extraction kit, according to manufacturer's protocol (Cinagen, Tehran, Iran). The quality and quantity of RNA was assessed respectively by 1% agarose gel electrophoresis (detection of 18S and 28S rRNA bonds) and UV spectrophotometer (Picodrop, Cambridge, UK) at the absorbance of 260/280. Single-stranded complementary DNA was synthesized from total RNA using TAKARA kit (Japan) containing oligo dT and random hexamer primers, and reverse transcriptase.

Quantitative PCR

Reactions of qPCR were performed on a real-time PCR system (Illumina, CA, USA) by using SYBR Green MasterMix (Amplicon, Odense, Denmark), according to the manufacturer's instructions. After mixing 5 µl MasterMix, 0.4 µl primer mix and 3.6 µl H_2O together for each reaction, 1 µl of each sample template was added to a 10 µl final reaction volume per well. Each assay was carried out in triplication for each sample. Amplification conditions for both *KMT2B* and *GAPDH* (as a endogenous reference gene) were as follows: 10 min at 95°C on holding stage and then 40 cycles of 25 s at 95°C, 30 s at 61°C and 30 s at 72°C with a final holding stage of 3 min at 72°C. Values of gene expression levels for tumorous tissues are reported as ratios between amplification levels of interest gene and that of reference gene as a normalization factor for the amount of RNA extracted from each tissue. This was subsequently normalized with the ratios obtained from marginal (control) samples.

Statistical analysis

The expression levels of *KMT2B* in breast cancer tissues relative to adjacent nontumor tissues were analyzed using REST software. The association between *KMT2B* expression and clinicopathological parameters was analyzed using the SPSS software by parametric t-test and one-way ANOVA. The p-values of ≤ 0.05 were considered statistically significant, and data were shown as means ± standard deviation. Moreover, receiver operating characteristic (ROC) curves was constructed to evaluate the specificity and sensitivity of predicting breast cancer and normal tissue by *KMT2B*, and the sensitivity/specificity at various cutoff values was calculated using SigmaPlot 12.5.

Results

Expression of *KMT2B* in breast cancer tissues & adjacent normal tissues

KMT2B expression levels were evaluated using qRT-PCR. The results of REST analysis showed that *KMT2B* expression levels were significantly downregulated in breast cancer tissues compared with marginal tissues (95% CI: 0.110–2.551; $p = 0.02$; Figure 1).

Association between expression levels of *KMT2B* & clinicopathological results

Data related to clinicopathological features was prepared and the associations of *KMT2B* expression levels and clinicopathological features, including age, stage, grade, side, tumor size, number of involved lymph nodes, ER, PR and HER2, were assayed. However statistical analysis demonstrated that *KMT2B* expression was not significantly associated with the clinicopathological features ($p > 0.05$; Table 1).

Capability of KMT2B to function as a breast cancer tumor marker

The analysis of ROC curved *KMT2B* expression showed an AROC of 0.63 ($p = 0.091$), indicating that this gene is likely a weak candidate for use as a biomarker (Figure 2).

Discussion

Studies have shown that epigenetic alterations play a crucial role in various human diseases, including cancer. Since epigenetic alterations occur in early stages of tumor evolution, the identification of epigenetic enzyme misregulations has undeniable effects on early detection, prevention and treatment response of cancer [20]. Mutations or expression changes in methyltransferase genes can cause methylation abnormalities in cancers [21]. Studies reported that KMT2 families are involved in different cancers. For example, *KMT2C* and *KMT2D* mutations are prevalent

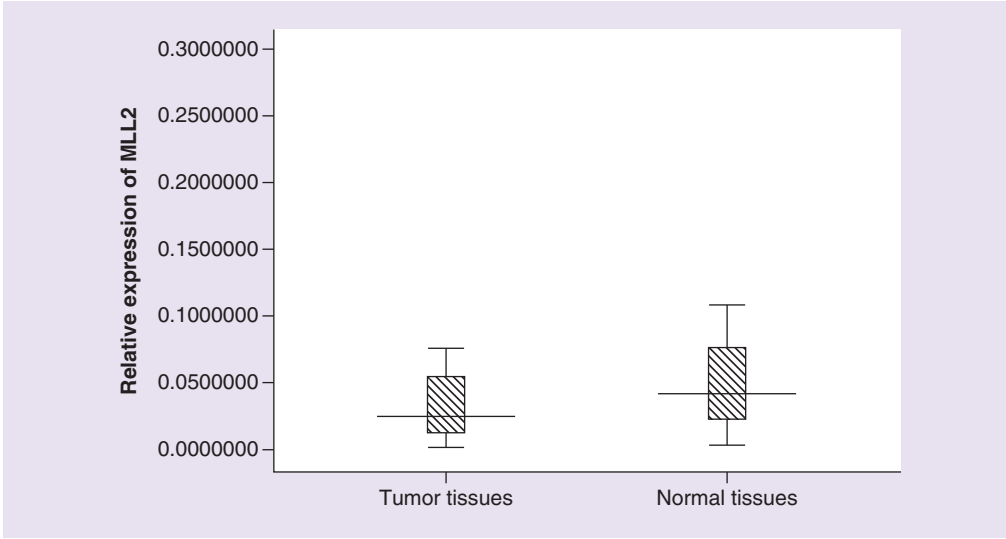

Figure 1. Misregulation of *KMT2B* in tumor and adjacent tissues.

Table 1. *KMT2B* expression and clinicopathological characteristics of patients with breast cancer.

Variable	N	*KMT2B* relevant expression ($2^{-\Delta ct}$) mean ± standard deviation	Statistical significance	p-value
Age (years)				
– <51	21	0.045 ± 0.046		
– ≥51	21	0.043 ± 0.059	NS	0.915
Tumor size (cm)				
– <2.5	18	0.048 ± 0.061		
– ≥2.5	18	0.036 ± 0.037	NS	0.475
Side				
– Right	18	0.040 ± 0.041		
– Left	23	0.046 ± 0.062	NS	0.695
Lymph nodes				
– <3	26	0.043 ± 0.055		
– 3–10	9	0.052 ± 0.065	NS	0.867
– >10	7	0.038 ± 0.025		
Stage				
– I	15	0.044 ± 0.0642		
– II	11	0.042 ± 0.0426	NS	0.982
– III	16	0.046 ± 0.0507		
Histological grade				
– Poorly differentiated	9	0.038 ± 0.0248		
– Moderately differentiated	26	0.031 ± 0.0322	NS	0.478
– Well differentiated	4	0.052 ± 0.0545		
ER (%)				
– <30	16	0.027 ± 0.0221		
– >30	16	0.055 ± 0.0648	NS	0.113
PR (%)				
– <30	19	0.042 ± 0.0580		
– >30	13	0.041 ± 0.0365	NS	0.953
Her2				
– Positive	8	0.024 ± 0.0263		
– Negative	24	0.047 ± 0.0546	NS	0.269

ER: Estrogen receptor; NS: Not significant; PR: Progesterone receptor.

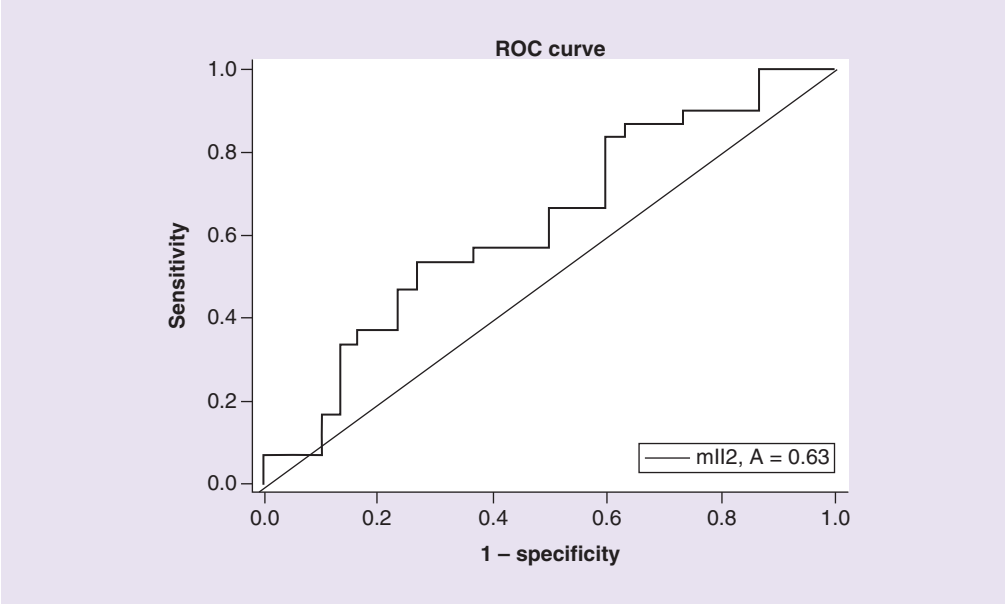

Figure 2. Receiver operating characteristic curve.

in lung, large intestinal, breast and endometrial cancers [9]. *KMT2A* and *KMT2B* share similar domains in both structure and function. Despite the similarity, they are directed to nonoverlapping target genes [22]. Studies have shown that mutations and rearrangements of *KMT2A*, as well as human leukemias, plays a role in development of some solid tumors such as colon, lung, bladder, endometrial and breast. Also, mutations of *KMT2B* have been reported in endometrium, large intestine, lung, brain and liver cancers [9]. The recent studies of the various *KMT2* families in larynx carcinoma and clear cell renal cell carcinoma stages showed an insignificant downregulation of *KMT2B* gene expression in larynx carcinoma; however, no significant correlation between *KMT2B* expression was found in clear cell renal cell carcinoma stages [23,24]. In addition, studies in five members of the *KMT2* family (*KMT2A*–*KMT2E*) in breast cancer cell lines and just eight tumorous breast tissues showed diminished expression of these genes compared with nontumor tissues [25]. Our studies of *KMT2B* gene expression on 43 tumorous tissues compared with nontumorous marginal tissues showed the significant downregulation of *KMT2B* and its association with breast cancer. Although overall mechanisms in which *KMT2B* downregulation contributes to cancer are unknown, one probable mechanism is the decline in recruitment of the KMT2B protein to CpG island promoters. KMT2B proteins contain several evolutionary conserved domains such as SET domain, which is responsible for methyltransferase action of KMT2B [26], and the CxxC domain, which bind to the nonmethylated CpG islands that are promoters of multiple genes [27]. Therefore, KMT2B has a critical role in regulation of some genes whose promoters are CpG islands. Studies on mouse embryonic stem cells showed that the methylation of H3K4 by the KMT2B enzyme in *MagohB* CpG island promoter not only leads to RNA polymerase II association, but also prevents DNA methylation on CpG island promoters. This process leads to the initiation and elongation of *MagohB* gene expression [28]. There are many tumor suppressor genes with CpG promoter islands and DNA hypermethylation of these promoters is known as an important mechanism in gene inactivation in cancer [29]. By considering CpG regions as target sequences of DNA methyltransferases, trimethylation of H3K4 by KMT2B likely causes the suppression of DNA methylation in active CpG island promoters [28]. This shows *MLL2* downregulation can induce DNA methylation of CpG island promoters in tumor suppressor genes and disregulation of their transcription. Our study upheld this mechanism of *MLL2* downregulation in breast cancer. In this study, we showed there is no significant correlation between *KMT2B* gene expression and clinicopathological parameters. It seems there is no mechanistic role of *KMT2B* expression change in ER/PR/HER2 pathways on breast cancer but further studies are needed to confirm. In conclusion, this research suggests that downregulation of *KMT2B* is involved in breast cancer and denotes its importance in tumorigenesis. However, further investigation is needed to reveal its mechanism in cancer.

Summary points

- Breast cancer is a heterogeneous disease in which several genetic and epigenetic ingredients contribute to its occurrence.
- Methylation of lysine 4 in histone H3 is done by members of the histone methyltransferase family known as lysine methyltransferase-2 (KMT2).
- Until now, mutations and disregulations of these methyltransferases have been observed in different cancers, but studies of *KMT2B* members are limited in breast cancer.
- In this study, 43 fresh samples of surgically removed tumorous tissues and 43 marginal breast tissues were collected from female patients.
- The expression of *KMT2B* gene is investigated in breast tumorous tissues compared with its marginal tumor free tissues.
- The statistical analysis showed the expression of *KMT2B* is downregulated significantly in breast tumor tissues compared with normal ones ($p = 0.02$).
- There was no significant change of *KMT2B* genes in relation to the clinicopathological parameters of breast cancer ($p > 0.05$).
- These results conclude that KMT2B as a histone methyltransferase has an important role in tumorigenesis.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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