Altered expression of *MLL* methyltransferase family genes in breast cancer

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Abstract. The histone lysine methyltransferases contain a SET domain, which catalyzes the addition of methyl groups to specific lysine residues. The *MLL* family of genes encodes histone-modifying enzymes with histone 3-lysine 4 methyltransferase activity that can regulate gene transcription. The MLL family exists in multi-protein complexes and has been implicated in a variety of processes including normal development and cell growth. Although some of the *MLL* family members have already been described to be involved in cancer, a clear relationship of these genes with breast cancer is not determined to date. In the present study, we used quantitative PCR to investigate the expression profile of all five *MLL* genes [*MLL (ALL-1), MLL2, MLL3, MLL4* and *MLL5*] in 7 breast cancer cell lines, 8 breast tumors and adjacent non-tumor tissues and in 12 normal tissues. We observed a diminished expression of all five genes in the breast cancer cell lines when compared to normal breast tissue. We found a significantly decreased expression of *MLL2* in the tumor samples compared to the non-tumor controls. In tumor samples, *MLL5* also showed a clear suppression tendency. Among the normal tissues analyzed, all genes showed a markedly higher expression in skeletal muscle and brain. Although further studies are required to determine the exact role of these methyltransferases in cancer development, our results indicate that the suppression of *MLL* genes, especially *MLL2* and *5*, take part in modulating breast carcinogenesis. Our assessment of the *MLL* family gene expression patterns in a diverse set of breast cancer cell lines and in a multitude of tissue types and breast tumors should lead to increasingly detailed information on the involvement of these genes in cancer progression.

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Introduction

Breast cancer is the most frequent cancer and the most common cause of cancer-related death in women (1). In Brazil alone, ~50,000 new cases of breast cancer are expected per year, with an estimated risk of 52 cases for every 100,000 women (2). Several studies have suggested that different genetic alterations are linked to different histological types of breast cancer, such as estrogen receptor expression/lobular type, c-ErbB2 expression/invasive type, among others (3,4). Recently, molecular studies have shown that breast cancer development involves not only alterations in oncogenes and tumor suppressor genes, but also epigenetic alterations in genes related to cellular growth, survival, motility and differentiation (5). Disturbance of epigenetic regulation of gene expression through methylation might contribute to tumorigenesis. In this context, altered histone modifying enzymes such as methyltransferases may play important roles in the process of carcinogenesis.

The mixed lineage leukemia (MLL) family is characterized by a conserved catalytic SET domain, responsible for the methyltransferase activity and comprises five genes [*MLL (ALL-1), MLL2, MLL3, MLL4* and *MLL5*].

The mixed lineage leukemia gene (*MLL* or *ALL-1, HRX* and *Htrx1*) (Gene ID: 4297) is located in human chromosome 11q23 and is frequently involved in chromosomal translocations in leukemia (6,7). *MLL* seems to function as a mammalian counterpart of *Drosophila trx*, which maintains the expression of multiple *Hox* genes during development. *Mll*-deficiency in mice results in failure to express *Hox* genes and in embryonic lethality. Heterozygosity for disrupted *MLL*, by homologous recombination in mouse embryonic stem cells, leads to retarded growth, hematopoietic abnormalities and skeleton malformations (8).

The *MLL2* gene (Gene ID: 8085) is located on human chromosome 12q13.12 and encodes a protein with domains similar to *MLL*. MLL2 was shown to be associated with Pax7 (paired-box transcription factor), forming the Wdr5-Ash2L-MLL2 histone methyltransferase (HMT) complex that directs tri-methylation of histone H3 lysine 4, when bound to Myf5. These chromatin modifications stimulate transcriptional activation of target genes to regulate entry into the myogenic developmental program (9). Another study, based on

muscle-specific *myogenin* gene, suggested that transcriptional activators Mef2d and Six4 mediate recruitment of trithorax group proteins Ash2L/MLL2 and UTX to MyoD-bound promoters to overcome the polycomb-mediated repression of muscle genes, allowing transcriptional activation of muscle-specific genes (10). *MLL* and *MLL2* genes show highly similar exon/intron structures. The proteins encoded by these genes are ubiquitously expressed among adult human tissues and although they share similar domains, they seem to act in a different manner. *MLL2* could not compensate for the heterozygosity of the disrupted *MLL*, by homologous recombination in mice (8), which indicates that their functions seem to be at least partially non-overlapping.

MLL3 (Gene ID: 58508) maps to chromosome 7q36.1, a region usually deleted in hematological malignancies (11). *MLL4* (Gene ID: 9757) is located on chromosome 19q13.1. *MLL3* and *MLL4* are found as part of a complex named ASCOM (for *ASC-2 COMplex*), that acts as a p53 co-activator. This complex is required for the expression of endogenous p53-target genes in response to the DNA damage (12). Deletions or truncations in *Mll, Mll2* and *Mll3* in mice show different phenotypes, suggesting that MLL protein functions are not redundant (8,13).

MLL5 (Gene ID: 55904) is located in human chromosome 7q22.1. The nuclear protein encoded shows single PHD and SET domains and lacks other conserved sequence elements, like its *Drosophila* homolog CG9007. *MLL5* shows ubiquitous tissue expression and is highly conserved. It is the latest addition to the mammalian Trithorax/*MLL* gene family and recent studies show that the MLL5 may function in a different manner, without methyltranferase activity, which is consistent with its different sequence of the SET domain compared to the rest of the MLL family of H3K4 methyltransferases (14). It was suggested that MLL5 would have an indirect mechanism regulating expression of histone-modifying enzymes, such as LSD1 and SET7/9 (15). MLL5 was shown to promote myogenic differentiation through positive regulation of the pro-myogenic genes *Pax7*, *Myf5* and *myogenin*, which are impaired in *MLL5* knockdown, leading to serious differentiation defects (15). *Mll5* deficiency in mice leads to growth retardation, male infertility and defects in multiple hematopoietic lineages (16). Therefore, the defects found could be associated with deregulation of cell cycle control.

Some studies have shown that MLLs are critical co-activators in estrogen (E2)-dependent regulation of E2-responsive genes, through LXXLL motifs (NR boxes) (17,18). The suppression of *MLL2* decreased expression of estrogen receptor (ER) target genes (18). MLL3 and MLL4 coordinate with ERs and act in transcriptional regulation of HOXC10 in the presence of estrogen (19). MLL3 and MLL4, as members of the ASCOM complex, play essential roles in gene activation mediated through nuclear receptors, which bind hormone responsive elements (12). The activity of the MLL family in hormone-mediated gene activation and signaling could be helpful to understand its involvement in breast carcinogenesis.

Here we present an analysis of the patterns of *MLL* family gene expression in seven breast cancer cells lines, twelve human normal tissues and eight breast tumors and their adjacent normal breast tissues. Our results may help to provide a better understanding of the role of the *MLL* genes in breast carcinogenesis.

Materials and methods

Cell lines and culture. Seven breast cancer lines were used on this study: HCC-1954, MCF-7, CAMA-1, SKBR-3, MDA-MB-231, MDA-MB-436 and MDA-MB-468. The cell lines HCC-1954, MCF-7 and MDA-MB-231, obtained from the ATCC, were cultured in our laboratory in recommended media and checked periodically for mycoplasma contamination. The main characteristics of the cell lines are summarized in Table I (20). Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum (Sigma-Aldrich) and penicillin/streptomycin (100 U/ml; Life Technologies, Carlsbad, CA, USA) at 37˚C and 5% CO_2. Total RNA from these cells was extracted and used for expression analysis as described below. Additionally, total RNA from the remainder cells were kindly provided by the Ludwig Institute for Cancer Research, São Paulo, and were included in the gene expression analysis.

Normal tissues. A commercial panel (OriGene Technologies, Rockville, MD, USA) of total RNA from twelve human normal tissues from different organs (lung, small intestine (SI), brain, colon, kidney, muscle, liver, spleen, heart, testis, stomach and placenta) was used in this study. Total RNA of each normal tissue was used to verify the expression levels of the genes studied.

Tumor specimens. The sample collection protocol was approved by the Research Ethics Committee of the Faculty of Health Sciences, University of Brasília, Brazil, based on resolution 196/96 of the National Health Council/Brazilian Ministry of Health, project no. 025/09. Written informed consent was obtained from all participants. The samples were collected from surgically removed breast tissue from female patients diagnosed with breast cancer at the Hospital of the University of Brasilia. A total of 14 samples (6 normal and 8 tumor samples) was used. The identification of tumor tissue from the removed samples was based on the histopathological examination and all types of breast cancer were included in our analyses. Samples were selected based on tumor content (minimally 80% tumor) as determined by microscopic pathological analysis. A sample of normal tissue was collected from each patient whenever possible. The clinical and histopathological characteristics of the patients are summarized in Table IV.

RNA extraction and cDNA synthesis. Total RNA was isolated using TRIzol LS reagent, according to the manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). Single-stranded complementary DNA was generated from total RNA obtained from cell lines, normal tissues and clinical samples with reverse transcriptase and random primers, using the Applied Biosystems High Capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA).

Quantitative PCR (qPCR). Reactions of qPCR were performed on a StepOnePlus™ Real-Time PCR System (Applied

Table I. Characteristics of the breast cancer cell lines.

Cell lines	Origin	ER	PR	Cerb-B2	Tumor type	Characteristics
HCC-1954	Primary breast cancer	-	-	+	DC	Ductal carcinoma with no lymph node metastases
MCF-7	Pleural effusion	+	+	*	IDC	Invasive ductal carcinoma; weakly invasive
CAMA-1	Pleural effusion	+	-	*	AC	Metastatic adenocarcinoma
SKBR-3/AU565	Pleural effusion	-	-	+	AC	Metastatic adenocarcinoma
MDA-MB-231	Pleural effusion	-	-	*	AC	Metastatic adenocarcinoma; highly invasive
MDA-MB-436	Pleural effusion	-	-	*	IDC	Metastatic adenocarcinoma; highly pleomorphic
MDA-MB-468	Pleural effusion	-	-	*	AC	Metastatic adenocarcinoma

ER, estrogen receptor; PR, progesterone receptor; Cerb-B2, epidermal growth factor receptor 2 overexpression; DC, ductal carcinoma; IDC, invasive ductal carcinoma; AC, adenocarcinoma; *Unavailable data.

Table II. Relative quantification of *MLL* genes in normal human tissues.

	MLL	*MLL2*	*MLL3*	*MLL4*	*MLL5*
Kidney	1	1	1	1	1
Lung	**2**	**2**	**2**	1	**2**
Small intestine	1	3	3	3	5
Brain	**5**	**3**	**3**	1	**3**
Colon	1	2	1	3	1
Muscle	**9**	**10**	**8**	**3**	**6**
Liver	8	1	163	1	20
Spleen	1	1	1	2	1
Heart	1	**2**	50	1	6
Testis	**3**	**2**	**2**	**5**	1
Stomach	2	1	23	1	6
Placenta	14	1	12	1	28
Breast	1	1	1	3	**7**

Sample calibrator, kidney (1X sample). Bold, upregulated expression in fold changes; underlined, downregulated expression in fold changes.

Biosystems) using TaqMan Universal PCR MasterMix and TaqMan Gene Expression Assays (Hs01061596_m1 for *MLL*, Hs00231606_m1 for *MLL2*, Hs00419011_m1 for *MLL3*, Hs00207065_m1 for *MLL4* and Hs00218773_m1 for *MLL5*, Applied Biosystems), according to the manufacturer's instructions. Briefly, 2 μl of each sample template was added to a 10 μl final reaction volume per well. Amplification conditions were as follows: 2 min at 50˚C and 10 min at 95˚C on holding stage and then 40 cycles of 15 sec at 95˚C and 1 min at 60˚C. Gene expression values are reported as ratios between the amplification levels of genes of interest and that of endogenous control gene (Hs99999903_m1 for β-actin, Applied Biosystems), which provides a normalization factor for the amount of RNA isolated from a specimen. This was subsequently normalized with the ratio obtained in control samples (relative expression level). Each assay was performed in triplicate for each sample.

qPCR data analysis. To determine the relative quantification (RQ) of gene expression, the data were analyzed using the comparative quantification Ct method (ΔΔCt) (Applied Biosystems). Briefly, the mean of Ct (cycle threshold) values of the replicates were calculated and normalized by subtracting the Ct value of the co-amplified endogenous control gene to yield a ΔCt value. The ΔCt of a random control calibrator sample (1X sample) was subtracted from the ΔCt of the other samples to yield a ΔΔCt value. The amount of target gene, normalized to an endogenous reference and relative to a calibrator was converted into relative quantification by the formula: $2^{-\Delta\Delta CT}$. For the cell lines, normal tissues and clinical samples used, the *MLL* genes expression was normalized using the β-actin as an endogenous control. Expression level was considered altered when augmented or diminished ≥2X compared to the control calibrator sample, chosen for each analysis. To check the statistical significance of the relative quantification of the genes, the non-parametric Mann-Whitney U test was performed for the pools of normal (n=6) and tumor (n=8) samples. The one-sample Wilcoxon signed-rank test was used for the expression results of the *MLL* genes family in seven cancer cell lines, to test if the samples means significantly differed from the hypothesized value (sample calibrator relative quantification: 1) The statistical tests were considered significant when the P-value was <0.05 (CI 95%). Calculations were performed using the software SPSS version 20 (SPSS Inc., Chicago, IL, USA). Additionally, we searched for *MLL* gene expression profiles in breast cancer datasets, available in the database Oncomine, to compare with our most significant findings.

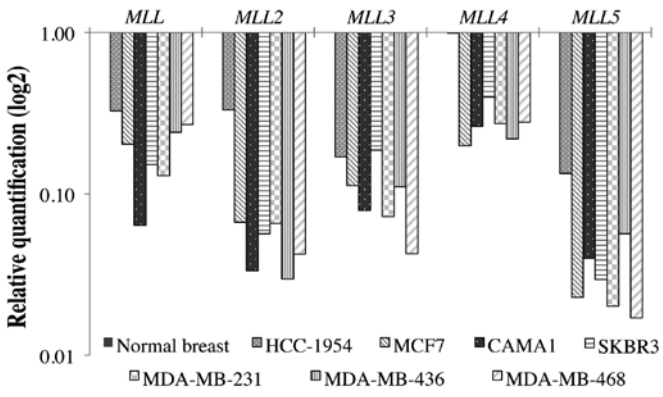

Figure 1. Expression levels of the *MLL* genes in 7 breast cancer cell lines, using the ΔΔCt method. Sample calibrator, normal breast clinical sample (relative quantification = 1). All genes showed expression levels below the calibrator sample (P<0.05).

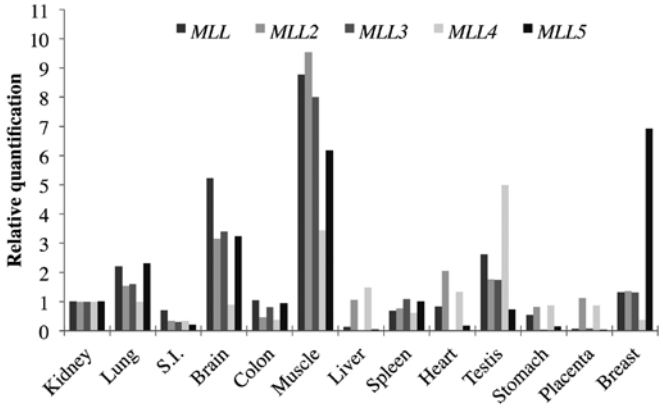

Figure 2. Expression levels of the *MLL* genes in 12 normal human tissues and in a normal breast clinical sample. Random sample calibrator, normal kidney (relative quantification = 1).

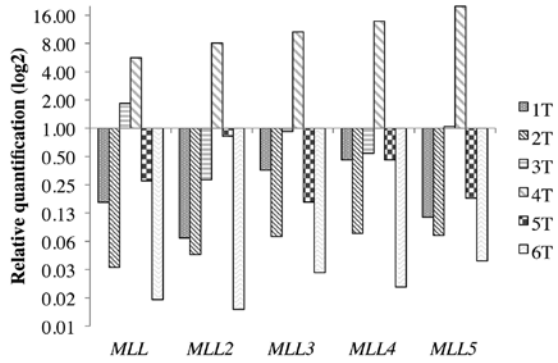

Figure 3. Relative quantification of the *MLL* genes by qPCR using the comparative quantification method ΔΔCT in 6 paired clinical samples. Sample calibrator, normal, non-tumor tissue from the same donor patient (relative quantification = 1).

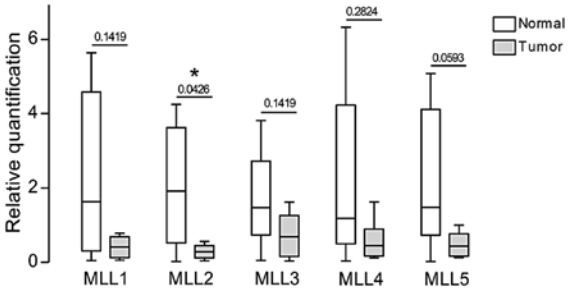

Figure 4. Relative quantification of the *MLL* genes in pools of normal (n=6) and tumor (n=8) clinical samples, compared using the independent Mann-Whitney U test (P<0.05 and CI 95%). The P-values for each gene are shown above the bars.

Results

MLL genes are downregulated in breast cancer cell lines. The results of the *MLL* gene expression analysis in seven cancer cell lines using the comparative quantification method ΔΔCT are shown in Fig. 1. All genes showed expression levels diminished in comparison to the normal breast sample calibrator (1X sample) (P<0.05). The *MLL2* and *MLL5* were the genes with the lowest expression level in all cell lines, with the exception of HCC-1954. The HCC-1954 lineage is the least aggressive among the breast cancer cell lines studied, with no lymph node metastases (Table I). Although all *MLL* genes were also downregulated in HCC-1954 cells in relation to the normal breast calibrator control, this cell line showed the least reduced expression among the breast cancer cell lines.

MLL genes are differentially expressed among normal tissues. In order to determine the *MLL* genes expression pattern in normal human tissues, quantitative real-time polymerase chain reaction (qPCR) was performed using a commercial panel of total RNA from normal tissues obtained from OriGene®. Expression analysis using the comparative quantification method ΔΔCT was performed. The kidney tissue, which showed a median expression level, was chosen as a sample calibrator (1X sample) and a normal breast clinical sample was included for the relative quantification. Relative quantification for the normal tissues can be seen in Fig. 2 and in Table II. These results evidence a heterogeneous tissue expression for the *MLL* family genes, suggesting that MLL proteins functions are not redundant, but acting in a tissue-specific manner.

Table III. Relative quantification of *MLL* genes in tumor samples compared to their normal counterparts.

	MLL	*MLL2*	*MLL3*	*MLL4*	*MLL5*
1T	6	15	3	2	9
2T	30	22	14	13	14
3T	**2**	4	1	2	1
4T	**6**	**8**	**11**	**14**	**20**
5T	4	1	6	2	6
6T	67	85	34	49	26

Sample calibrator, normal tissue from the same donor patient (1X sample). Bold, upregulated expression in fold changes; underlined, downregulated expression in fold changes.

Table IV. Clinical and histopathological characteristics of the 8 breast cancer patients.

Samples	Breast affected	Age	Histological type	Histological grade	Stage	Neoadjuvant chemotherapy	C-erb-B2	ER	PR
1	Left	58	DCI	High	T3N2AM0	Yes	+	-	-
2	Left	54	DCI	Intermediate	T4DN3M0	Yes	-	+	+
3	Left	64	DCI	Intermediate	YPT4PN1M0	Yes	-	-	-
4	Right	43	DCI	High	T2N1M0	Yes	*	*	*
5	Left	56	DCI	High	T2N1AM0	Yes	-	-	-
6	Right	60	DCI	High	T2N3M0	Yes	-	+	+
7	Right	61	LCI	Intermediate	T2N0M0	No	+	+	+
8	Right	32	DCI	Low	T2N0M0	Yes	+	+	+

DCI, ductal carcinoma *in situ*; LCI, lobular carcinoma *in situ*; Cerb-B2, epidermal growth factor receptor 2; ER, estrogen receptor; PR, progesterone receptor. *Unavailable data.

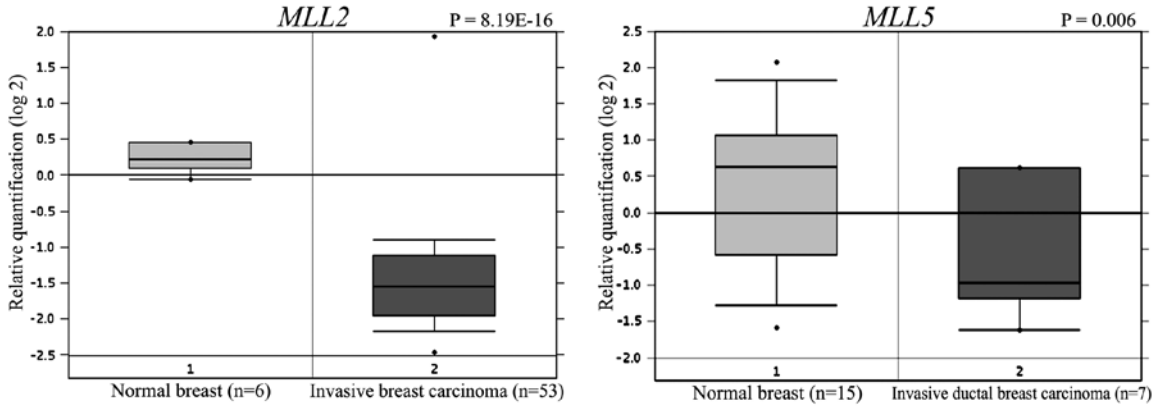

Figure 5. *MLL2* and *MLL5* are downregulated in breast cancer. Gene expression data in Oncomine was analyzed. The thick bars in the boxes are average expression levels and the boxes represent 95% of the samples. The error bars are above or below the boxes and the range of expression levels is enclosed by two dots.

MLL2 is significantly downregulated in clinical samples. The paired expression analysis between normal and tumor samples from the same patient can be seen in Fig. 3 and the relative quantification values in Table III. The quantification analysis for the pools of normal (n=6) and tumor (n=8) clinical samples showed a decreased expression level of all genes in the tumors when compared to the pool of normal samples (Fig. 4). The expression of *MLL* was reduced in 5 of the 8 (62.5%) tumor samples and remained the same in the 3 other (37.5%) samples. The expression of *MLL2* was reduced in all the 8 tumor samples (100%). The expression of *MLL3* was reduced in 4 of the 8 (50%) tumor samples, remained the same in the 3 other (37.5%) samples and was increased in 1 sample (12.5%). The expression of *MLL4* was reduced in 5 of the 8 (62.5%) tumor samples, remained the same in the other 2 (25%) samples and was increased in 1 sample (12.5%). For the *MLL5*, the expression was reduced in 6 of the 8 samples (75%) and remained the same in 2 (25%). Although this observation reveals a significantly suppressed expression of *MLL2* and *MLL5* in the majority of tumors, the small number of cases does not allow strong associations with the clinical and pathological characteristics of the patients (Table IV).

To overcome our limited clinical breast cancer cohort, we searched for *MLL* genes expression profile available in the Oncomine microarray database. We found that *MLL2* was significantly downregulated in a set of 53 invasive breast carcinomas compared to 6 normal breast tissues (P=8.19E-16) (21). *MLL5* was found to be 2-fold downregulated in a set of 7 invasive breast carcinomas compared to 15 normal breast tissues (P<0.005) (22). These data corroborate our findings indicating that the levels of *MLL2* and *MLL5* are widely downregulated in breast carcinomas (Fig. 5).

Discussion

Despite the advances that have been made in uncovering potential roles for protein methyltransferases in human cancers, it is still largely unknown how they contribute to carcinogenesis. Deregulated methylation mechanisms in cancer cells may be due to enzyme mutations or altered expression (23,24). Recent studies have shown a relationship between SMYD3, a lysine methyltransferase and breast carcinogenesis (25). SMYD3 modulates the chromatin structure through specific methylation of lysine 4 of histone 3 (H3K4), an epigenetic alteration

that leads to uncontrolled cell proliferation (25). Several other lysine methyltransferases have already been shown to be involved in cancer. For example, the gene *EZH2* was overexpressed in different cancer tissues, including breast and prostate (26). The *SETD2*, a potential tumor suppressor gene, was downregulated in breast cancer samples and in high-grade tumors (27). Deregulation of *PRDM14* gene has been associated to several human tumors, including an overexpression state in breast cancer cells (28). Although little is known about the exact roles of *MLL* family genes in breast carcinogenesis, some recent studies have linked alterations in individual *MLL* genes with cancer progression. The present study provides the first comparative expression analysis of the five MLL family genes in breast cancers. In this study, we investigated the differential expression of *MLL* genes in breast tumor samples and cell lines, as well as in normal tissues. All *MLL* genes had a significantly decreased expression in the seven breast cancer cell lines analyzed. It is noteworthy that although these cell lines represent different stages of the disease, presenting different receptors and characteristics of aggressiveness (Table I), they all showed a similar expression profile for all five *MLL* genes. In the HCC-1954 cell line, which represents a less aggressive breast cancer, with no lymph node metastases, a subtle higher expression for the *MLL* genes when compared to the other cell lines was found (Fig. 1). This observation suggests that expression of *MLL* family genes may decrease as disease progresses. Cell lines exhibit substantial genomic, transcriptional and biological heterogeneity found in primary tumors and represents a good system to study primary breast tumor features (20). Indeed, the results obtained in cell lines were in accordance to those obtained in clinical samples.

The analysis of the *MLL* genes in normal tissues showed highly heterogeneous expression among the different tissues studied. This finding indicates a possible tissue-specific function for each individual gene. The only consensus seems to be the high expression of all *MLL* genes in skeletal muscle and brain. For instance, *MLL2* gene showed a markedly increased expression in skeletal muscle (>10-fold) and heart (>2-fold), which is in agreement with other studies showing the involvement of this gene with myogenic developmental program (9,10). It is of interest that in normal breast tissue, *MLL5* has a markedly higher expression among all *MLL* family genes. The analysis of the primary breast clinical samples clearly pointed to decreased expression of the genes *MLL2* and *MLL5* in breast cancer. The other *MLL* genes did not show a significant difference in expression between tumor and normal samples.

The *MLL* gene had a slightly decreased expression in the tumor samples, compared to the normal ones (P=0.142). In all the cancer cell lines *MLL* was downregulated in comparison to the pool of normal samples (P<0.05). Mammalian *MLL* and *MLL2* have a role in long-term maintenance of *Hox* and other gene expression patterns during development (8,13). Several rearrangements of the *MLL* gene underlie the majority of infant acute leukemias, as well as of therapy-related leukemias occurring in cancer patients treated with inhibitors of topoisomerase II (29). Higher transcript levels of *MLL* were associated with patients who remained disease-free compared to those with bone metastasis. (30). Liu *et al* identified *MLL* as a key constituent of the DNA damage response pathway, showing that deregulation of the S-phase checkpoint caused by *MLL* translocations may contribute to the pathogenesis of human *MLL* leukemias (31).

The comparative analysis for *MLL2*, revealed a significantly decreased expression of this gene in tumor samples when compared to normal ones (P=0.043). In the analysis using breast cancer cell lines, *MLL2* was the second gene with the most prominent downregulation, after *MLL5* (P<0.05). The Oncomine database results corroborated our data, showing that *MLL2* was found markedly downregulated in a set of 53 invasive breast carcinomas compared to 6 normal breast tissues (P=8.19E-16) (21). These results are consistent with several other studies on this gene, in different types of cancer. Curiously, Huntsman *et al* (32) showed that *MLL2* is amplified in some solid tumor cell lines (pancreatic and glioblastoma cell lines), with the exception of breast cancer cell lines. Morin *et al* (33) detected, in lymphoma patients, several somatic mutations distributed across *MLL2*, with some mutations affecting both *MLL2* alleles or leading to loss of heterozygosity (LOH), which is consistent with the complete, or almost complete, loss of *MLL2* in the tumor cells studied. They suggested that since the majority of the somatic mutations found in *MLL2* were inactivating, *MLL2* probably acts as a tumor suppressor of significance in lymphomas. Pasqualucci *et al* (34) found that the *MLL2* gene was the most frequently mutated gene in diffuse large B-cell lymphoma (DLBCL) with most cases of inactivating mutations, generating truncated proteins lacking the entire or part of the C-terminal cluster of conserved domains (including the SET domain). Since most of the cases affected a single allele, they suggested a role for *MLL2* as a haploinsufficient tumor suppressor. Dalgliesh *et al* (35) demonstrated that the *MLL2* is frequently mutated in clear cell renal cell carcinoma (ccRCC), with silence, missense and truncating mutations. It was identified as a likely ccRCC cancer gene in statistical analyses. Finally, *MLL2* was shown to be involved in several cellular signaling pathways, such as p53 and cAMP, regulating different sets of genes. The link of *MLL2* to the p53 pathway corroborates a possible role of *MLL2* as tumor suppressor gene. Also, among the categories of genes regulated by *MLL2*, were those that respond to nuclear hormones, including genes from the retinoic acid signaling (36).

MLL3 did not show a statistical significant differential expression between the tumor and normal samples used in this study (P=0.142), although in Fig. 4, this tendency can be noted. In the seven breast cancer cell lines analyzed (Fig. 1), there is a clear decreased expression of *MLL3* (P<0.05). The *MLL3* seems to be frequently mutated in many types of human tumors and a recent study detected that 40% of the primary breast tumor samples showed reduced expression of this gene, suggesting its possible role in the development of breast cancer as a tumor suppressor (37). Parsons *et al* (38) found, that *MLL3*, as well as *MLL2*, harbors several inactivating mutations in medulloblastoma that seem to be driver mutations. In the case of *MLL2*, 67% of the mutations were predicted to truncate the encoded proteins. This observation led to the conclusion that both genes act as tumor suppressors inactivated by mutation in medulloblastoma. *MLL3* also showed inactivating mutations in colorectal cancer samples (5,39) and reduced expression in primary breast tumor samples, suggesting a role in cancer development (37).

We found no significant difference between normal and tumor sample expression of *MLL4* (P=0.282). In the seven breast cancer cell lines (Fig. 1), *MLL4* showed decreased expression, although it was the less downregulated gene among the other members of the family (P<0.05). *MLL4* is mostly reported as a member of the complex ASCOM (for *ASC-2 COMplex*), that acts as a p53 co-activator. Nonetheless, it is suggested that MLL3 and MLL4 are found in distinct ASC-2-containing complexes rather than in a common ASC-2 complex, with only partially redundant functions (12).

For the *MLL5* gene, our results revealed a clear tendency of diminished expression in the clinical tumor samples (P=0.059). It also showed a significant decreased expression in the cancer cell lines, representing the most downregulated gene among the *MLL* family for the seven lineages (P<0.05). The Oncomine database results for *MLL5* expression in breast cancer also corroborated the tendency of our data, with a 2-fold downregulation of *MLL5* in a set of 7 invasive breast carcinomas compared to 15 normal breast tissues (P<0.005) (22). *MLL5* gene is the latest addition to the mammalian Trithorax/*MLL* gene family and recent studies show that the MLL5 may function in a different manner, without histone methyltransferase activity (16). MLL5 lacks DNA-binding motifs of A-T hooks and methyltransferase homology motifs, both common to other MLL protein members. Instead of binding directly to DNA, MLL5 may modulate transcription indirectly via protein-protein interactions through its PHD (plant homeodomain, a nuclear protein-interaction domain) and SET domains (14). Overexpression of *MLL5* prevented cell cycle progression pointing to a role of this gene in the cell cycle regulatory network (6). MLL5 seems to form intranuclear protein complexes, similar to other tumor suppressors, that may play a role in chromatin remodeling and cellular growth suppression (6). In cell line transfection studies, overexpression of *MLL5* transcripts inhibits cell cycle progression, suggesting a role for *MLL5* in tumor suppression (6,16). Moreover, *MLL5* was identified as a candidate suppressor gene in 7q22, a chromosomal site frequently deleted in patients with aggressive acute myeloid leukemia and high *MLL5* transcript levels seem to be associated with a favorable outcome in this disease (40). Therefore, it is possible that in cancer, the truncated or absent MLL5 protein is unable to form the intranuclear protein complexes to interact with chromatin, allowing the cell cycle to progress.

Although the complete genome screening is essential to unveil the genetic profile of complex diseases, the comparative analysis of a specific group of genes can be valuable to better understand the complexity of cancer mechanisms. In the present study, we analyzed the expression profiles of the *MLL* family in different breast cancer cell lines and clinical samples. Our results demonstrate a significant decreased expression of the *MLL2* and a clear tendency of *MLL5* downregulation in tumor samples, which could indicate a possible role as tumor suppressor genes. Since *MLL2* has a role in maintenance of *Hox* genes expression during development and *MLL5* seems to have a role in cell cycle regulation, they may both be involved in the regulation of other genes and pathways (8,13). Furthermore, MLLs seem to have other roles in regulating gene activation beyond their methyltransferase activities, including responses under a hormonal environment (12,17-19). Our results are consistent with several studies on these genes in different cancer types, as discussed above, confirming that our high-throughput approach was reliable enough for validation of the genes expression profiles. Although these results point to an involvement of *MLL2* and *MLL5* in breast cancer, the number of cases does not enable robust statistical analyses neither associations with the clinical characteristics. Therefore, further studies using a larger clinical breast cancer cohort are required to determine the exact role of these methyltransferases as epigenetic regulators in cancer development.

The correlation between various methyltransferases and breast cancer highlights the importance of this protein family in the progression of this disease. Further study is required to reinforce the importance of the *MLL* family as a target for breast cancer therapy and will help elucidate the mechanisms involved in the regulation of its activity. Our findings reveal the importance of these genes in breast carcinogenesis and may contribute to the identification of novel strategies to treat breast tumors.

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