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Frequent epigenetic inactivation of *KIBRA*, an upstream member of the Salvador/Warts/Hippo (SWH) tumor suppressor network, is associated with specific genetic event in B-cell acute lymphocytic leukemia

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The WW-domain containing protein KIBRA has recently been identified as a new member of the Salvador/Warts/Hippo (SWH) pathway in Drosophila and is shown to act as a tumor suppressor gene in Drosophila. This pathway is conserved in humans and members of the pathway have been shown to act as tumor suppressor genes in mammalian systems. We determined the methylation status of the 5' CpG island associated with the *KIBRA* gene in human cancers. In a large panel of cancer cell lines representing common epithelial cancers *KIBRA* was unmethylated. But in pediatric acute lymphocytic leukemia (ALL) cell lines *KIBRA* showed frequent hypermethylation and silencing of gene expression, which could be reversed by treatment with 5-aza-2'-deoxycytidine. In ALL patient samples *KIBRA* was methylated in 70% B-ALL but was methylated in <20% T-ALL leukemia ($p = 0.0019$). In B-ALL *KIBRA* methylation was associated with *ETV6/RUNX1* [t(12;21)(p13;q22)] chromosomal translocation ($p = 0.0082$) phenotype, suggesting that *KIBRA* may play an important role in t(12;21) leukemogenesis. In ALL paired samples at diagnosis and remission *KIBRA* methylation was seen in diagnostic but not in any of the remission samples accompanied by loss of *KIBRA* expression in disease state compared to patients in remission. Hence *KIBRA* methylation occurs frequently in B-cell acute lymphocytic leukemia but not in epithelial cancers and is linked to specific genetic event in B-ALL.

Introduction

The Salvador/Warts/Hippo (SWH) network was originally identified in *D. melanogastor* as a pathway responsible for regulating cellular proliferation and apoptosis through upregulation of cyclin E, diap1 and bantam.[1] The core components of the fly network, Hippo, Sav, Wts, Mats are conserved in mammals as MST1/2, WW45, LATS1/2 and MOB1. The core of the hippo pathway has been well studied.[2] The sterile-20 kinase Hippo forms a complex with Salvador, a WW-repeat scaffolding protein. This in turn phosphorylates and activates the DBF family kinase Warts. Activated Warts in association with MATS phosphorylates and inhibits the transcriptional co-activator Yorkie. This in turn leads to a reduction in cell number owing to the downregulation of transcriptional targets of Yorkie, the anti-apoptotic molecules diap1 and the microRNA bantam and the cell cycle regulator cyclin E. Hence the SWH pathway activation leads to smaller organs with fewer cells, and the reverse happens, increase in cell number and tissue size when the pathway is downregulated. Upstream components of the pathway include Merlin and Expanded. Very recently three groups identified the WW-domain containing protein Kibra as an upstream component of the SWH pathway and demonstrated that it acts as a tumor suppressor gene in Drosophila.[3-5]

RASSF1A tumor suppressor gene belongs to a family of proteins containing a RAS-association domain in either their C (RASSF1-RASSF6) or N-terminals (RASSF7-RASSF10).[6-8] It has been demonstrated that in mammalian cells RASSF1A forms a complex similar to the SWH network in Drosophila.[9] RASSF1A interacts with MST1 and MST2 and this binding

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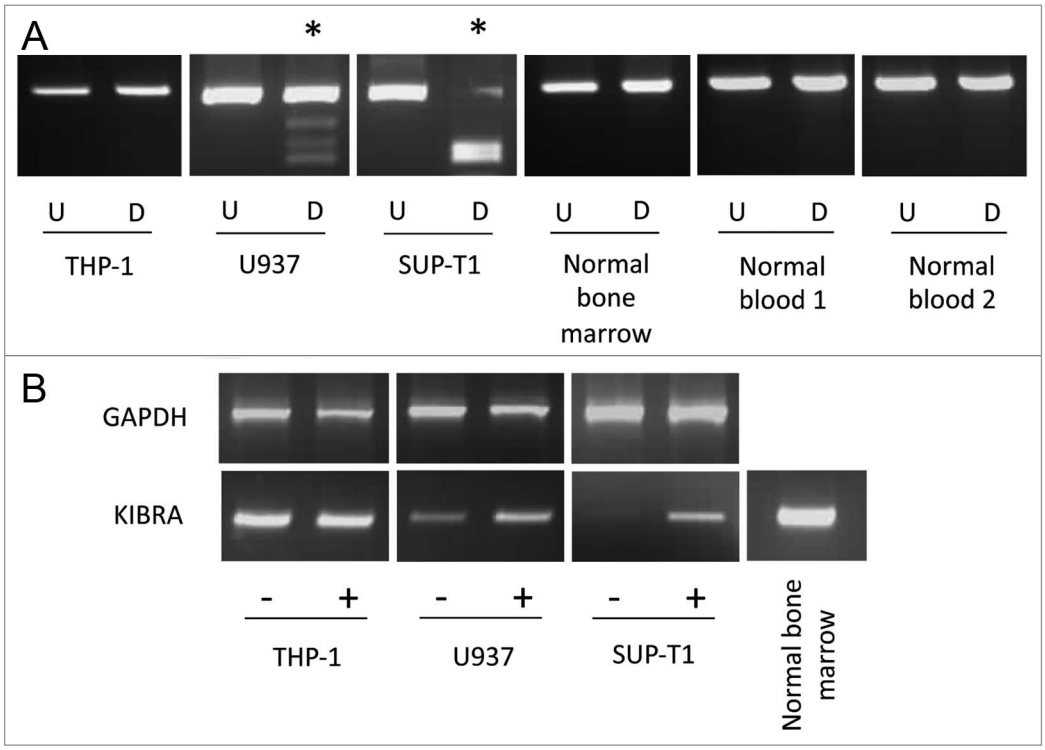

Figure 1. Leukemia cell line methylation and expression status. *KIBRA* methylation and re-expression in leukemia cell lines. (A) Undigested PCR product (U) is shown next to digested PCR product (D) for three leukemia cell lines, one normal bone marrow sample and two normal blood samples. (B) GAPDH and *KIBRA* RT-PCR results are shown for unmethylated cell line THP-1, partially methylated cell line U937 and completely methylated cell line SUP-T1 pre (-) and post (+) treatment with 5-aza-2'-deoxycitidine. * indicates methylated samples.

is important for some of its functional properties.[10-12] We previously undertook a methylation screen of all *RASSF* members (*RASSF1A-RASSF10*) in acute lymphocytic leukemia. We demonstrated that *RASSF6* and *RASSF10* were the only members that were frequently and specifically methylated in ALL. *RASSF6* was very frequently methylated in B-ALL (94%) and in 41% T-ALL, while *RASSF10* was frequently methylated in 88% T-ALL but showed <20% methylation in B-ALL.[13] In this report we demonstrate that the latest member of the SWH pathway, *KIBRA* is frequently methylated in B-ALL and this methylation is associated with the most common genetic event occurring in B-ALL.

Results

Methylation analysis in common epithelial cancers. A large panel of tumor cell lines (n = 49) consisting of major epithelial cancers was analyzed for methylation in the 5'CpG island associated with the *KIBRA* gene. We designed primers encompassing part of the *KIBRA* 5'CpG island and used COBRA assay to detect methylation. The cell line panel consisted of breast, colorectal, glioma, kidney, lung and prostate cancer cell lines. One out of seven glioma cell lines showed partial methylation, rest of the cancer cell lines in the panel showed no methylation (**Sup. Fig. S1**). We went on to analyze 20 glioma tumors, *KIBRA* was unmethylated in all cases (**Sup. Fig. S1**). Hence *KIBRA* is unmethylated in these epithelial cancers.

Methylation and expression analysis in leukemia cell lines. We next examined a panel of acute lymphocytic leukemia cell lines for *KIBRA* methylation. Among six cell lines, three were completely and one was partially methylated for *KIBRA* and two cell lines were unmethylated (**Fig. 1A**). In order to demonstrate biological significance of this methylation, the leukemia cell lines were treated with 5-aza-2'-deoxycytidine. *KIBRA* gene expression could be seen after 5-aza-2'-deoxycytidine treatment in the methylated cell lines, while no change was seen in unmethylated lines (**Fig. 1B**).

Methylation analysis in primary ALL leukemia samples. In acute lymphocytic leukemia *KIBRA* methylation was detected in 33/47 (70%) B-cell ALL and much less frequently in T-cell ALL, 2/12 (16.7%) (**Fig. 2A and B**) (p = 0.0019). In order to confirm the COBRA results and to determine the extent and pattern of *KIBRA* CpG island methylation, we sequenced the DNA from bisulfite-modified ALL samples. As seen in **Figure 3** the CpG region analyzed was heavily methylated throughout giving high values for the methylation index (MI). No methylation was detected in DNA from age matched peripheral blood lymphocytes from healthy individuals or in healthy bone marrow (**Fig. 3**). In B-cell ALL patient samples *KIBRA* methylation was strongly associated with *ETV6/RUNX1* translocation (p = 0.0082), the most common translocation occurring in this leukemia sub-type. Our data suggests that *KIBRA* may play an important role in t(12;21) leukemogenesis. Survival analysis was not possible in the ALL cohort since very few individuals

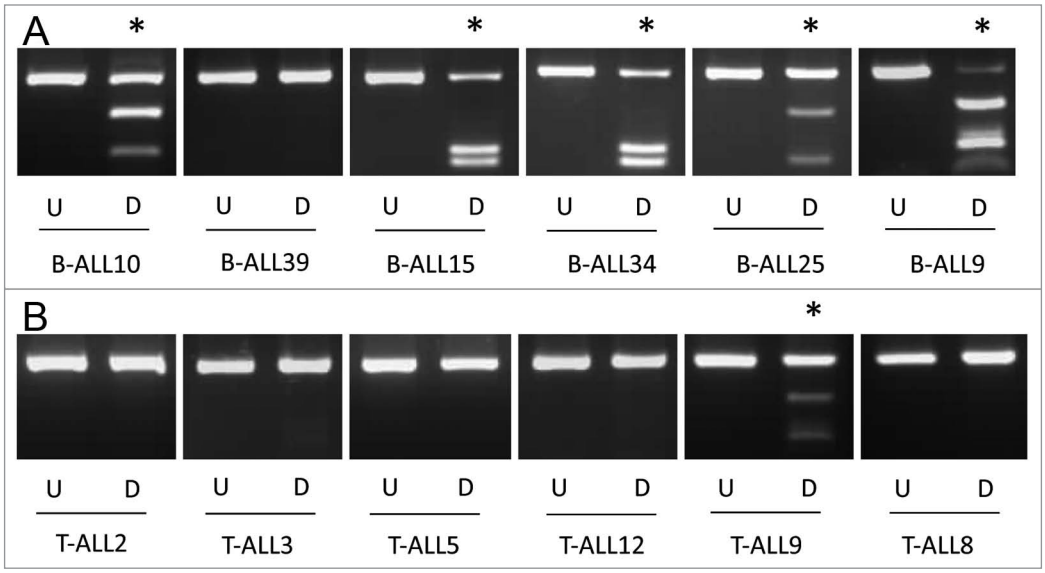

Figure 2. *KIBRA* methylation in ALL samples. CoBRA results are shown for six childhood B-ALL samples (A) and six childhood T-ALL samples (B). Undigested products (U) are shown next to BstUI digested products (D) for each sample. * indicates methylated samples.

underwent relapse. We also analyzed other members of the SWH pathway (*FAT1*, *LATS1* and *LATS2*, *MOBs*, *MST1* and *MST2* and *SAV1*) for epigenetic inactivation in acute lymphocytic leukemia. Only *FAT1* was found to be frequently methylated in B-ALL (65%) and less so in T-ALL (25%) (**Sup. Figs. 2 and 3**). *FAT1* methylation in B-ALL associated with *ETV6/RUNX1* translocation ($p < 0.05$).

Methylation and expression analysis in paired ALL samples at diagnosis and remission. We analyzed five paired ALL samples with the *ETV6/RUNX1* translocation for *KIBRA* methylation in matched DNA at diagnosis and in remission. *KIBRA* was methylated in all five diagnostic samples but was unmethylated in all five matched remission samples (**Fig. 4A**). This provides further evidence that KIBRA methylation is cancer specific and is associated with the malignant state. We also examined *KIBRA* expression in the above matched samples. *KIBRA* expression was reduced or absent in the disease state compared to patients in remission (**Fig. 4B**). Hence loss of *KIBRA* is highly associated with the disease state in these patients and is biologically relevant.

Discussion

Acute lymphocytic leukemia is the most common form of childhood leukemia in the western World. ALL is characterized by specific translocations, including the most common translocation *ETV6/RUNX1* [t(12;21)(p13;q22)], occurring in approximately 50% of childhood cases. *ETV6/RUNX1* translocation is associated with good prognosis in individuals with this disease.[14] Aberrant DNA methylation has been well documented for epithelial and hematological cancers and shown to play a strong role in tumorigenesis. Hypermethylation of tumor suppressor gene promoters are a well characterized hallmark of many cancers.[15,16] There have been several recent genome-wide studies to identify hypermethylated genes in acute lymphocytic leukemia.[17-19] Our laboratory recently carried out a comprehensive analysis of all RASSF gene family members (*RASSF1-RASSF10*) with an associated 5' CpG island in acute lymphocytic leukemia. We demonstrated that *RASSF6* was the most frequently methylated family member in B-cell ALL (94%), while the newly characterized *RASSF10* was most frequently methylated in T-cell ALL (88%).[13] RASSF1A has recently been linked with a highly conserved pathway between Drosophila and mammals, Salvador-Warts-Hippo (SWH) tumor suppressor network. RASSF1A binds to a core component of the pathway, MST1 and MST2 (Hippo in Drosophila) and the subsequent biological role of this association has been determined in various cellular contexts.[10,11,20,21] The SWH pathway was originally identified in the fly to control organ size. The core components of the pathway (Hippo, Salvador, Warts/Lats, Mats, Yorkie and very recently Kibra) are evolutionally conserved in mammals (MST1 and 2, SAV1, LATS1 and 2, MOB1, YAP and KIBRA). Recent studies have demonstrated a key role for the SWH pathway in regulating cell contact inhibition, organ size control and development of cancer in mammals.[22-26] Kibra was recently identified as upstream member of this pathway and a potential tumor suppressor in Drosophila. Human KIBRA has been reported to play a role in cell migration and is known to be phosphorylated by protein kinase C (PKC) zeta.[27-29]

MSTs and LATs members of the SWH pathway have previously been shown to be epigenetically inactivated in certain human cancers. Frequent methylation of *MST2* (96%) was found in head and neck squamous cell carcinoma and less frequently for *LATS1* (24%) and *LATS2* (8%).[30] *MST1* and *MST2* were also methylated in soft tissue sarcomas.[31] *LATS1* and *LATS2* were frequently methylated in human astrocytomas and in breast cancer.[32,33] *LATS2* was also methylated in 24% of acute lymphocytic leukemia.[34] A very recent study highlighted the importance of the hippo signaling in the pathogenesis of B-cell

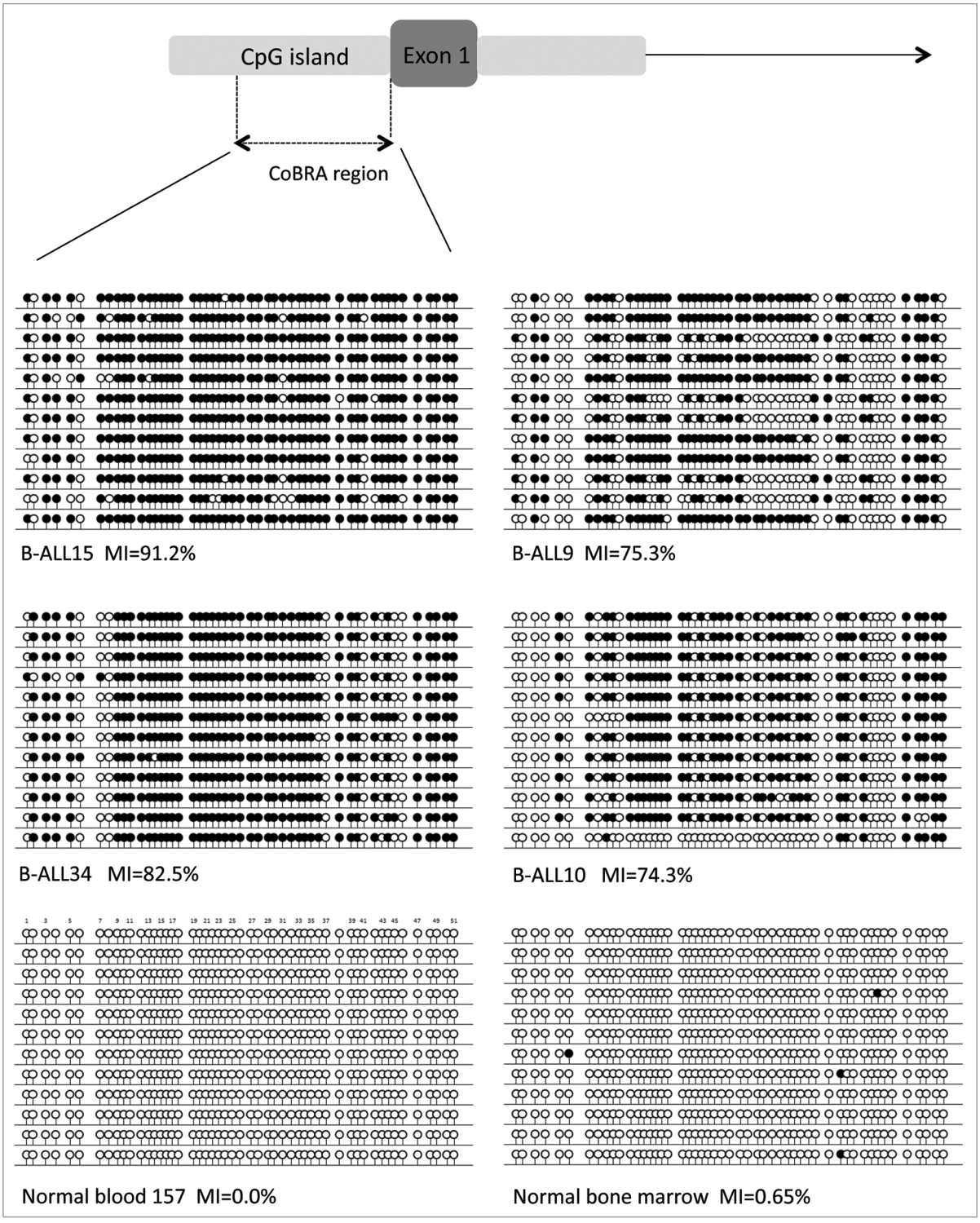

Figure 3. Sequencing of ALL samples and control blood and bone marrow. A schematic of the CpG island encompassing the first exon of *KIBRA* is shown with the CpG island in light grey, exon 1 in dark grey and the region analyzed for methylation by CoBRA and clone sequencing represented by dashed lines. Clone sequencing results are shown for four childhood B-ALL samples (B-ALL15, B-ALL9, B-ALL34 and B-ALL10), one normal blood sample and one normal bone marrow sample. Black circles represent methylated CpGs and white circles represent unmethylated CpGs. The methylation index (MI) is given as a percentage for each sample.

lymphomas and demonstrated for the first time the Hippo pathway as tumor suppressor genes contributing to lymphoma tumorigenesis.[35] Decreased expression of Hippo members *MOBKL2A*, *MOBKL2B* and *LATS2* was associated with inferior survival in mantle cell lymphoma (MCL); furthermore, loss of the genomic regions where the three genes reside was observed in 38% of

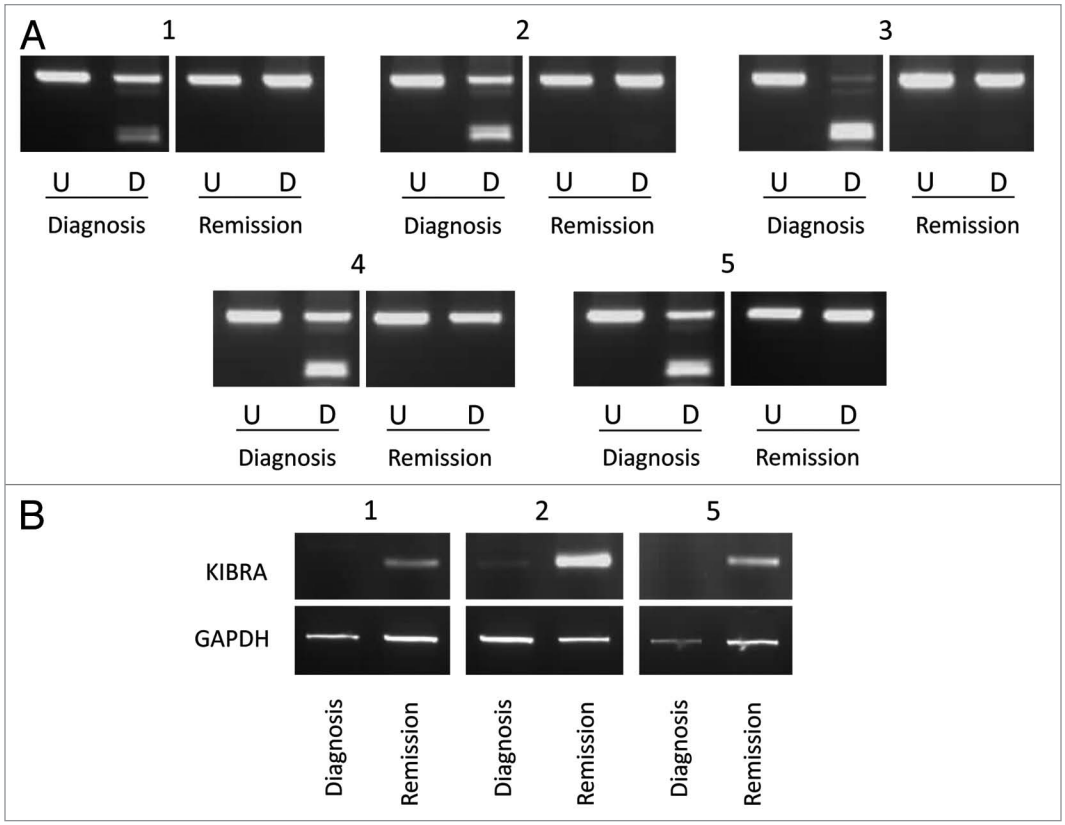

Figure 4. Paired diagnosis and remission analysis. (A) CoBRA results are shown for five childhood ALL patients at diagnosis and remission stages of disease. All samples are shown with undigested product (U) next to digested product (D). (B) Expression results are shown for three of the five patients shown in (A) at diagnosis and remission stages of disease for *KIBRA* and *GAPDH*.

MCLs. *MOBKL2A* and *MOBKL2B* are homologs of the *MOB1* gene that interacts with LATS in inhibition of YAP, a growth promoter.

There has been no comprehensive analysis of the SWH pathway genes in both epithelial and hematological malignancies. Therefore, we have undertaken a detailed epigenetic analysis of *KIBRA* a newly identified member of the SWH pathway in both epithelial and hematological cancers. We describe for the first time frequent methylation of *KIBRA* in B-cell ALL, but not in major epithelial cancers. *KIBRA* gene methylation in childhood B-cell ALL is associated with the most common translocation (*ETV6/RUNX1*) found in this subtype of leukemia and leads to loss of *KIBRA* expression in the malignant state. Roman-Gomez et al. have previously shown that a set of four genes (*DKK3*, *sFRP2*, *PTEN* and *P73*) show methylation specificity for *ETV6/RUNX1* positive B-ALL and suggest that these genes may play important roles in t(12;21) leukemogenesis. Furthermore they demonstrated that patients carrying the *ETV6/RUNX1* translocation showed a higher degree of genes simultaneously methylated compared to *ETV6/RUNX1* negative cases.[36] This suggests that epigenetic inactivation plays a more important role in t(12;21) leukemogenesis compared to other forms of B-ALL. Morrow et al. demonstrated that *ETV6/RUNX1* translocation is necessary but insufficient for leukemia development.[37] The above data suggests that subsequent molecular events occurring in early childhood are required for leukemogenesis, one such mechanism could be epigenetic inactivation.

Our results further indicate that *KIBRA* is a potential tumor suppressor gene not only in Drosophila but also in mammalian cells and plays an important role in B-cell leukemogenesis and may provide a potential new therapeutic target for clinical applications especially due to the reversible nature of promoter hypermethylation. It will be interesting to see if *KIBRA* is methylated in other B-cell leukemias and lymphomas and to understand what role does KIBRA play in B-cell development/differentiation. Our results add further evidence of the Hippo tumor suppressor pathway dysregulation in hematological cancers.

Materials and Methods

Cell lines and primary patient samples. A panel of 49 epithelial cancer cell lines (five breast, seven colorectal, seven glioma, ten kidney, 15 lung and five prostate) and seven leukemia cell lines were used for initial analysis [Jurkat (JKT), NALM6, U937, SUP-T1, CCRF-CEM (CEM), DND-41 and THP-1], in addition 20 glioma tumors were also analyzed. A total of 70 childhood ALL samples were used comprising 52 B-ALL, 1 pre-B-ALL, 12 T-ALL and five unclassified. Characteristics of the ALL samples have been described previously in reference 17. Five diagnosis and remission paired samples were also used for childhood ALL

methylation and expression analyses. Ethical guidelines were followed for all collected samples.

Methylation analysis. DNA was bisulfite-modified as described previously in reference 38. We analyzed a 368 bp region of the *KIBRA* CpG island just upstream of the transcription start site using the following semi-nested primers, forward: 5'-GTA TTT GGY GGA GGT AGA AGT TAT AAA TT-3', reverse nested: 5'-CAT AAT CCR AAA AAT AAC RCC CRC AAA TAA-3' and reverse: 5'-TAT CCC RCR AAT CRA CCA ACT AAT AAT A-3'. Primary and secondary PCR reactions were touchdown PCRs with a final annealing temperature of 55°C and total cycle number of 42 and 47 respectively with 5 μl primary PCR product transferred to the secondary reaction. 15–20 μl of PCR product was digested with BstUI (CGCG) at 37°C overnight prior to visualization on a 2% agarose gel.

Clone sequencing was used to quantify selected CoBRA results. Selected PCR products were cloned into the pGEM-T-Easy vector according to manufacturers' instructions. Single colony PCR was carried out on up to 12 colonies for each cloned sample using the following forward primer: 5'-TAA TAC GAC TCA CTA TAG GG-3'; and reverse primer: 5'-ACA CTA TAG AAT ACT CAA GC-3'. PCR products were then sequenced and methylation indexes (MIs) calculated for each sample as a percentage of the number of methylated CpGs out of the total number of CpGs analyzed per sample.

Expression analysis. Leukemia cell lines were maintained in RPMI1640 (Sigma) with 10% FCS, 2 mM glutamine, 20 mM HEPES, 1 mM sodium pyruvate, 12.6 mM glucose monohydrate at 37°C, 5% CO_2. Genome demethylation was achieved by treatment with 5 μM 5-aza-2'-deoxycitidine over five days with daily media changes. RNA-bee (AMS biotechnology) was used to extract RNA according to manufacturers' instructions. Control bone marrow RNA was purchased from AMS biotechnology. cDNA was synthesized from 1 μg total RNA using Superscript III (Invitrogen) and random hexamer primers (Fermentas). Expression was assessed using a touchdown PCR with a total cycle number of 40 and final annealing temperature of 56°C using the following primers: F: 5'-AAA CAG AGC AGG GAG CTC AA-3' and R: 5'-CCC ATC CAT ATC AGG TGA GG-3'. GAPDH analysis was carried out concurrently with a lower cycle number (32 instead of 40) using the forward primer: 5'-TGA AGG TCG GAG TCA ACG-3' and reverse primer: 5'-CAT GTG GGC CAT GAG GTC C-3'.

Statistical analysis. Statistical analysis was performed as indicated. $p < 0.05$ was considered significant.

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Note

Supplemental materials can be found at:
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