Progressive Chromatin Repression and Promoter Methylation of CTNNA1 Associated with Advanced Myeloid Malignancies

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Abstract

Complete loss or deletion of the long arm of chromosome 5 is frequent in myelodysplastic syndrome (MDS) and acute myelogenous leukemia (AML). The putative gene(s) deleted and responsible for the pathogenesis of these poor prognosis hematologic disorders remain controversial. This study is a comprehensive analysis of previously implicated and novel genes for epigenetic inactivation in AML and MDS. In 146 AML cases, methylation of CTNNA1 was frequent, and more common in AML patients with 5q deletion (31%) than those without 5q deletion (14%), whereas no methylation of other 5q genes was observed. In 31 MDS cases, CTNNA1 methylation was only found in high-risk MDS (2RAEB2), but not in low-risk MDS (-RAEB2), indicating that CTNNA1 methylation might be important in the transformation of MDS to AML. CTNNA1 expression was lowest in AML/MDS patients with CTNNA1 methylation, although reduced expression was found in some patients without promoter methylation. Repressive chromatin marks (H3K27me3) at the promoter were identified in CTNNA1-repressed AML cell lines and primary leukemias, with the most repressive state correlating with DNA methylation. These results suggest progressive, acquired epigenetic inactivation at CTNNA1, including histone modifications and promoter CpG methylation, as a component of leukemia progression in patients with both 5q- and non-5q- myeloid malignancies. [Cancer Res 2009;69(21):8482–90]

Introduction

Myelodysplastic syndrome (MDS) represents a heterogeneous group of clonal bone marrow (BM) failure disorders with significant clinical morbidity and mortality. MDS typically progresses in severity over time with increased BM and peripheral blood (PB) blasts. Cases with >20% blasts are considered acute myeloid leukemia (AML) with tri-lineage dysplasia. The molecular events associated with progression from MDS to AML with tri-lineage dysplasia transformation are unknown (1, 2).

Loss of all (∼5–) or interstitial loss of the long arm of chromosome 5 [del(5q)], either as the sole karyotypic abnormality or part of more complex karyotypes, is frequent and has distinct implications for MDS and AML. The 5q- syndrome has unique clinical features, isolated 5q deletion and <5% BM blasts. With a low probability of leukemic transformation, it has a good prognosis compared with other ∼5/del(5q) disorders (3, 4). In contrast, del(5q) occurring with additional chromosomal abnormalities, or complete loss of chromosome 5, has a median survival of 45 months compared with 146 months with isolated del(5q) (ref. 5).

The recurrent nature of chromosomal deletion suggests that 5q contains tumor suppressor gene(s) important for hematologic transformation. Detailed cytogenetic and molecular analyses have shed light on this complex genomic region (6, 7). Boultwood and colleagues (8) narrowed the common deleted region for good prognosis 5q- syndrome to an ∼1.5-mb interval at 5q33.1, flanked by DSS413 and GLRA1. This region is distinct from the proximal 5q deletion(s) at 5q31 in advanced MDS or AML (9–11). Despite efforts over the past 30 years, no biallelic deletions or point mutations within commonly deleted regions have been found in either 5q- syndrome or complex del(5q), suggesting alternative mechanisms of gene alteration.

One proposed mechanism is haploinsufficiency. Loss of a single allele of EGR1 on 5q31 cooperates with mutations induced by alkylating agents in mouse models of malignant lymphoid and myeloid diseases (12). Distinct from this commonly deleted region, the ribosomal subunit protein RPS14 on 5q33 was identified as a candidate 5q- syndrome gene using RNA interference screening (13), with partial loss of RPS14 phenocopying components of human disease in normal hematopoietic progenitor cells, and forced expression of RPS14 rescuing the disease phenotype in patient-derived BM cells.

Epigenetic changes, including promoter hypermethylation and posttranslational histone modifications, may inactivate tumor suppressor genes. Genes, including p15INK4b/CDKN2B, CDH1/E-cadherin, HIC1, and ER are inactivated by DNA methylation in hematopoietic malignancies (14, 15). The activity of two DNA methyltransferase inhibitors, 5-azacitidine and 2′-deoxy-5-azacytidine (5-aza-dC) in patients with MDS provides an additional rationale for the study of 5q epigenetic changes.

Using a large cohort of hematologic malignancies and a multimodal gene discovery approach, we examine implicated 5q genes, including catenin α-1 (CTNNA1). CTNNA1, identified as a putative ∼5/del(5q) hematopoietic tumor suppressor gene

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/). Requests for reprints: Michael A. McDevitt, Division of Hematology, The Johns Hopkins University School of Medicine, Ross Research Building, Room 1033, 720 Rutland Avenue, Baltimore, MD 21205. Phone: 410-555-0185; Fax: 410-955-0185; E-mail: mnmcdevi1@jhmi.edu and James G. Herman, The Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, 1650 Orleans Street, Room 5M59, Baltimore, MD 21231. Phone: 410-955-8506; Fax: 410-614-9884; E-mail: hermanji@jhmi.edu.

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(16), is expressed at lower levels in the leukemia-initiating stem cells in del(5q) myelogenous leukemia or MDS (17), but previous studies have supported (17) or not detected (18) DNA methylation. Our studies identify CTNNAI as a specific epigenetically inactivated tumor suppressor gene on 5q through multiple repressive mechanisms.

Materials and Methods

Patient samples. PB or BM samples from 146 patients with de novo or secondary AML, 31 MDS, 19 acute lymphocytic leukemia (ALL), 14 chronic myelogenous leukemia, and 15 normal controls were obtained with informed consent as part of Institutional Review Board–approved protocols at Johns Hopkins Sidney Kimmel Comprehensive Cancer Center, University Hospital of Aachen Germany, or the Cleveland Clinic Taussig Cancer Center. BM and PB mononuclear cells (MNC) were Ficoll-Hypaque–purified (Sigma).

Cell culture. HL-60, HNT34, KG1a, KG1, ML-L, and U937 (American Type Culture Collection) were maintained in 90% RPMI 1640 (Invitrogen) with 10% fetal bovine serum, 100 μg/mL of penicillin, and 100 μg/mL of streptomycin. Cells were treated with either 5-aza-dC (Sigma) at a concentration of 1 μmol/L for 3 d with replacement of the medium and 5-aza-dC every 24 h or suberoylanilide hydroxamic acid (SAHA; Upstate Biotechnology) at 2.5 μmol/L for 24 h.

DNA preparation. Genomic DNA from BM or PB MNCs and from AML cell lines were prepared using the previously described proteinase-K method (19).

RNA isolation and semiquantitative reverse transcription-PCR. Total RNA was isolated using Trizol (Life Technologies). First-strand cDNA was synthesized from 5 μg of total RNA using random hexamers with the Superscript First-Strand Synthesis System (Invitrogen). Completed cDNA was diluted to 100 μL with double-distilled water and 2.5 μL of diluted cDNA was used in a 25 μL PCR reaction. Primer sequences and PCR conditions (Supplementary Table S1) spanned intronic sequences between adjacent exons.

Methylation-specific PCR. Genomic DNA from primary leukemia and cell lines was bisulfite-modified by EZ DNA Methylation Kit (Zymo Research). Primer sequences and PCR conditions (Supplementary Table S1) for each methylation-specific PCR (MSP) reaction included ~100 ng of bisulfite-treated DNA, 25 pmol of each primer, 100 pmol of deoxynucleotide triphosphates, 10X PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma) in a final 25 μL volume. MSP products were analyzed on 6% polyacrylamide gels.

Bisulfite sequencing. Bisulfite-treated DNA was amplified with sequencing primers in the CTNNAI promoter; CTNNAI-BTS-forward, 5′-TAGGCTTTTATTACAGTGGTATAATGAGGG-3′; CTNNAI-BTS-reverse, 5′-TACTTTTTCTCCTTCCCCATCCTCTAAAAG-3′ for each methylation-specific PCR (MSP) reaction included ~100 ng of bisulfite-treated DNA, 25 pmol of each primer, 100 pmol of deoxynucleotide triphosphates, 10X PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma) in a final 25 μL volume. MSP products were analyzed on 6% polyacrylamide gels.

Western blot. Whole cell lysates were prepared using radioimmunoprecipitation assay lysis buffer (Sigma-Aldrich) with complete protease inhibitors (Roche Diagnostics GmbH). Protein concentrations were determined by Bio-Rad assay, 40 μg protein electrophoresed on 4% to 12% Bis-Tris Gels (NuPAGE Novex), electrotransferred onto Immobilon-P membrane (Millipore) and blocked with 5% milk/TSA buffer. Anti-CTNNAI (1:7954 (Santa Cruz Biotechnology) 1:100 was fixed by 1:5000 secondary antibody-horseradish peroxidase conjugate, and visualized using Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

Human CTNNAI promoter and luciferase reporter assays. The human CTNNAI promoter, -492 to +394 bp (pGL3-P1.0) from transcription initiation was amplified with primers CTNNAI forward 2, 5′-CTGGGGTACCGTTGTTCCATATGGGATTGA-3′; CTNNAI reverse, 5′-CTTGAATCTCCTGCGGCTATAGTTTCTCC-3′, gel purified, subcloned into the pGL3-Basic vector (Promega) via KpnI and BglII sites, and sequence verified. Promoter activity was measured as described (20), with 5 × 10⁴ HEK293T cells seeded in 24-well plates 24 h before transfection. Human CTNNAI promoter constructs or empty vector were transfected using FuGENE 6 (Roche Applied Science) at 100 ng/well, and pRL-TK vector (Promega) was cotransfected at 8 ng/well as an internal reporter. For in vitro methylated assays, methylated and mock-methylated constructs were transfected at 200 ng/well. Seventy-two hours posttransfection, cells were washed and lysed in passive lysis buffer (Promega). Luciferase activity was measured and transfection efficiency normalized to Renilla luciferase activity using the Dual Luciferase Reporter Assay (Promega).

In vitro DNA methylation. Luciferase reporter plasmid DNA (20 μg) was digested with KpnI and BglII and CTNNAI promoter fragments were gel-purified. KpnI-BglII fragments were treated with M.SsoI methylase in the presence (methylated) or absence (mock-methylated) of 5-adenosylmethionine (New England Biolabs). DNA was phenol-chloroform–extracted and ethanol-precipitated, and complete DNA methylation confirmed by digestion with the methylation-sensitive restriction endonuclease Hpall. Methylated and mock-methylated promoter fragments were ligated into pGL3-Basic vector and used directly for transfection.

Chromatin immunoprecipitation. Cultured cells or MNCs from BM or PB by Ficoll-Hypaque (Sigma) were washed with PBS, resuspended in PBS, and crosslinked in 1% formaldehyde for 10 min at room temperature. Glycine to a final concentration of 0.125 mol/L for 5 min at room temperature quenched crosslinking. Cells were washed with 1× PBS and processed with chromatin immunoprecipitation (ChIP) Assay kit (Upstate). Sonicated DNA (100–130 μg) was used for each immunoprecipitation, using anti–H3 dimethyl-K4, anti–acetyl-Histone H3 (Lys 9), anti–H3 dimethyl-K9, anti–H3 trimethyl-K27 (Upstate), anti–DNMT1 (Imgenex), and anti–DNMT3b (Imgenex), with normal rabbit IgG (2 μg/immunoprecipitated; Upstate) as a control. Fifty microliters of sonicated, preimmunoprecipitated DNA was used as input control. Real-time PCR conditions and primers used are available on request. Twenty-microliter PCR reactions used 2 μl of either immunoprecipitated (bound) DNA or 1:100 dilution of nonimmunoprecipitated (input) DNA. Enrichment compared with input is the average from at least two independent ChIP experiments and multiple PCR analyses (three PCR reactions per independent ChIP).

Statistical analysis. Comparisons were made with Fisher’s exact and χ² tests using STATA statistical software. Results are reported as odds ratios with 95% confidence intervals.

Results

CTNNAI is silenced by hypermethylation and can be induced by 5-aza-dC in myeloid cell lines. Methylation of the promoter regions of five published candidate pathogenesis genes IRF1 (21), SMAD5 (22, 23), EGR1 (12), CTNNAI (16), and NPM1 (24) on chromosome 5q31-5 was examined using MSP. CTNNAI was completely methylated in KG1a (Fig. 1A). CTNNAI was partially methylated in U937 cells, although it was unmethylated in HL60, HNT34, and ML-1 cell lines. There was partial methylation of SMAD5 in U937, but the remaining genes, EGR1, IRF1, and NPM1, were unmethylated in all cell lines. The NPM1 results are consistent with Oki and colleagues (24).

mRNA expression of these genes at baseline shows complete loss of CTNNAI expression only in KG1a, consistent with DNA methylation patterns (Fig. 1B). CTNNAI mRNA was re-expressed after 5-aza-dC treatment of KG1a. The other four candidate genes EGR1, IRF1, SMAD5, and NPM1, were all expressed at baseline in all cell lines, and no major changes in expression were observed following 5-aza-dC.
treatment (Fig. 1B). Using real-time reverse transcription-PCR, KG1a indeed showed complete loss of CTNNA1 expression, whereas CTNNA1 mRNA is expressed at varying levels in leukemia cell lines without DNA methylation (Fig. 1C). CTNNA1 was re-expressed after 5-aza-dC treatment of KG1a, but other cell lines also increased CTNNA1 expression after 5-aza-dC treatment. Western blot confirmed that CTNNA1 mRNA correlated with CTNNA1 protein (Fig. 1D). Expression was highest in HNT34, decreased in HL60, and absent in KG1a, consistent with quantitative mRNA analysis. CTNNA1 protein was detected in KG1a after 5-aza-dC treatment.

The promoter region of CTNNA1 was characterized to determine the mechanisms underlying loss of expression (Fig. 2A). Genomic bisulfite sequencing was used to comprehensively examine DNA methylation in leukemia cell lines (Fig. 2B). Cell lines with detectable CTNNA1 expression by reverse transcription-PCR (HL60, HNT34, and ML-1) showed no CTNNA1 allelic methylation by MSP, and bisulfite sequencing of 5 to 13 individual clones of PCR products revealed methylation of only rare CpGs within the promoter region. In contrast, KG1a had nearly complete 5′ CpG island methylation. Bisulfite sequencing of U937 alleles revealed a hemimethylated status, confirming MSP results. To determine whether methylation of this region was functionally resulting in gene repression, we created a CTNNA1 promoter luciferase reporter transfected into HEK293T cells. Transient transfection of −692/+394 of the human CTNNA1 gene inserted in the pGL3-Basic vector (pGL3-C1.0) showed strong promoter activity (12-fold higher than vector; Fig. 2C). However, in vitro methylation of this construct (pGL3-C1.0M) almost completely abolished promoter activity (Fig. 2C, similar results with −836/+394 construct), suggesting that extensive CpG methylation within this region represses CTNNA1 promoter activity.

CTNNA1 methylation is common in AML patients with del (5q). We examined CTNNA1 methylation in primary myeloid leukemia, in which del(5q) is most common. In 146 individual AML cases, including 26 patients with del(5q) and 120 without del(5q), CTNNA1 methylation was more frequent in del(5q) AML patients (Fig. 3A), compared with those without del(5q) (Fig. 3B; 31% versus 14%, \( \text{P} = 0.047 \); Table 1). No CTNNA1 methylation was present in 15 normal controls. Bisulfite sequencing of primary AML samples (Fig. 3C) is consistent with MSP, showing no CpG methylation in MSP-negative patients (C1 and C2) and variated hypermethylation in MSP-positive patients (C9 and C10). The degree and heterogeneity of methylation in primary leukemias suggests that repression of CTNNA1 progresses through transformation. Although CTNNA1 methylation was more frequent in AML patients with preexisting MDS, unfavorable karyotypes, and secondary AML, none of these associations reached independent statistical significance. In multivariate analysis, only 5q deletion was associated with CTNNA1 methylation (Table 1), with an adjusted odds ratio of 3.5 (95% CI, 1.1 to 11.2; \( \text{P} = 0.043 \)).
CTNNA1 Methylation in AML Transformation

To determine whether CTNNA1 methylation was related to del(5q), or alternatively related to higher methylation frequencies in del(5q) disease, we examined CDKN2B and CDH1 methylation. CDKN2B was methylated in 38% (10 of 26) of AML patients with del(5q) and 55% (62 of 113) without del(5q); CDH1 was methylated in 0% (0 of 26) in AML patients with del(5q) and 16% (18 of 113) in the non-del(5q) group, suggesting that del(5q) are less frequently methylated at other loci. In fact, the lack of methylation of CDH1 in patients with genetic and epigenetic alterations of CTNNA1 is of interest because both genes are part of the same adhesion complex.

To further investigate CTNNA1 as a primary target of inactivation on 5q, published microarray expression data from primary AML cases was examined (25). An individual patient's expression level for genes is represented by a single data point. For CDH1, a biphasic pattern of expression is present, consistent with known repression of CDH1 in AML (ref. 26; Supplemental Fig. S1). A similar pattern is seen for CTNNA1, although with fewer cases (10%) with low expression. This is consistent with the frequency of promoter methylation in AML without −5/del(5q) of 14% because only 4 of 287 cases in this data set have 5q deletions. Among other genes examined for methylation (EGRI, SMAD5, IFI, and NPM1) or the 5q syndrome candidate gene RPS14, only EGRI showed differential expression among the samples, but was not the biphasic pattern seen with CDH1 or CTNNA1. For eight other 5q genes in the deleted region, there was no evidence of gene repression in AML (Supplemental Fig. S2).

CTNNA1 methylation was examined in 31 primary MDS samples. In these 31 samples, whereas 17 have −5/del(5q), methylation of CTNNA1 was detected in only 3 of 31 MDS patients (Table 1). Of note, all three cases of MDS with CTNNA1 methylation had advanced MDS (14–20% blasts), with the difference in CTNNA1 methylation between high-risk MDS (≥RAEB2, 3 of 10; 30%) and low-risk MDS (<RAEB2, 0 of 21; 0%) reaching statistical significance (P = 0.03). This suggests that CTNNA1 promoter methylation may be associated with progression within MDS and from MDS to AML.

Figure 2. Bisulfite sequencing of CTNNA1 and direct repression by DNA methylation. A, schematic of the CTNNA1 promoter CpG island. Vertical lines, individual CpG sites. TS, transcriptional start site; double-headed arrows, amplification for MSP, bisulfite sequencing (BSS), and location for ChIP primers. The region of the human CTNNA1 promoter for luciferase assays (−692 to −394 bp). B, bisulfite sequencing of CTNNA1 in AML cell lines. Filled circles, methylated CpG sites; open circles, unmethylated CpG sites. CpG sites are numbered relative to the transcription start site. C, inhibition of CTNNA1 promoter by CpG methylation. Top, luciferase activity of wild-type human CTNNA1 promoter, with empty pGL3 as negative control. Fold increase was calculated relative to control cells. Bottom, luciferase activity of the in vitro–methylated construct, pGL3-C1.0M compared with mock-methylated, pGL3-C1.0U (percentage relative to mock). Columns, average of two independent experiments performed in triplicate; bars, SD.
(≥RAEB2, 8 of 10 MDS; 80%) compared with low-risk MDS (-RAEB2, 9 of 21 MDS; 43%), consistent with previous reports.

To further investigate the specificity of CTNNA1 methylation among 5q genes, we examined primary AML samples for other 5q genes. No EGR1 methylation was detected in 24 primary MDS cases (8 with 5q loss) nor in 86 primary AML (15 with 5q loss) cases. In addition, methylation was not seen at the promoter regions for IRF1, SMAD5, and NPM1 in 34 AML samples (15 with 5q loss). Although loss of 5q is of particular clinical significance for MDS and AML, recurrent loss of the long arm of chromosome 5 is also found in other malignancies (30–32) and diminished expression of CTNNA1 has been reported in gastric and esophageal cancer (33–35). We examined whether CTNNA1 was hypermethylated in other cancer phenotypes. In 41 solid tumor cancer cell lines and in 99 primary gastric and esophageal cancer samples (Table 1), no methylation of CTNNA1 was detected, suggesting that methylation of CTNNA1 is specific to myeloid malignancies.

Reduced CTNNA1 expression associated with DNA methylation and chromatin changes. The observed correlation of promoter methylation to repression of CTNNA1 mRNA in KG1a, led us to examine CTNNA1 mRNA expression in a subset of AML cases with sufficient RNA. Normal MNCs express high levels of CTNNA1 (Fig. 3D). In contrast, nine AML patient samples with CTNNA1 methylation had much lower expression. Unexpectedly, primary AML without CTNNA1 methylation had a mean expression lower than normal MNCs, and a number of these AML had repressed CTNNA1.

These results suggest multiple genetic (deletion) and epigenetic alterations leading to CTNNA1 inactivation in hematologic malignancies. One model to integrate these observations would be progressive inactivation of the CTNNA1 locus, with CpG promoter methylation as the final and most definitive inactivation. To test this hypothesis, we investigated the KG1/KG1a system because the more well-differentiated KG1 is the origin of KG1a (36), and there is progressive inactivation of CDKN2B (37). Both cell lines have identical karyotypic abnormalities including monosomy 5 (38), but KG1a cells are morphologically and functionally less differentiated than KG1. KG1 has no DNA methylation, but nearly undetectable CTNNA1 expression (Fig. 4A and B). However, unlike KG1a, expression could be restored either by 5-aza-dC or the HDAC inhibitor SAHA, an observation consistent with a repressed but unmethylated gene (39). In contrast, in KG1a, CTNNA1 is completely methylated and silenced, and could only be induced by 5-aza-dC. This suggests that transcriptional repression in KG1 “progressed” to complete silencing associated with DNA methylation in KG1a.

ChIP was used to examine the CTNNA1 promoter using two active (H3K9Ac and H3K4me2) and two inactive (H3K9me2 and H3K27me3) histone marks in leukemia cell lines (Fig. 4C). The unmethylated cell line HNT34, with high CTNNA1 expression, has enrichment of the active mark H3K4me2, and to a lesser degree, H3K9Ac, whereas inactive marks were absent. In contrast, CTNNA1 methylation in KG1a was accompanied by the enrichment of inactive marks, H3K9me2 and H3K27me3, and depletion of active marks. Of greatest interest were HL60 and KG1, in which CTNNA1 is unmethylated and decreased expression was accompanied by a mix of active and inactive marks. This mixed chromatin phenotype has recently been termed bivalent chromatin (40, 41), which is

Figure 3. CTNNA1 methylation in AML and diminished expression. A, representative MSP results of CTNNA1 in primary AML patients with del(5q). B, representative MSP results of CTNNA1 in primary AML patients without del(5q). C, bisulfite sequencing of CTNNA1 in AML patients. C1 and C2 were AML patients without CTNNA1 methylation detected by MSP; C9 and C10 were AML patients with CTNNA1 methylation. D, quantitative analysis of CTNNA1 expression levels in MNCs from normals and individuals with or without CTNNA1 methylation. Real-time PCR was performed as described in Materials and Methods. CTNNA1 expression levels were normalized to GAPDH and calculated relative to HNT34. The results were presented by box and whisker plot. The plots show CTNNA1 expression levels in two patient groups with different CTNNA1 methylation status and one healthy control group. Lines within boxes, median values; diamonds, mean; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the maximum and minimum value, respectively.
characteristic of cancer genes predisposed to aberrant DNA methylation (42). Our results are consistent with histone deacetylation and/or methylation establishing condensed chromatin and transcriptional repression of CTNNA1 (HL60 and KG1), which may result in promoter DNA methylation and complete gene inactivation (KG1a).

To explore the mechanism of CTNNA1 activation by 5-aza-dC in unmethylated cell lines, we performed ChIP for DNA methyltransferases 1 (DNMT1) and DNMT3b (Fig. 4D). Although as expected, DNMT1 is present at the CTNNA1 promoter in DNA-methylated KG1a, it was also present, but with less enrichment, in unmethylated HNT34, HL60, and KG1. DNMT1 enrichment directly correlates with

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<sup>†</sup>Those two MDS cases are RAEB-t, which were reclassified by the WHO as AML.
<sup>‡</sup>There was no age information available for 14 patients.
<sup>§</sup>Karyotypic features: favorable, t(8;21); inv(16)/t(16;16); t(15;17); intermediate, normal karyotype and less than three chromosomal abnormalities; adverse, complex karyotype, ~7–5, del(5q), and MLL gene rearrangements (11q23). There were seven patients lacking karyotypic data.
<sup>§</sup>Model adjusted by MDS status, 5q deletion, age, and AML subtype.
CTNNA1 repression. In contrast, ML-1, with no increase in CTNNA1 expression with 5-aza-dC treatment, DNMT1 is absent. DNMT3b was not detected at the CTNNA1 promoter in any cell line. This suggest that DNMT1 plays a critical role in initiating and/or maintaining DNA repression of CTNNA1 in AML cell lines, which is relieved by 5-aza-dC. This occupancy may position DNMT1 to initiate promoter DNA methylation, leading to complete gene silencing.

**In vivo chromatin at the CTNNA1 promoter in primary AML.**

We examined chromatin at the CTNNA1 promoter in primary AML using ChIP. Ten freshly collected samples with an initial diagnosis of AML had sufficient number and purity of blasts, including cases with del(5q) (n = 3) and with CTNNA1 methylation (n = 2; Supplemental Table S2). High CTNNA1 expression cases lack del(5q), and lack CTNNA1 DNA methylation (Fig. 5). Consistent with high CTNNA1 expression, H3K9Ac was enriched (active chromatin mark) and H3K27me3 depleted (repressive mark). In contrast, seven AML cases with lower CTNNA1 expression (samples 4–10) had lower levels of H3K9Ac and greater enrichment of H3K27me3. The greatest enrichment of H3K27me3 was found in sample 10, which also had CTNNA1 promoter methylation (Fig. 5B–D). Thus, repressive chromatin is present at the CTNNA1 promoter in some primary AML, and in the most repressed transcriptional state, associated with promoter DNA methylation.

**Discussion**

The search for the gene(s) associated with deletions of −5/del(5q) and responsible for the pathogenesis of MDS and AML has been long, and has not reached consensus. Complicating this search are clinical differences between 5q loss in "the 5q syndrome" and del(5q) associated with complex karyotypes. Through examination of epigenetic alterations, our data point to CTNNA1 as a critical target for loss of function in 5q deletions associated with MDS/AML.

CTNNA1 is a cytoplasmic adhesion protein forming a trimolecular complex with CDH1 and β-catenin. CTNNA1 was proposed as a candidate 5q tumor suppressor gene (16), but additional evidence has been challenging. Reduced expression of CTNNA1 in MDS and AML patients with del(5q) compared with those without del(5q) has been reported but without a molecular explanation (43). Studies associating reduced CTNNA1 expression with DNA methylation (17) or without any evidence of DNA methylation (18) have recently been published. These studies were relatively small in sample size.

**Figure 4.** Progressive repression and silencing of CTNNA1. A, MSP of CTNNA1 in KG1 and KG1a before and after treatment with 5-aza-dC or SAHA. B, quantitative real-time expression of CTNNA1 in KG1 and KG1a before and after treatment with 5-aza-dC or SAHA. CTNNA1 expression was normalized to GAPDH and calculated relative to HNT34 (columns, mean from at least two independent PCR in triplicate; bars, SD). CTNNA1 is induced by 5-aza-dC or SAHA in KG1, but only by 5-aza-dC in KG1a. C, ChIP of histone modifications at the CTNNA1 promoter in AML cell lines. Cell lines are displayed in order of descending expression of CTNNA1, with DNA methylation (bottom). The CTNNA1 promoter has enrichment of repressive chromatin in a DNA hypermethylated and silent state (KG1a) compared with when unmethylated and expressed (HNT34), with reciprocal enrichment of active marks. H3K27me3 was also enriched in KG1, in which CTNNA1 is not methylated but expression is low. Enrichment was normalized to total input (columns, mean from at least two independent ChIP experiments and multiple independent PCR analyses; bars, SD). D, ChIP of DNMT1 and DNMT3b occupancy at the CTNNA1 promoter in AML cell lines. DNMT1 shows greatest enrichment at the promoter of CTNNA1 in KG1a, lower enrichment in HNT34, HL60, and KG1 cell line, and is not present in ML-1. DNMT3b was not present at the CTNNA1 promoter (columns, mean from at least two independent ChIP experiments and multiple independent PCR analyses; bars, SD).
size, with 12 patients with del(5q) and 10 without del(5q) analyzed in the former study, and only 6 del(5q) samples examined for methylation in the latter study.

Our comprehensive analysis of CTNNA1 for copy number, DNA methylation, chromatin, and gene expression in a large cohort of 31 MDS and 146 AML patients resolves this controversy and provide a molecular explanation. CTNNA1 methylation occurs more frequently in patients with del(5q) AML, but is not exclusive to del (5q). Reduced expression of CTNNA1 is more frequent than DNA methylation and is associated with repressive chromatin marks. This unique picture of progressive silencing is supported by leukemia cell lines, and for the first time, primary leukemia ChIP analysis. CTNNA1 methylation exclusively in high-risk, but not in low-risk MDS, provides an unusual insight into progression of this disease, because more often, loci are methylated in both MDS and AML (29, 44, 45).

Repression and silencing of CTNNA1 results from an interplay between DNA methylation and changes in histone marks, notably enrichment of H3K27me3. Our results show the functional role of DNA methylation in this region in silencing CTNNA1 expression. However, repression with low levels of methylation and in leukemias without DNA methylation suggest that progressive epigenetic silencing does not initiate with DNA methylation. Methylated H3K27 serves as an anchorage point for the recruitment of EZH2-containing polycomb group proteins (46), the binding of which contributes to the formation of repressive chromatin. CTNNA1, but not other 5q genes (IRF1, SMAD5, EGR, and NPM1) without DNA methylation in AML is polycomb group–marked (47), providing further support for repressive marks promoting epigenetic silencing (48). Interaction of polycomb group complexes with DNA methyltransferases could facilitate CpG methylation (46). Demonstration of DNMT1 occupancy of the CTNNA1 promoter revealed no significant difference in enrichment. D, enrichment of the repressive mark (H3K27me3) was seen in patients with diminished expression, with or without CTNNA1 DNA methylation.

Disclosure of Potential Conflicts of Interest
J.G. Herman: consultant, OncoMethylome Sciences. The other authors disclosed no potential conflicts of interest.

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References

Figure 5. ChIP assay of histone marks at the CTNNA1 promoter in primary AML patients. A, quantitative expression of CTNNA1 in AML patients. 1, 2, and 3, patients with normal CTNNA1 expression and without methylation; 4, 5, 6, 7, and 8, patients with decreased CTNNA1 expression and without methylation; 9 and 10, low CTNNA1 expression and methylation. CTNNA1 expression levels were normalized to GAPDH and calculated relative to HNT34. B, enrichment of active mark H3K9Ac on the CTNNA1 promoter (500 bp upstream of the transcriptional start site) was greatest in patients with normal CTNNA1 expression and without DNA methylation and least in patients with decreased CTNNA1 expression and with methylation. C, enrichment of H3K4me2 on the CTNNA1 promoter revealed no significant difference in enrichment. D, enrichment of the repressive mark (H3K27me3) was seen in patients with diminished expression, with or without CTNNA1 DNA methylation.
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