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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 hematological 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%), while no methylation of other 5q genes was observed. In 31 MDS cases, *CTNNA1* methylation was only found in high risk MDS (≥RAEB2), but not in low risk MDS (<RAEB2), indicating *CTNNA1* methylation may 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.

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Keywords

del(5q); monosomy 5; *CTNNA1*; methylation; myelodysplastic syndrome; acute myelogenous leukemia; methylation; Progressive silencing; AML transformation

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 greater than 20% blasts are considered acute myeloid leukemia with tri-lineage dysplasia (AML-TLD). The molecular events associated with progression from MDS to AML-TLD transformation are unknown (1,2).

Loss of all (−5) or interstitial loss of the long arm of chromosome (Ch)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 to other −5/del(5q) disorders (3,4). In contrast, del (5q) occurring with additional chromosomal abnormalities, or complete loss of Ch 5, has a median survival of 45 months compared to 146 months with isolated del(5q) (5).

The recurrent nature of chromosomal deletion suggests that 5q contains tumor suppressor gene(s) important to hematologic transformation. Detailed cytogenetic and molecular analyses have shed light on this complex genomic region (6,7). Boultwood et al. (8) narrowed the common deleted region (CDR) for good prognosis 5q-syndrome to an approximately 1.5-megabase interval at 5q33.1, flanked by *D5S413* 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 CDRs have been found in either 5q- syndrome or complex del (5q), suggesting alternative mechanisms of gene alteration.

One proposed mechanism is haploinsufficency. 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 CDR, 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 post-translational histone modifications, may inactivate tumor suppressor genes. Genes, including $p15^{INK4b}$*/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 rational for the study of 5q epigenetic changes.

Using a large cohort of hematological malignancies and a multimodal gene discovery approach, we examine implicated 5q genes, including *Catenin, alpha-1*(*CTNNA1).* *CTNNA1*, identified as a putative −5/del(5q) hematopoietic tumor suppressor gene (16), is expressed at lower levels in the leukemia-initiating stem cells in del(5q) ML or MDS (17), but previous studies have supported (17) or not detected (18) DNA methylation. Our studies

identify CTNNA1 as a specific epigenetically inactivated tumor suppressor gene on 5q through multiple repressive mechanisms.

Materials and Methods

Patient Samples

Peripheral blood (PB) or bone marrow (BM) samples from 146 patients with *de novo* or secondary AML, 31 MDS, 19 acute lymphocytic leukemia (ALL), 14 chronic myelogenous leukemia (CML), and 15 normal controls were obtained with informed consent as part of IRB 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 (MNCs) were Ficoll-Hypaque purified (Sigma).

Cell Culture

HL-60, HNT34, KG1a, KG1, ML-1, and U937 (ATCC) were maintained in 90% RPMI 1640 medium (Invitrogen) with 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were treated with either 5-aza-dC (Sigma) at a concentration of 1 μM for three days with replacement of the medium and 5-aza-dC every 24 hours or Suberoylanilide Hydroxamic Acid (SAHA, Upstate Biotechnology) at 2.5μM for 24 hours.

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 Semi-quantitative RT-PCR

Total RNA was isolated using Trizol (Life Technologies). First strand cDNA was synthesized from 5μg total RNA using random hexamers with the Superscript™ First-Strand Synthesis System (Invitrogen). Completed cDNA was diluted to 100μl with ddH_2O and 2.5 μl diluted cDNA used in a 25μl PCR reaction. Primer sequences (Supplementary Table 1) spanned intronic sequences between adjacent exons. *GAPDH* was amplified with 25 cycles. Amplified products were analyzed on 2% agarose gels.

Methylation-Specific PCR (MSP)

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 1) for each MSP reaction included approximately 100ng of bisulfite-treated DNA, 25pmoles of each primer, 100pmoles dNTPs, 10X PCR buffer, and 1 unit of JumpStart Red Taq Polymerase (Sigma) in a final 25μl volume. MSP products were analyzed on 6% polyacrylamide gels.

Bisulfite Sequencing

Bisulfite-treated DNA was amplified with sequencing primers in the CTNNA1 promoter: CTNNA1-BTS-forward, 5′-TAGGGGTTATTTTYGGTTTAAGTTTTTATTAGGGG-3′; CTNNA1-BTS-reverse, 5′-TACTTTATCTCCCTCCAATCCRACTAAAAA. PCR products were gel purified and cloned into pCR2.1-TOPO vector (Invitrogen). Plasmids from single colonies were purified using QIAprep Spin Miniprep Kit (Qiagen) and sequenced with M13 reverse primers (Johns Hopkins Sequencing Facility).

Real time PCR

Real-time RT-PCR used the QuantiTecti™ SYBR Green PCR kit (Qiagen) in an iCycler Optical Module (Bio-Rad), with 2.5μL cDNA per reaction in a volume of 25 μL. Experiments were performed in triplicate with primers forCTNNA1 and glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*). CTNNA1 expression was normalized to *GAPDH*: relative expression = $2^{-(\text{Sample } \Delta Ct - \text{Control } \Delta Ct)}$, where ΔCt= average *C*t (CTNNA1) − average *C*t (*GAPDH*).

Western Blot

Whole cell lysates were prepared used RIPA lysis buffer (Sigma-Aldrich) with complete protease inhibitors (Roche Diagnostics GmbH). Protein concentrations were determined by BIO-RAD assay (Bio-Rad), 40μg protein electrophoresed on 4–12% Bid-Tris Gels (NuPAGE Novex), electrotransferred onto Immobilon-P membrane (Millipore) and blocked with 5% milk/TSA buffer. Anti-CTNNA1 c-7894 (Santa Cruz Biotechnology) 1:100 was followed by 1:5000 secondary antibody-horseradish peroxidase conjugate, and visualized using Supersignal West Femto Maximum Sensitivity Substrate (Pierce).

Human CTNNA1 promoter and luciferase reporter assays

The human *CTNNA1* promoter, −692 to +394 bp (pGL3-P1.0) from transcription initiation, was amplified with primers CTNNA1forward 2: 5′-CTGGGGTACCGGTGTTTCCATCTGTGGAGTGA-3′; CTNNA1reverse: 5′-CTGAAGATCTCGCTGGGCCTATAGTTTCTCC-3′, gel purified, subcloned into the pGL3-Basic vector (Promega) via *Kpn*I and *Bgl*II sites, and sequence verified. Promoter activity was assessed as described (20), with 5×10^4 HEK293T cells seeded in 24-well plates 24h before transfection. Human *CTNNA1* 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. 72 hours post-transfection, 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 *Kpn*I and *Bgl*II, and *CTNNA1* promoter fragments gel purified. *Kpn*I-*Bgl*II fragments were treated with M. *SssI* methylase in the presence (methylated) or absence (mock-methylated) of S-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 *Hpa*II. Methylated and mock-methylated promoter fragments were ligated into pGL3-Basic vector and used directly for transfection.

Chromatin Immunoprecipitation (ChIP)

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 (RT). Glycine to a final concentration of 0.125M for 5 min at RT quenched crosslinking. Cells were washed with 1x PBS and processed with ChIP Assay kit (Upstate). One hundred to 130 μg of sonicated DNA were used for each immunoprecipiration, using anti-H3 dimethyl-K4 (07–030), anti-acetyl-Histone H3 (Lys 9) (07–352), anti-H3 dimethyl-K9 (07–441), anti-H3 trimethyl-K27 (07–449) (Upstate), anti-DNMT1(IMG-261A, IMGENEX) and anti-DNMT3b (IMG-184A, IMGENEX), with normal rabbit IgG (2 μg/IP; Upstate) as a control. Fifty μl of sonicated, pre-IP DNA was used as input control. Real Time PCR

conditions and primers used are available upon request. 20 μL PCR reactions used 2 μL of either immunoprecipitated (bound) DNA or 1:100 dilution of non-immunoprecipitated (input) DNA. Enrichment compared to 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 χ^2 tests using STATA statistical software. Results are reported as odds ratios (OR) with corresponding 95% confidence intervals (CIs).

Results

CTNNA1 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), *CTNNA1* (16), and *NPM1* (24) on chromosome 5q31-5 was examined using MSP. *CTNNA1* was completely methylated in KG1a (Figure 1A). *CTNNA1* was partially methylated in U937 cells, while 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 et al. (24).

mRNA expression of these genes at baseline demonstrates complete loss of *CTNNA1* expression only in KG1a, consistent with DNA methylation patterns (Figure 1B). *CTNNA1* mRNA was re-expressed after 5-aza-dC treatment of KG1a. The other 4 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 (Figure 1B). By real-time RT-PCR, KG1a indeed has complete loss of *CTNNA1* expression, while *CTNNA1* mRNA is expressed at varying levels in leukemia cell lines without DNA methylation (Figure 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 (Figure 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 (Figure 2A). Genomic bisulfite sequencing was used to comprehensively examine DNA methylation in leukemia cell lines (Figure 2B). Cell lines with detectable *CTNNA1* expression by RT-PCR (HL60, HNT34, and ML-1) demonstrated no *CTNNA1* allelic methylation by MSP, and bisulfite sequencing of 5–13 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 hemi-methylated 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) demonstrated strong promoter activity (12 fold higher than vector, Figure 2C). However, *in vitro* methylation of this construct (pGL3-C1.0M) almost completely abolished promoter activity (Figure 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, where 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 (Figure 3A), compared to those without del(5q) (Figure 3B), (31% vs. 14%, p=0.047, Table 1). No *CTNNA1* methylation was present in 15 normal controls. Bisulfite sequencing of primary AML samples (Figure 3C) is consistent with MSP, showing no CpG methylation in MSP negative patients (C1 and C2) and variegated 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 3.13, (95% CI 1.07–9.13, p=0.037). No methylation of *CTNNA1* was found in either CML or ALL.

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% (10/26) of AML patients with del(5q) and 55% (62/113) without del(5q); *CDH1* was methylated in 0% (0/26) in AML patients with del(5q) and 16% (18/113) in 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, since 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 (26) (Supplemental Figure 1). 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%, since only 4 of 287 cases in this data set have 5q deletions. Among other genes examined for methylation (*EGR1*, *SMAD5*, *IF1*, *NPM1*) or the 5q syndrome candidate gene *RPS14*, only *EGR1* demonstrated 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 Figure 2).

CTNNA1 methylation was examined in 31 primary MDS samples. In these 31 samples, while 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% to 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. To determine whether these samples were suitable for detection of *CTNNA1* methylation, *p15/CDKN2B* methylation was examined, since it is frequent in MDS and AML (27–29). Seventeen of 31 (55%) MDS samples had *CDKN2B* methylation, with a trend towards a higher frequency of methylation in high risk MDS (≥RAEB2, 8 of 10 MDS, 80%), than 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* (Figure 3D). In contrast, 9 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 have repressed *CTNNA1*.

These results suggest multiple genetic (deletion) and epigenetic alterations leading to *CTNNA1* inactivation in hematological 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, since 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 (Figure 4A and 4B). 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 can be induced only 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 (Figure 4C). The unmethylated cell line HNT34, with high *CTNNA1* expression, has enrichment of the active mark H3K4me2, and to a lesser degree, H3K9Ac, while inactive marks were absent. In contrast, *CTNNA1* methylation in KG1a was accompanied by enrichment of inactive marks, H3K9me2 and H3K27me3, and depletion of active marks. Of greatest interest were HL60 and KG1, where *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 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, 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 (Figure 4D). While 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 *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 the 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 2). High *CTNNA1* expression cases lack del(5q), and lack *CTNNA1* DNA methylation (Figure 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 (Figure 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 to 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 been recently published. These studies were relatively small in sample size, with 12 patients with del(5q) and ten 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 resolve 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, but not low risk MDS, provides an unusual insight into progression of this disease, since 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 demonstrate 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 progressive epigenetic silencing does not initiate with DNA methylation. Methylated H3K27 serves as an anchorage point for the recruitment of EZH2-

containing Polycomb group (PcG) proteins (46), whose binding contributes to formation of repressive chromatin. *CTNNA1*, but not other 5q genes (*IRF1*, *SMAD5*, *EGR*, and *NPM1*) without DNA methylation in AML, is Polycomb marked (47), providing further support for repressive marks promoting epigenetic silencing (48). Interaction of PcG complexes with DNA methyltransferases could facilitate CpG methylation (46). Demonstration of DNMT1 occupancy of the *CTNNA1* promoter in cell lines with repressed, but unmethylated *CTNNA1*, support this association. The discovery of intermediate stages of decreased *CTNNA1* expression and repressed chromatin marks without DNA methylation has important implications for epigenetic therapy, particularly those combining histone deacetylase and DNA methylation inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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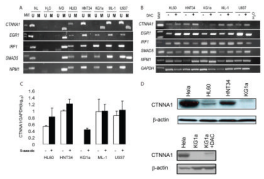

Figure 1. *CTNNA1* is hypermethylated and repressed in AML cell lines

(A) Methylation of 5q31-5 genes in AML cell lines using MSP. (U), unmethylated allele, (M), methylated allele. Normal human lymphocytes (NL) and *in vitro* methylated DNA (IVD) are negative and positive methylated controls. (B) Expression of 5q31-5 genes in AML cell lines before (−) and after (+) treatment with 1 μmol/L 5-aza-dc for 72 hours by semi-quantitative RT-PCR, with *GAPDH* expression for control. (C) Quantitative real-time expression of *CTNNA1* in AML cell lines, normalized to *GAPDH* and calculated relative to HNT34. Data is the mean±SD from at least two independent PCR in triplicate. (D) Western blot of CTNNA1 protein in AML cell lines. Hela is the positive control for CTNNA1 protein expression, and β-actin is a loading control.

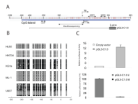

Figure 2. Bisulfite sequencing of *CTNNA1* and direct repression by DNA methylation

(A) Schematic of the *CTNNA1* promoter CpG island. Vertical lines, individual CpG sites. Transcriptional start site (TS) and double-headed arrow show 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) is shown. (B) BSS of *CTNNA1* in AML cell lines. Filled circles represent methylated CpG sites, and open circles denote 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 to mock-methylated, pGL3-C1.0U (percentage relative to mock). Data represent the average ± SD of two independent experiments performed in triplicate.

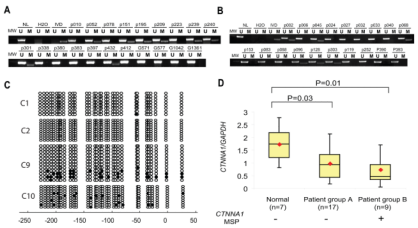

Figure 3. *CTNNA1* methylation in AML and diminished expression

(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) BSS 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 normal 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. In these plots, lines within boxes represent median values and the diamonds indicate the 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.

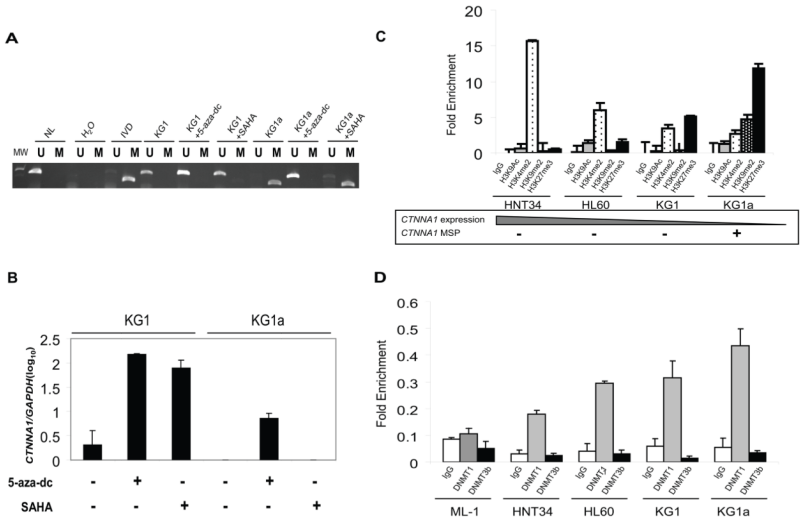

Figure 4. Progressive repression and silencing of *CTNNA1*

(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 (mean±SD from at least two independent PCR in triplicate). *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 shown on the bottom. The *CTNNA1* promoter has enrichment of repressive chromatin in a DNA hypermethylated and silent state (KG1a) compared to 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 (mean±SD from at least two independent ChIP experiments and multiple independent PCR analyses). (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. Data are the mean±SD from at least two independent ChIP experiments and multiple independent PCR analyses.

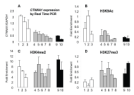

Figure 5. ChIP assay of histone marks at the *CTNNA1* promoter in primary AML patients

(A) Quantitative expression of *CTNNA1* in AML patients. One, 2, and 3 are patients with normal *CTNNA1* expression and without methylation; 4,5,6,7, and 8 are patients with decreased *CTNNA1* expression and without methylation; 9 and 10 have 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 (500bp upstream 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.

Table 1

CTNNA1 Methylation

	Methylated	Unmethylated	Crude			Adjusted		
Normal Lymphocytes (n=15)	0 (0%)	15 (100%)						
Hematological malignancies								
MDS(n=31)								
5q deletion (n=18)	2 (11%)	16 (89%)						
<10% blasts(n=12)	0	12						
10%<blasts<19%(n=3)	1	2						
>20%blasts (n=3)	1 [1]	2						
non 5q deletion (n=13)	1 (8%)	12 (92%)						
<10% blasts(n=9)	0	9						
10%<blasts<19%(n=2)	0	2						
>20%blasts (n=2)	1 [1]	1						
AML (n=146)			OR	95% CI	p value	OR[4]	95% CI	p value
non 5q deletion (n=114)	16 (14%)	98(86%)	1.00	referent		1.00	referent	
5q deletion (n=26)	8 (31%)	18 (69%)	2.72	1.01 – 7.30	0.047	3.13	1.07 – 9.13	0.037
AML w/o preexisting MDS (n=87)	13 (15%)	74 (85%)	1.00	referent		1.00	referent	
MDS/AML (n=59)	11 (19%)	48 (81%)	1.30	0.54 – 3.15	0.55	0.72	0.20 – 2.67	0.63
De novo (n=65)	8 (12%)	57 (82%)	1.00	referent		1.00	referent	
Secondary AML (n=81)	16 (20%)	65 (80%)	1.75	0.70 – 4.40	0.23	1.61	0.43 – 6.01	0.48
<60[2] (n=54)	9 (17%)	45 (83%)	1.00	referent		1.00	referent	
≥60 (n=78)	13 (17%)	65 (83%)	1.04	0.41 – 2.63	0.94	1.09	0.39 – 3.00	0.87
Favorable Karyotype[3] (n=13)	0 (0%)	13 (100%)						

Intermediate Karyotype(n=61)	9 (15%)	52 (85%)	
Adverse Karyotype(n=65)	15 (23%)	50 (77%)	
CML (n=14)	0 (0%)	14 (100%)	
ALL (n=19)	0 (0%)	19 (100%)	
Non-Hematological malignancies			
Colon cell lines (n=8)	0 (0%)	8 (100%)	
Lung cell lines (n=9)	0 (0%)	9 (100%)	
Liver cell lines (n=6)	0 (0%)	6 (100%)	
Cervix cell lines (n=6)	0 (0%)	6 (100%)	
Breast cell lines (n=7)	0 (0%)	7 (100%)	
Gastric cancer (n=51)	0 (0%)	51 (100%)	
Esophageal cancer (n=48)	0 (0%)	48 (100%)	

[1]Those two MDS cases are RAEB-t which were reclassified by WHO as AML.

[2]There are no age information available for 14 patients.

[3]Karyotypic features: Favorable, t(8;21); inv(16)/t(16;16):t(15;17); Intermediate, normal karyotype and less than 3 chromosomal abnormalities; Adverse, Complex karyotype, −7, −5, del(5q), and MLL gene rearrangements (11q23). There are 7 patients lacking karyotypic data.

[4]Model adjusted by MDS status, 5q deletion, Age, and AML subtype

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