t(11;14)-positive mantle cell lymphomas lacking cyclin D1 (CCND1) immunostaining because of a CCND1 mutation or exclusive expression of the CCND1b isoform

Ingram Iaccarino,[1,2] Lamis Afify,[1] Sietse M. Aukema,[1] Katharina Reddemann,[1] Philipp Schütt,[3] Martin Flür[4] and Wolfram Klapper[1]

[1]Department of Pathology, Hematopathology Section and Lymph Node Registry, University of Kiel, Kiel, Germany; [2]Institute of Genetics and Biophysics, "A. Buzzati-Traverso", Consiglio Nazionale delle Ricerche, Naples, Italy; [3]Onkologische Schwerpunktpraxis Gütersloh, Germany and [4]Helios Klinikum Schwelm, Germany

Correspondence: iiaccarino@medgen.uni-kiel.de
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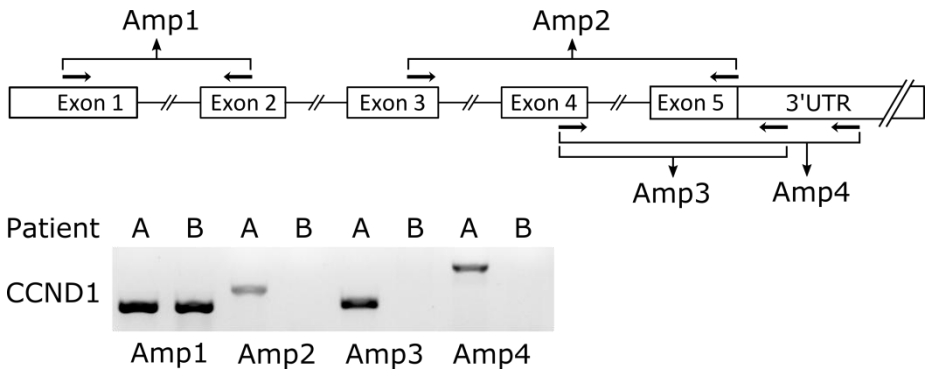
Supplementary Figure 1



Supplementary Figure 1: PCR analysis of CCND1 expression in Patient A & B. PCR analysis of CCND1 expression in FFPE extracted RNA from patients A and B. One microliter of cDNA was amplified in a 20 µl reaction containing 4 mM MgCl2, 1 X Q solution (Qiagen), 0.25 mM dNTPs, 0.25 µM each primer, 1.5 Units of AmpliTaq Gold™ DNA Polymerase (ThermoFisher Scientific) and 1 X reaction buffer II with 60 °C as annealing temperature and 45 cycles. Amplicon 1 (Amp1) is a measure of total CCND1 and was amplified using primers D1EX1_fw and D1EX2_rev2. Amplicons 2, 3 and 4 are meant to investigate the extent of exon 5 expression in RNA extracted from both patient A and B. Amp2 was amplified using primers D1EX3_fw2 and D1CDS_rev. Amp3 was amplified using primers D1EX4_fw2 and D1EX5_rev and Amp4 was amplified using primers D1EX4_fw2 and D13UTR_rev. The sequence of the primers used is provided in supplementary Table 1.

Supplementary Materials and Methods

Immunohistochemical analysis. The expression of cyclin D1 (clone SP4, Thermo Fisher Scientific), CD20 (clone L26, Dako), CD5 (clone 4C7, Novocastra), CD23 (clone 1B12, Novocastra) and SOX11 (clone MRQ-58, CellMarque) were investigated in paraffin-embedded sections. IHC was performed on a fully automated IHC/ISH stainer (Leica BOND) according to the company's protocol. Antibodies were used at the following dilutions and using the following epitope retrieval (ER) solutions: CCND1, 1:20, ER2; CD20, 1:400, ER1; CD5, 1:25, ER1; CD23, 1:20, ER1; SOX11, 1:50, ER1.

FISH analysis. Locus-specific interphase FISH was performed on paraffin-embedded tissue sections by using a CCND1 break apart probe (Abbott).

Real Time quantitative PCR analysis. RNA was extracted from FFPE material using the ExpressArt FFPE Clear RNAready kit (AmpTec) and concentration was measured using a Qubit fluorometer (ThermoFisher Scientific). For cDNA synthesis, 800 ng of purified RNA was reverse transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). Quantitative PCR was performed with a Light Cycler 480 (Roche) using the QuantiNova SYBR Green PCR Kit (Qiagen). Each cDNA (1µl per reaction) was amplified in a 15 µl reaction volume using QuantiTect Primer Assay (Qiagen) for CCND1 (QT00495285) and CCND2 (QT00057575). Expression was normalized using as internal controls either GUSB (Qiagen, QT00046046) or HPRT1 (primer sequences in Supplementary Table 1). Relative gene expression was calculated using the formula: 2^(mean Cp reference gene)/2^(mean Cp gene of interest). Values reported are the mean of three independent experiments.

Mutation analysis. Genomic DNA was extracted from FFPE material using the GeneRead DNA FFPE kit (Qiagen) and concentration was measured using Qubit fluorometer (ThermoFisher Scientific). Each of the five exons of CCND1 (NM_053056.2) was PCR amplified from the genomic DNA of each patient using the primers listed in Supplementary Table 1. Each reaction contained: 100 ng DNA, 4 mM MgCl2, 1 X Q solution (Qiagen), 0.25 mM dNTPs, 0.25 µM each primer, 1.5 Units of AmpliTaq Gold™ DNA Polymerase (ThermoFisher Scientific) and 1 X reaction buffer. PCR products were column purified using the QIAquick PCR Purification Kit (Qiagen) and 1 µl of each was used in a sequencing reaction using the BigDye™ Terminator v1.1 Cycle Sequencing Kit (ThermoFisher Scientific) according to the manufacturer. Purified sequencing products were separated on a Genetic Analyzer 3130 (Applied Biosystems).

CCND1 cloning and expression in HEK 293 cells. CCND1 coding sequence was amplified from a cDNA obtained from the colorectal cancer cell line HCT116, a cell line expressing high levels of CCND1. WT and mutant forms were obtained by PCR using the primers shown in Supplementary Table 1. Forward primers inserted an EcoRI restriction site upstream of the second codon of the CCND1 cDNA and the reverse primer inserted an XhoI restriction site downstream of the STOP codon of the CCND1 cDNA. The mutations found in Patients A and B were introduced using primers carrying each of the mutation. PCR products were cloned in pcDNA3N3xHA (gift of Tencho Tenev, Institute of Cancer Research, London, UK). The presence of the desired mutations and the absence of undesired, PCR-introduced mutations were confirmed by sequencing. Two micrograms of plasmid DNA were transfected in HEK 293 cells (400000/well in a 6 well plate) using Lipofectamine 2000. After 48 hours cells were lysed in RIPA buffer and further processed for western blotting analysis. Antibodies used are the following: CCND1 Ab1=anti-Cyclin D1 antibody clone SP4 (Thermo Fisher Scientific, Cat. RM-

9104); CCND1 Ab2= anti-Cyclin D1 antibody EP272Y (abcam, Cat. ab40754); anti GAPDH antibody (GeneTex, Cat. GTX627408)

PCR analysis of isoform expression. cDNA was produced from FFPE extracted RNA as described above. One micro liter of cDNA was amplified in a 20 µl reaction containing 4 mM MgCl2, 1 X Q solution (Qiagen), 0.25 mM dNTPs, 0.25 µM each primer, 1.5 Units of AmpliTaq Gold™ DNA Polymerase (ThermoFisher Scientific) and 1 X reaction buffer II using a Biorad T100 thermocycler with 60 °C as annealing temperature and 45 cycles. For the amplification of total CCND1 we used primers D1EX1_fw and D1EX2_rev2, annealing respectively to exon 1 and exon 2 of the CCND1 gene. For the amplification of the CCND1a isoform we used primers D1EX4_fw2 and D1EX5_rev, annealing respectively to exon 4 and exon 5 of the CCND1 gene. For the amplification of the CCND1b isoform we used primers D1EX3_fw2 and D1EX4_rev, annealing respectively to exon 3 and to the beginning of intron 4 of the CCND1 gene. PCR reactions were resolved on 2% agarose gels. The sequence of the primers used is provided in Supplementary Table 1.

Supplementary Table 1

HPRT1_fw	TGTTGTAGGATATGCCCTTGACT	qPCR analysis of HPRT1 expression
HPRT1_rev	GGCTTTGTATTTTGCTTTTCCA	qPCR analysis of HPRT1 expression
D1EX1_fw	CAGCCAGGACCCACAGCCCTCC	PCR amplification and sequencing of exon 1 of CCND1
D1EX1_rev	CAGGGAAGTCTTAAGAGAGCCG	PCR amplification and sequencing of exon 1 of CCND1
D1EX2_fw	CTCCGTAGGTCTGCGAGGAACAGAAG	PCR amplification and sequencing of exon 2 of CCND1
D1EX2_rev	TCCAGTGGTTACCAGCAGCTCCTCG	PCR amplification and sequencing of exon 2 of CCND1
D1EX3_fw	CCTCCCCTGATGGCCGCTCAC	PCR amplification and sequencing of exon 3 of CCND1
D1EX3_rev	CCTAAGGAGCCGGCTCTCAAGG	PCR amplification and sequencing of exon 3 of CCND1
D1EX4_fw	CCTGAGAGGGTCCCCTGCTCAC	PCR amplification and sequencing of exon 4 of CCND1
D1EX4_rev	CCGGCAAGGCTGCCTGGGACATC	PCR amplification and sequencing of exon 4 of CCND1
D1EX5_fw	TGCAGGCCCCTTCTAAGGACC	PCR amplification and sequencing of exon 5 of CCND1
D1EX5_rev	GACTGTCAGGGGAGCACCTGG	PCR amplification and sequencing of exon 5 of CCND1
D1CDS_fw2	cccgaattcGAACACCAGCTCCTGTGCTGC	Cloning of WT and Mut1 CCND1
D1CDS_wt_rev	ccggctcgagTCAGATGTCCACGTCCCGCACG	Cloning of WT and Mut2 CCND1
D1CDS_Mut1_rev	ccggctcgagTCAGATGTCCACGcCCCGCACG	Cloning of Mut1 CCND1
D1CDS_Mut2_fw	cccgaattcGAACACCAGCTCCcGTGCTGC	Cloning of Mut2 CCND1
D1EX2_rev2	CGCACTTCTGTTCCTCGCAG	PCR amplification of CCND1 isoforms
D1EX4_fw2	CCTCTCCAGAGTGATCAAGTG	PCR amplification of CCND1 isoforms
D1EX3_fw2	CACGCGCAGACCTTCGTTGCC	PCR amplification of CCND1 isoforms
D1CDS_rev	TCAGATGTCCACGTCCCGCACG	PCR amplification of CCND1 isoforms
D1EX4_fw2	CCTCTCCAGAGTGATCAAGTG	PCR amplification of CCND1 isoforms
D13UTR_rev	CTATCATCTGTAGCACAACCCTCC	PCR amplification of CCND1 isoforms

Supplementary Table 1: list of PCR primers used