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著者別表示	前田 大地
journal or publication title	Oncology Reports
volume	30
number	2
page range	584-588
year	2013-06-03
URL	http://doi.org/10.24517/00062677

doi: 10.3892/or.2013.2515



Cyclin D1 harboring the T286I mutation promotes oncogenic activation in endometrial cancer

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Received February 26, 2013; Accepted April 15, 2013

DOI: 10.3892/or.2013.2515

Abstract. Cyclin D1 is an important regulator of cell cycle progression. Phosphorylation of cyclin D1 at Thr286 by GSK3 β triggers its nuclear export and cytoplasmic proteolysis via the 26S proteasome. Cyclin D1 overexpression is a common event in various types of human cancers; however, reports of mutations are extremely rare. We analyzed mutations of the cyclin D1 gene, *CCND1*, in 88 endometrial cancer tissue specimens and detected mutations in 2 cases (2.3%). Both were unreported mutations with substitution of threonine to isoleucine at codon 286 (T286I). These two tumors harbored coexisting mutations in *K-ras*, *PIK3CA* and/or *PTEN* and showed accumulation of cyclin D1 in the nucleus by immunohistochemistry. Furthermore, we analyzed the functions of mutant cyclin D1 (T286I) by luciferase assays, immunofluorescence, western blotting and clonogenic cell survival assays in HEK-293T cells. We found that exogenous mutant cyclin D1 (T286I) accumulated in the nuclei in HEK-293T cells, and that it inhibited the expression of pRb. Additionally, the number of colonies was increased by introduction of mutant cyclin D1 (T286I) compared to that of wild-type cyclin D1. In conclusion, we identified an unreported *CCND1* mutation (T286I) in two endometrial cancers and revealed that the mutation was functional for inducing cell proliferation in human cells.

Introduction

Endometrial cancer is the eighth leading cause of cancer-related mortality among women (1). Approximately 80% of

endometrial carcinomas are endometrioid endometrial cancers (EECs) and are generally considered as estrogen-dependent tumors (2). EECs frequently possess mutations in PI3K (phosphatidylinositol 3-kinase) pathway genes including *PTEN*, *PIK3CA*, *K-ras* and *AKT1* (3-7).

Cyclin D1, encoded by the *CCND1* gene, is located on chromosome 11q13 and plays a crucial role in cell cycle regulation (8). Cyclin D1 interacts with cyclin-dependent-kinase 4 and 6 (CDK4/6), and the complex phosphorylates retinoblastoma protein (pRb) to promote cell cycle progression from G1 to S phase (9-11). Cyclin D1 is activated by upstream inputs, including the Wnt- β -catenin and Ras/PI3K signaling pathways (11,12). Degradation of cyclin D1 is regulated by GSK3 β , a downstream target of the PI3K pathway. The phosphorylation of cyclin D1 at Thr-286 by GSK3 β triggers its nuclear export and cytoplasmic proteolysis via the 26S proteasome (13). A polymorphism of *CCND1* (G/A870), encoding cyclin D1b, was reported to influence cancer risk and prognostic outcome (14). Cyclin D1b has a different configuration at the C-terminal domain, which includes Thr286, and is constitutively localized to the nucleus (15,16). Thus, this polymorphism (G/A870) is suggested to increase the risk of endometrial cancer, possibly through nuclear accumulation of cyclin D1 (17). Overexpression of cyclin D1 is a common event in various types of human cancers, including those of the breast, lung, bladder, esophagus and endometrium (13). However, mutations of *CCND1* have only been reported in endometrial and esophageal cancer (18,19). In the present study, we detected a novel *CCND1* mutation in endometrial cancer, and analyzed whether this T286I mutation causes gain-of-function.

Materials and methods

Tumor samples and genomic DNA. Surgical samples were obtained from 88 patients with primary endometrial carcinoma who underwent resection of their tumors at the University of Tokyo Hospital. All of the patients provided informed consent for the collection and use of their samples, and the use of

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Key words: cyclin D1, T286I, mutation, endometrial cancer

Table I. Clinicopathological background of all cases (n=88).

Variables	No. of patients (%)
Median age (range) in years	55 (28-79)
FIGO stage	
I	39 (44)
II	9 (10)
III	31 (35)
IV	9 (10)
Histological subtype	
Endometrioid adenocarcinoma	80 (91)
Grade 1	43 (49)
Grade 2	28 (32)
Grade 3	9 (10)
Adenosquamous carcinoma	3 (3)
Squamous cell carcinoma	1 (1)
Clear cell carcinoma	1 (1)
Mixed type	3 (3)
Lymph metastasis	20 (23)
Lymph-vascular invasion	39 (44)
Muscle invasion (>1/3)	53 (60)

tissues for the present study was approved by the appropriate institutional ethics committees. Histopathologically, 80 out of 88 cases (91%) were EECs (Table I). Genomic DNA was isolated from the tumor sections or lymphocyte pellets as previously described (7).

PCR and sequencing. The primer sequences of exon 5 for the *CCND1* gene were as follows: forward 5'-TGCTGGAGTCAAGCCTGCG-3' and reverse 5'-ACTGTCAGGGGAGCACCTG-3'. The PCR products were sequenced using the BigDye (Applied Biosystems, Foster City, CA, USA) terminator method on an autosequencer. Mutations for *PTEN* (exons 1-8), *K-Ras* (exons 1 and 2), *PIK3CA* (exons 9 and 20) and *CTNNB1* (exon 3) were analyzed as previously described (3,4,7,20).

Immunohistochemistry (IHC). Immunohistochemical staining was performed according to standard techniques using a Ventana Benchmark XT autostainer (Ventana Medical Systems Inc., Tucson, AZ, USA). Anti-cyclin D1 rabbit monoclonal antibody (#N161987; Dako, Carpinteria, CA, USA) was applied at a dilution of 1:100.

Cell lines and transfection. The human embryonic kidney HEK293T cell line was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The T286I cyclin D1 mutant vector was generated using the QuickChange Lightning Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA, USA). Transfection was performed using Effectene transfection reagent (Qiagen, Valencia, CA, USA).

Western blotting. HEK293T cells were lysed as previously described (3). Preparations of nuclear and cytoplasmic extracts

were constructed using NucBuster Protein Extraction kit (Merck, Darmstadt, Germany). Western blotting was performed as per standard protocols. The expression of proteins was examined using antibodies against cyclin D1 (ab16663; Abcam, Cambridge, UK), HA (#2367; Cell Signaling Technology, Inc., Beverly, MA, USA) and p84 (ab487; Abcam). Antibody against β -actin (A2228; Sigma, St. Louis, MO, USA) was used as an internal control. The secondary antibodies used were NA931V and NV934V (GE Healthcare, Sydney, Australia). All antibodies were used at the recommended concentrations as directed by the manufacturer. The proteins were visualized using an ECL Western Blot Detection kit (Amersham Biosciences, Piscataway, NJ, USA).

Immunocytochemistry. Immunocytochemistry was performed as previously described (21). The primary antibodies used were the same as those for western blotting. The secondary antibodies were Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexa Fluor 555-conjugated goat anti-rabbit IgG (Invitrogen, Carlsbad, CA, USA). The primary and secondary antibodies were used at a 1:100 dilution. All slides were briefly counterstained with DAPI (1 μ g/ml) for 5 min for nuclear determination and analyzed by confocal fluorescence microscopy (Carl-Zeiss MicroImaging Inc., Oberkochen, Germany).

Luciferase assays. HEK293T cells were transfected with wild-type (WT) cyclin D1 or T286I cyclin D1 expression plasmids (0.4 μ g) together with a reporter plasmid (pRb-TA-luc) (0.25 μ g). As an internal control, cells were co-transfected with pRL CMV-*Renilla* vector (Promega, Madison, WI, USA) (0.01 ng). Luciferase activity was detected using the Microplate Luminometer LB96V (EG&G Brethelod, Wildbad, Germany). Each experiment was repeated three times.

Clonogenic cell survival assays. HEK293T cells were seeded in 6-well plates. After transfection with WT- or T286I-cyclin D1, cells were incubated for 1 week with DMEM supplemented with G418 (1,000 μ g/ml) (Invitrogen). The colonies were stained with Giemsa.

Statistical analysis. Statistical analyses of data from luciferase and clonogenic cell survival assays were carried out using the Student's t-test. $P < 0.05$ was considered to indicate a statistically significant result.

Results

T286I *CCND1* mutation in endometrial cancer. Clinicopathological characteristics are examined and are presented in Table I. Briefly, sequencing of the *CCND1* gene identified somatically acquired (non-germline) mutations in 2 out of 88 (2.3%) endometrial cancer specimens (Fig. 1A). Both mutations were a substitution of amino acid 286 from threonine to isoleucine (T286I). These 2 patients died of the disease within 11 and 26 months of diagnosis, respectively. One case was stage IV endometrial carcinoma (grade 3), whereas the other was mixed adenocarcinoma with stage IIIc. Immunohistochemical staining of these 2 tumors showed accumulation of cyclin D1 in the nucleus (Fig. 1B).

Table II. Clinicopathological background and mutation status of the two cases harboring the *CCND1* mutation.

Case no.	Age (years)	Histology	Grade	Stage	Survival (months)	Mutation status			
						<i>CCND1</i>	<i>K-Ras</i>	<i>PIK3CA</i>	<i>PTEN</i>
1	52	Endometrioid	3	4	11	T286I	G13D	H1047R	Del 135-141 (Del 19 bp, stop 140)
2	55	Mixed	1	3c	26	T286I	G12D	G1007R	R130G, Del 267 (Del A, stop 275)

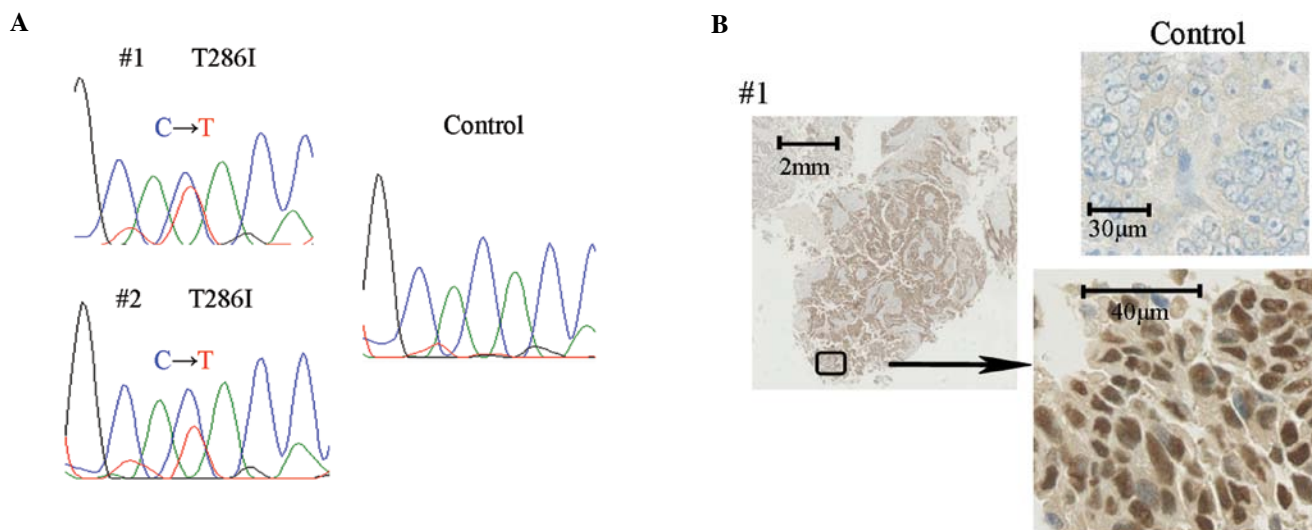


Figure 1. *CCND1* mutations in 2 endometrial carcinomas and correlation with cyclin D1 nuclear accumulation. (A) PCR-direct sequence analysis of *CCND1* in endometrial cancer tumors. Two of 88 samples (2.3%) showed a single-base substitution in amino acid 286 from threonine to isoleucine. (B) Immunohistochemical analysis showed cyclin D1 expression in the nucleus in tumors harboring the cyclin D1-T286I mutation.

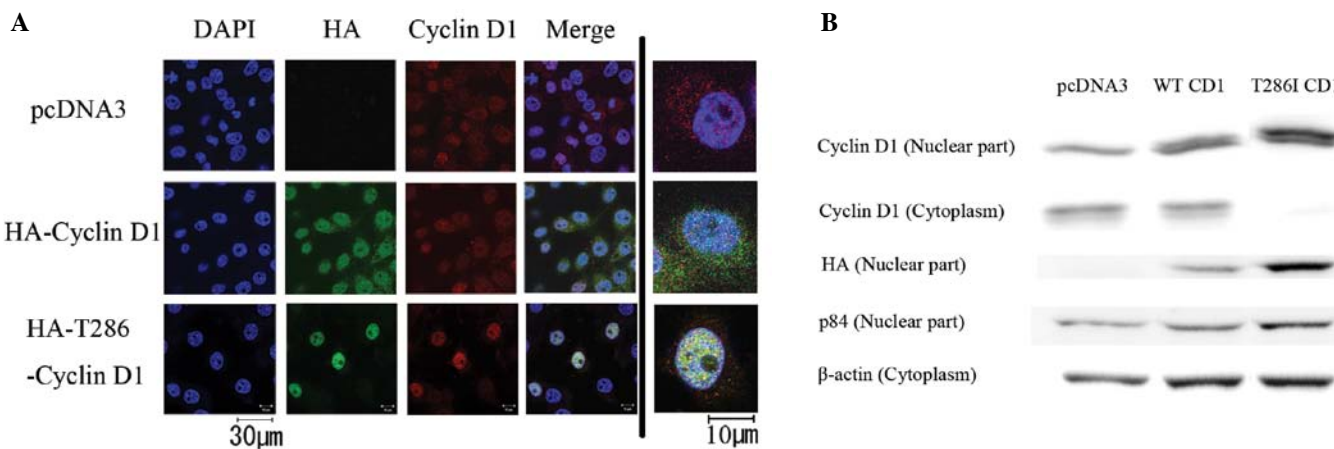


Figure 2. T286I mutation induces constitutive and stable nuclear cyclin D1 translocation in HEK293T cells. HEK293T cells were transfected with HA-tagged pcDNA3 (pcDNA3-CT) or HA-tagged WT cyclin D1 pcDNA3 (CD1-WT) or HA-tagged T286I cyclin D1 pcDNA3 (CD1-T286I). (A) Immunofluorescence showed cyclin D1 was overexpressed in the CD1-WT-expressing cells and was confined to the nucleus in the CD1-T286I-expressing cells. (B) Western blotting revealed that CD1-T286I transfection resulted in cyclin D1 stabilization in the nucleus and not in the cytoplasm.

CCND1 mutation at T286I coexists with triple mutations of *K-Ras*, *PIK3CA* and *PTEN*. Mutational analysis of *K-Ras*, *PIK3CA* and *PTEN* was performed in both samples with the *CCND1* mutation at T286I. Case no. 1 possessed overlapping mutations in *K-Ras* at G13D, in *PIK3CA* at H1047R and in *PTEN* (there was a deletion of 19 bp at codons 135-141). Case no. 2 also possessed these three mutations; in *K-Ras* at G12D,

in *PIK3CA* at G1007R and in *PTEN* at R130G and deletion of 'A' in codon 267 (Table II).

T286I mutation induces constitutive nuclear cyclin D1 accumulation. We introduced mutant cyclin D1 (T286I) into HEK293T cells, which do not possess *CCND1* mutations. We transfected three types of pcDNA3 plasmids: pcDNA3

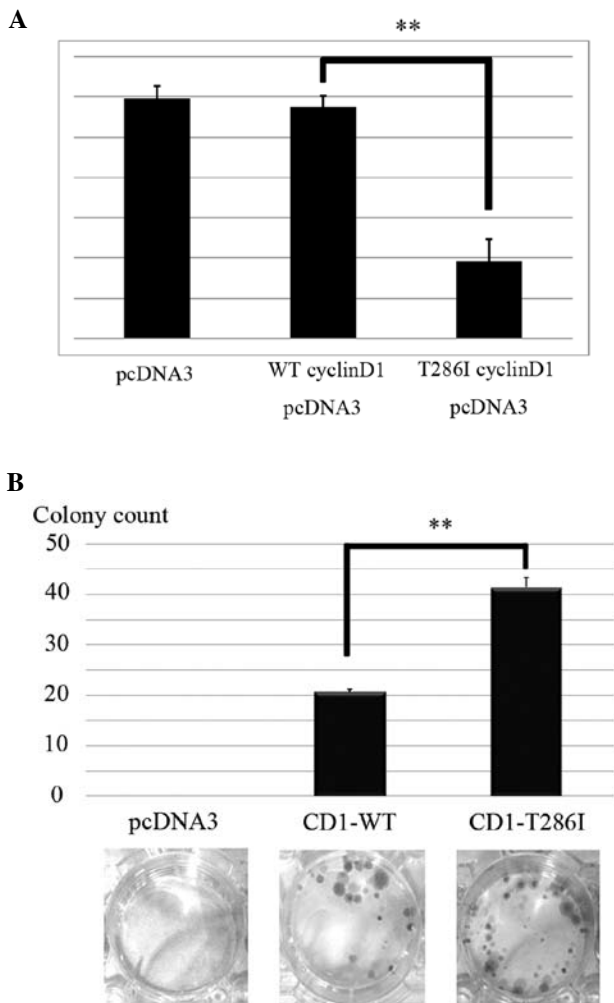


Figure 3. Cyclin D1-T286I mutation promotes oncogenic activation. (A) pRB transcriptional activity was examined in HEK293T cells transfected with CD1-WT, CD1-T286I or the control plasmid, by luciferase assays. CD1-T286I suppressed the expression of pRb protein. (B) Clonogenic cell survival assays were performed using similarly transfected HEK293T cells. After 1 week of selection in G418, colonies were stained with Giemsa and quantified. All experiments were repeated three times, and each value is shown as the mean of 3 experiments \pm SD (** $P < 0.01$). The result showed increased formation of colonies in cells with the CD1-T286I mutation.

as a control (pcDNA3-CT), HA-tagged WT cyclin D1 (CD1-WT) and HA-tagged T286I mutant cyclin D1 (CD1-T286I). Immunocytochemistry indicated that endogenous cyclin D1 was localized to both the nucleus and cytoplasm in the pcDNA3-CT-transfected cells (Fig. 2A). Exogenous CD1-WT was more clearly expressed in the nucleus than that in the cytoplasm. In the CD1-T286I-transfected cells, cyclin D1 was strongly accumulated in the nucleus, and endogenous cyclin D1 was undetectable in the cytoplasm. In western blot experiments, both nuclear and cytoplasmic expression of cyclin D1 was observed in the pcDNA3-CT- and CD1-WT-transfected cells (Fig. 2B). However, either endogenous or exogenous cyclin D1 was predominantly expressed in the nucleus in the CD1-T286I-transfected cells. These results suggest that the *CCND1* mutation at T286I prevents nuclear export of cyclin D1.

T286I mutation reduces Rb expression and promotes cell proliferation. As nuclear cyclin D1 and pRb expression are

antagonistic for cell cycle progression, we transiently transfected cyclin D1 vectors and examined pRb transcriptional activity using luciferase assays. No significant difference was observed in pRb transcriptional activity by introduction of CD1-WT, when compared with pcDNA3-CT. However, CD1-T286I clearly suppressed the transcriptional activation of pRb ($P = 0.002$) (Fig. 3A).

Finally, to investigate the effects of T286I on cell proliferation, we performed clonogenic cell survival assays. No colonies were observed in the pcDNA3-CT-transfected cells, whereas colonies were observed in the CD1-WT cells (Fig. 3B). In addition, CD1-T286I further increased the number of colonies in HEK293T cells ($P = 0.007$) (Fig. 3B). These results suggest that accumulation of mutant cyclin D1 (T286I) in the nucleus promotes cell cycle proliferation.

Discussion

Phosphorylation-dependent nuclear export of cyclin D1 at Thr286 is suggested to be critical for prevention of aberrant cell proliferation *in vitro* (22). This is the first report of *CCND1* mutations at Thr286 in endometrial cancer. We identified this mutation (T286I) in two tumors (2.3%) in association with accumulation of cyclin D1 protein in the nucleus.

Previously, Moreno-Bueno *et al* (19) reported the existence of *CCND1* mutations at P287T, P287S and a 12 bp in-frame deletion (289-292) in endometrial cancer. Benzeno *et al* (18) showed that these mutations specifically disrupt phosphorylation-dependent nuclear export of cyclin D1 and contribute to the genesis and progression of neoplastic growth. However, the function of the *CCND1* mutation at T286I (CD1-T286I) has not been well characterized. Our results showed that CD1-T286I prevents the nuclear export of cyclin D1 and significantly promotes cell proliferation. These findings suggest that this *CCND1* (T286I) mutation might promote tumor progression in certain endometrial carcinomas.

The functions of the *CCND1* mutation *in vivo* were reported by Gladden *et al* (23). They showed that transgenic mice expressing mutant cyclin D1 (T286A) induced oncogenic nuclear retention of cyclin D1. This report is compatible with our data that a missense mutation at Thr286 disrupts nuclear export of cyclin D1. The clinical outcome of patients with cyclin D1 mutations were not reported in previous studies (18,19). However, cyclin D1 overexpression is frequently observed in various types of cancers, and the prognostic impact of the overexpression was distinct among tumor types. Cyclin D1 overexpression is suggested to be induced by chromosomal translocation, gene amplification and protein stabilization (24,25). The prognosis of patients with cyclin D1 overexpression is poor in ovarian cancer (26) and favorable in colorectal cancer (27). These contradictory results may be caused by additional genetic alterations in each cancer type. Indeed, expression of wild-type cyclin D1 in murine fibroblasts or lymphocytes does not promote neoplastic transformation without co-expression of a cooperating oncogene such as Myc or Ras (28-31). In the present study, both patients with mutant cyclin D1 (T286I) had poor prognosis as they harbored coexisting triple mutations of *K-Ras*, *PTEN* and *PIK3CA*. Although the sample size was extremely small, our data may suggest that activation of both Ras/MAPK and PI3K

pathways cooperate with mutant cyclin D1 and promote tumor aggressiveness in certain endometrial carcinomas. Further study is warranted to clarify the significance of *CCND1* mutations in endometrial cancer.

Acknowledgements

We thank Keiko Shoji, Yuichiro Miyamoto, Yoko Matsumoto, Takahide Arimoto, Kensuke Tomio, Satoko Kojima and Reiko Kurikawa for their support and assistance.

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