Supplementary Materials: CCND1 Splice Variant as a Novel Diagnostic and Predictive Biomarker for Thyroid Cancer

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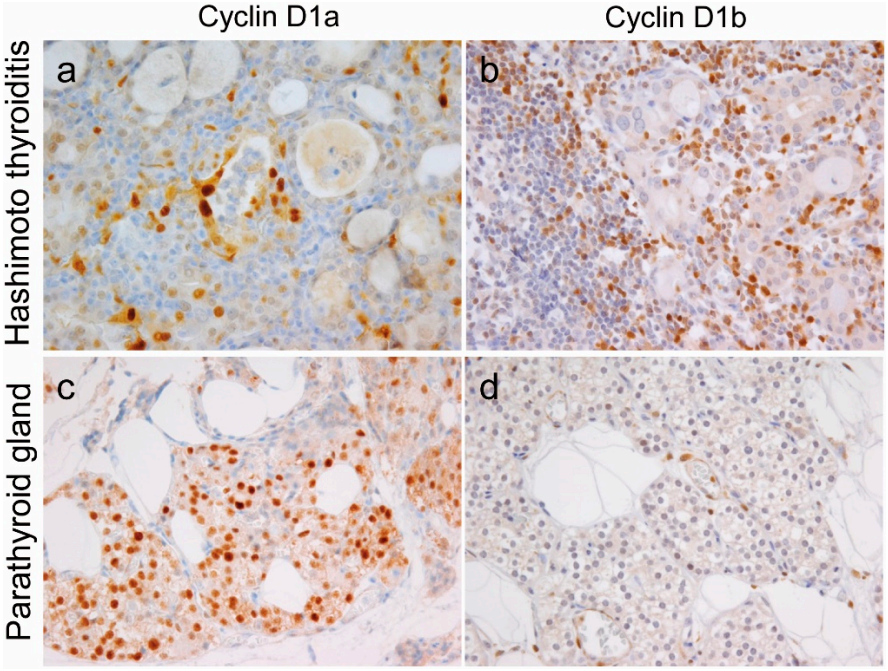

Figure S1. Hürthle cells in Hashimoto's thyroiditis are positive for cyclin D1a (**a**, × 400) and negative for cyclin D1b (**b**, ×400). Most chronic inflammatory cells mixed with Hürthle cells are negative for cyclin D1a and positive for cyclin D1b. Parathyroid cells are positive for cyclin D1a (**c**, ×400) and negative for cyclin D1b (**d**, ×400).

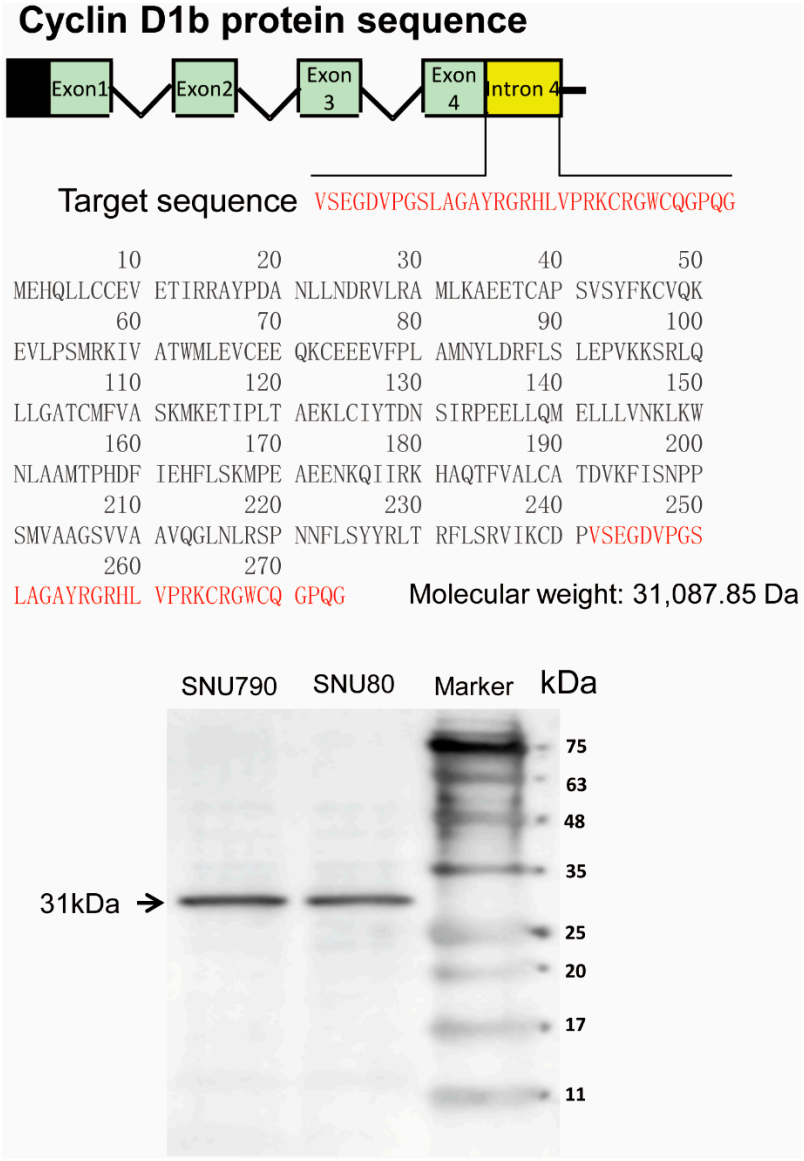

Figure S2. The rabbit polyclonal cyclin D1b antibody was derived from the intron 4 sequence (VSEGDVPGSLAGAYRGRHLVPRKCRGWCQGPQG). This rabbit polyclonal cyclin D1b antibody was used in Western blot to detect cyclin D1b in two thyroid cancer cell lines, SNU80 (anaplastic thyroid carcinoma) with *BRAF* G469R mutation and SNU790 (papillary thyroid carcinoma) with *BRAF* V600E mutation, which were obtained from the Korea Cell Line Bank (Seoul National University, Seoul, Korea). Cyclin D1b runs as a 31kDa molecule on 10% SDS-PAGE gel.

Table S1. Correlation between clinicopathologic features and expression of *CCND1* mRNA in The Cancer Genome Atlas (TCGA) dataset of papillary thyroid carcinoma.

Characteristic	Low Expression of *CCND1* mRNA	High Expression of *CCND1* mRNA	*p*-value
Age (years)			
<45	101 (47.9%)	110 (52.1%)	0.397
≥45	126 (51.9%)	117 (48.1%)	
Gender			
Male	159 (48.0%)	172 (52.0%)	0.17
Female	68 (55.3%)	55 (44.7%)	
Histologic type			
Classic	148 (47.4%)	164 (52.6%)	0.360
Follicular	59 (59.6%)	40 (40.4%)	
Tall cell	15 (44.1%)	19 (55.9%)	
Other	5 (55.6%)	4 (44.4%)	
Extrathyroidal extension			
None	161 (52.1%)	148 (47.9%)	0.284
Minimal (T3)	51 (43.6%)	66 (56.4%)	
Moderate/advanced (T4)	7 (46.7%)	8 (53.3%)	
Pathologic T (pT) stage			
pT1	77 (58.8%)	54 (41.2%)	0.09
pT 2	67 (44.1%)	85 (55.9%)	
pT 3	72 (47.7%)	79 (52.3%)	
pT 4	9 (50.0%)	9 (50.0%)	
Pathologic lymph node (pN) stage			
pN0	105 (50.5%)	103 (49.5%)	0.664
pN1	97 (48.3%)	104 (51.7%)	
pNX	25 (55.6%)	20 (44.4%)	
Metastasis (M) stage			
M0	116 (47.9%)	126 (52.1%)	0.371
M1	4 (50.0%)	4 (50.0%)	
MX	106 (52.2%)	97 (47.8%)	
American Joint Committee on Cancer tumor stage			
Stage I	131 (50.4%)	129 (49.6%)	0.974
Stage II	25 (51.0%)	24 (49.0%)	
Stage III	47 (48.0%)	51 (52.0%)	
Stage IV	23 (51.1%)	22 (48.9%)	
BRAF-RAS molecular type			
BRAF-like	111 (40.8%)	161 (59.2%)	<0.001
RAS-like	81 (68.1%)	38 (31.9%)	
Recurrence risk group			
Low	90 (54.2%)	76 (45.8%)	0.269
Intermediate	117 (46.4%)	135 (53.6%)	
High	13 (54.2%)	11 (45.8%)	

The mRNA expression levels of the *CCND1*mRNA were grouped as high or low based on the median value.

Table S2. Sequences of primers and probes for the analysis of CCND1 gene.

Name	Position	Sequence (5′→3′)
Endpoint real-time PCR for detection of CCND1 G/A870 polymorphism		
CCND1 Forward	Exon 4	CTTCCTGTCCTACTACCG
CCND1 Reverse	Intron 4	GTGTCTCCCCCTGTAAG
CCND1 Probe_A	Exon 4-Intron4	[HEX]CCTCACTTACTGGGTCACACT[BHQ1]
CCND1 Probe_G	Exon 4-Intron4	[6FAM]CCTCACTTACCGGGTCACACT[BHQ1]
Sanger sequencing for CCND1 G/A870 polymorphism		
CCND1 Forward	Exon 4	AGTTCATTTCCAATCCGCCC
CCND1 Reverse	Intron 4	TTTCCGTGGCACTAGGTGTC
Detecting mRNA expression of CCND1 isoforms by quantitative real-time PCR		
CCND1a Forward	Exon 4	GTCCTACTACCGCCTCACACG
CCND1a Reverse	Exon 5	TTCGATCTGCTCCTGGCAG
CCND1b Forward	Exon 4	TGAGGAGCCCCAACAACTTC
CCND1b Reverse	Intron 4	CCTGGGACATCACCCTCACTTA
CCND1a/1b Probe	Exon 4	[6FAM]TTCCTCTCCAGAGTGATCAAGTGTGACCC[BHQ1]
GAPDH Forward	Exon 3	AGTGGATATTGTTGCCATC
GAPDH Reverse	Exon 4	TTCCATTGATGACAAGCTT
GAPDH Probe	Exon 4	[6FAM]ATGGGTGGAATCATATTGGAACAT[BHQ1]

Table S3. Sequences of primers for the molecular analysis of *BRAF*, *NRAS*, *HRAS*, and *KRAS* genes.

Name	Forward	Reverse
BRAF	5′-TCATAATGCTTGCTCTGATAGGA-3′	5′-GGCCAAAAATTTAATCAGTGGA-3′
NRAS(codon61)	5′-CCCCTTACCCTCCACACC-3′	5′-GAGGTTAATATCCGCAAATGACTT-3′
NRAS(codon61)	5′-GTGAAACCTGTTTGTTGGAC-3′	5′-CCTGTAGAGGTTAATATCCG-3′
NRAS(codon61)	5′-ACACCCCCAGGATTCTTACAG-3′	5′-GCCTGTCCTCATGTATTGGTC-3′
HRAS(exon3)	5′-GTCCTCCTGCAGGATTCCTA-3′	5′-CGGGGTTCACCTGTACT-3′
KRAS(exon2)	5′-GGTGAGTTTGTATTAAAAGGTACTGG-3′	5′TCCTGCACCAGTAATATGCA-3′
KRAS(exon3)	5′-GGTGCACTGTAATAATCCAGAC-3′	5′TGATTTAGTATTATTTATGGC-3′

Table S4. Rabbit immunization protocol for polyclonal cyclin D1b antibody production.

Procedure	Protocol week	Description	Note
Pre-immune serum collection	Week 0	Bleed; Control serum	~ 1 mL serum/rabbit
Primary injection of antigen	Week 0	Immunize with 1mg antigen/rabbit in Complete Freund's Adjuvant	Subcutaneous injection
First booster	Week 4	Immunize with 500 μg/rabbit antigen in IFA	Subcutaneous injection
First serum collection	Week 5	Bleed; peptide-specific ELISA titration of antibody	~ 1 mL serum/rabbit
Second booster	Week 6	Immunize with 500 μg/rabbit antigen in IFA	Subcutaneous injection
Second serum collection	Week 7	Bleed; peptide-specific ELISA titration of antibody	~ 1 mL serum/rabbit
Third booster	Week 8	Immunize with 500 μg/rabbit antigen in IFA	Subcutaneous injection
Third serum collection	Week 9	Sacrifice (Heart puncture); peptide-specific ELISA titration of antibody	~ 50 mL serum/rabbit

IFA, Incomplete Freund's Adjuvant.