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Amygdalin Modulates Cell Cycle Regulator Genes in Human Chronic Myeloid Leukemia Cells

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Abstract

To determine the anticancer effect of D-amygdalin (D-mandelinitrole-β-D-gentiobioside) in human chronic myeloid leukemia cells K562, we profiled the gene expression between amygdalin treatment and control groups. Through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, the cytotoxicity of D-amygdalin was 57.79±1.83% at the concentration of 5 mg/mL for 24 h. We performed cDNA microarray analysis and compared the gene expression profiles between D-amygdalin (5 mg/mL, 24 h) treatment and control groups. Among the genes changed by D-amygdalin, we paid attention to cell cycle-related genes, and particularly cell cycle regulator genes; because arrest of cell cycle processing was ideal tactic in remedy for cancer. In our data, expressions of *cyclin-dependent kinase inhibitor 1B (p27, Kip1)* (*CDKN1B*), *ataxia telangiectasia mutated* (includes complementation groups A, C, and D) (ATM), *cyclin-dependent kinase inhibitor 1C (p57, Kip2)* (*CDKN1C*), and *CHK1 checkpoint homolog* (*CHEK1*, formally known as *CHK1*) were increased, while expressions of *cyclin-dependent kinase 2* (*CDK2*), *cell division cycle 25A* (*CDC25A*), and *cyclin E1* (*CCNE1*) were decreased. The pattern of these gene expressions were confirmed through RT-PCR. Our results showed that D-amygdalin might control cell cycle regulator genes and arrest S phase of cell cycle in K562 cells as the useful anticancer drug.

Keywords: Chronic myeloid leukemia, D-Amygdalin, cDNA microarray, Cell cycle regulator genes

Chronic myeloid leukemia (CML) is a hematological malignancy characterized by immature granulocytosis, basophilia, thrombocytosis, and anemia[1]. CML represents about 14% of all leukemias and occurs with a frequency of about 1 in 100,000. Ionizing radiation has been implicated in some cases of CML, but in most individual no cause is known. In despite of high responses of new anticancer drug such as Gleevec (imatinib mesylate) on CML, drug resistance occurred ultimately in chronic myeloid leukemia blast crisis[2,3]. In recent years, the development of new anticancer drug is a key issue for cancer chemotherapy, and herbal medicine has attracted a great deal of recent attention due to their low toxicity and costs.

Armeniacae semen is a seed of *Prunus armeniaca* Linnen var. *ansu* Maximowicz, which belongs to the Rosaceae family. This seed has been widely used to treat asthma, aplastic anemia and several cancers in Oriental medicine[4]. Amygdalin, ingredient of Armeniacae semen, has been reviewed as prevention and control of cancers. Amygdalin belonging to a family of compounds called cyanide would be broken down by an enzyme in cancerous tissue, and toxic cyanide to release from broken amygdalin would kill the cancer. It is further hypothesized that another enzyme, rhodanese, which has the ability to detoxify cyanide, is present in normal tissues but deficient in cancer cells. These two factors supposedly combine to effect a selective poisoning of cancer cells by the cyanide, while normal cells remain undamaged[5,6]. Nevertheless, there have been controversial views on the use of amygdalin because of its toxicity[7-9]. However, Kwon *et al.*[10] was demonstrated that the toxicity of amygdalin was due to inactivate isoform of amygdalin. Furthermore, it was reported that D-amygdalin (D-mandelinitrole-β-D-gentiobioside) used in our study selectively killed cancer cells at the tumor site without systemic toxicity, which is the usual problem when using general chemotherapeutic agents[4].

In this study, we determined whether D-amygdalin induced cell death of human chronic myeloid leukemia cells, K562 and compared the expression profiles between D-amygdalin-treated and control groups using cDNA microarray analysis. Additionally, the

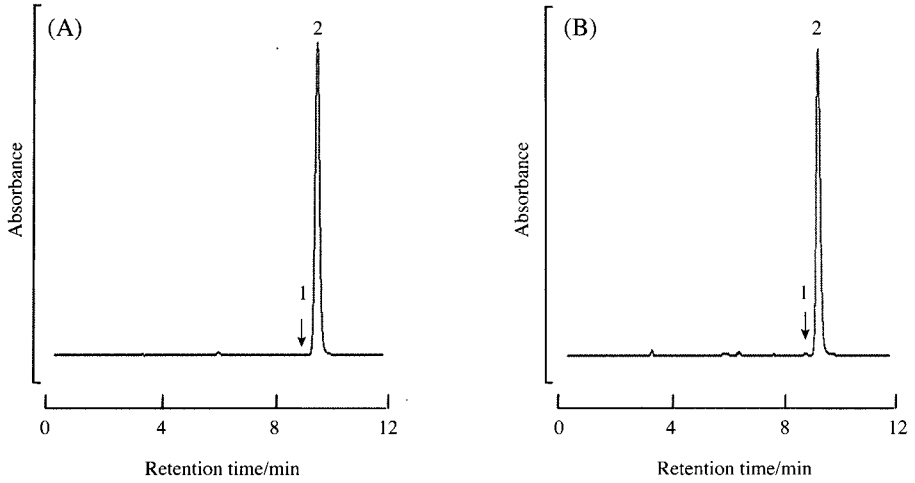

Fig. 1. Reverse-phase HPLC separation of amygdalin in phosphate buffer. (A) D-amygdalin standard. (B) D-amygdalin obtained by our method. Peak 1, neoamygdalin; peak 2, D-amygdalin.

genes selected by cDNA microarray analysis were confirmed through RT-PCR.

Cytotoxicity of D-amygdalin in K562 Cells

When treated with D-amygdalin of 0.5, 1.0, 2.5, and 5 mg/mL concentrations for 24 h, viabilities of K562 cells were 80.13±3.67%, 71.92±0.78%, 63.56±2.63%, and 57.79±1.83%, respectively, compared with those of nontreated cells (Fig. 2). MTT assay showed the dose-dependent cytotoxicity of D-amygdalin on K562 cells, and significant at concentration of 2.5 and 5 mg/mL. To compare the precise effect of D-amygdalin, further experiments were carried out using 5 mg/mL D-amygdalin for 24 h.

Analysis of Microarray Expression Data

In order to assess the expression profiles, D-amygdalin (5 mg/mL, 24 h)-treated group was compared to control group. The cDNA microarray that contained duplicate cDNA probes from 1 K leukemia cancer clones was used (Digital Genomics, Seoul, Korea). To normalize intensity ratio of each gene expression pattern, global M method was used in this study. First, the primary data were normalized by the total spots of intensity between two groups, and then normalized by the intensity ratio of reference genes, such as housekeeping genes in both groups. Finally, the expression ratio of D-amygdalin-treated group to control group was converted to $\log_2$ ratio of each gene. After normalizing the data, a difference of more than 2 fold in the normalized intensity ratio was selected and considered as significance. The expression ratios of 40 genes were upregulated more than 2 fold by D-amygdalin, whereas those of 25 genes were downregulated lower than 2 fold (data not shown).

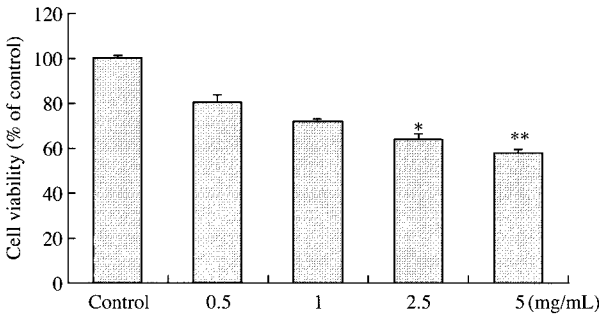

Fig. 2. Cytotoxicity of D-amygdalin. Human chronic myeloid leukemia, K562 cells were treated with various concentrations (0.5, 1.0, 2.5, and 5 mg/mL) of D-amygdalin for 24 h prior to the determination of cellular viability through 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Independent experiment was repeated three times. Results are presented as mean± S.E.M. ($*p<0.05$, $**p<0.01$).

We paid attention to genes belonging to cell cycle category. Particularly, expressions of cell cycle related genes such as *cyclin-dependent kinase inhibitor 1B (p27, Kip1)* (*CDKN1B*), *ataxia telangiectasia mutated* (includes complementation groups A, C, and D) (*ATM*), *cyclin-dependent kinase inhibitor 1C (p57, Kip2)* (*CDKN1C*), and *CHK1 checkpoint homolog* (*CHEK1*, formally known as *CHK1*) were upregulated more than 8-fold (Table 1), while the expression of the genes such as *cyclin-dependent kinase 2* (*CDK2*), *cell division cycle 25A* (*CDC25A*), and *cyclin E1* (*CCNE1*) were downregulated lower than 4-fold (Table 2).

Table 1. List of cell cycle-related genes upregulated by D-amygdalin.

Gene name	GenBank number	Chromosome	Title	Global M
CDKN1B	AA455410	12p13.1-p12	cyclin-dependent kinase inhibitor 1B (p27, Kip1)	4.111
ATM	U67093	11q22-q23	ataxia telangiectasia mutated (includes complementation groups A, C and D)	3.495
CDKN1C	AI088356	11p15.5	cyclin-dependent kinase inhibitor 1C (p57, Kip2)	3.285
CHEK1	AF016582	11q24-q24	CHK1 checkpoint homolog (S. pombe)	3.039
CDKN2C	AF041248	1p32	cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	2.983
NBS1	NM_002485	8q21	Nijmegen breakage syndrome 1 (nibrin)	2.953
ACPP	AA613916	3q21-q23	acid phosphatase, prostate	2.932
MDM2	Z12020	12q14.3-q15	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse)	2.854
CHES1	U68723	14q24.3-q31	checkpoint suppressor 1	2.766
CORO1A	X89109	16p12.1	coronin, actin binding protein, 1A	1.999
BRCA1	U14680	17q21	breast cancer 1, early onset	1.731
CCNA1	U66838	13q12.3-q13	cyclin A1	1.675
LIG4	X83441	13q33-q34	ligase IV, DNA, ATP-dependent	1.466
MSH2	AW004683	2p22-p21	mutS homolog 2, colon cancer, nonpolyposis type 1 (E. coli)	1.447
HUS1	Y16893	7p13-p12	HUS1 checkpoint homolog (S. pombe)	1.266
RGS2	AI675283	1q31	regulator of G-protein signalling 2, 24 kDa	1.144

Table 2. List of cell cycle-related genes downregulated by D-amygdalin.

Gene name	GenBank number	Chromosome	Title	Global M
CDC2	Y00272	10q21.1	cell division cycle 2, G1 to S and G2 to M	−1.129
CCNB1	AI972071	5q12	cyclin B1	−1.144
MAPK6	X80692	15q21	mitogen-activated protein kinase 6	−1.256
E2F1	NM_005225	20q11.2	E2F transcription factor 1	−1.26
BCL2	M14745	18q21.33	B-cell CLL/lymphoma 2	−1.446
EGFR	X00588	7p12	epidermal growth factor receptor (erythroblastic leukemia viral (v-erb-b) oncogene homolog, avian)	−1.463
FGF7	AI075338	15q15-q21.1	fibroblast growth factor 7 (keratinocyte growth factor)	−1.632
TGFA	X70340	2p13	transforming growth factor, alpha	−1.673
MCC	M62397	5q21-q22	mutated in colorectal cancers	−1.838
CDK2	AA789250	12q13	cyclin-dependent kinase 2	−2.158
CDC25A	M81933	3p21	cell division cycle 25A	−2.712
CCNE1	M73812	19q12	cyclin E1	−2.817

Confirmation of cDNA Microarray Findings by RT-PCR

Selecting upregulated 4 genes (*CDKN1B, ATM, CDKN1C*, and *CHEK1*) and downregulated 3 genes (*CDK2, CDC25A*, and *CCNE1*) by D-amygdalin treatment among cell cycle related genes, we observed the mRNA expressions using RT-PCR reproduced the results of cDNA microarray. The efficiency of the reaction was adjusted by GAPDH amplification. As shown on Fig. 3, the expressions of *CDKN1B, ATM, CDKN1C*, and *CHEK1* were increased (Fig. 3A), while the levels of *CDK2, CDC25A*, and *CCNE1* were decreased by D-amygdalin treatment (Fig. 3B).

Discussion

We referred to concentration of D-amygdalin observed in study of Kwon *et al.*[10] and examined its cytotoxicity in human chronic myeloid leukemia cells, K562. D-Amygdalin induced cell death as a dose-dependent manner, and showed significant cytotoxicity at the concentrations of 2.5 and 5 mg/mL. In this study, to determine the anticancer effect of D-amygdalin, we identified either decrease or increase of gene expression by D-amygdalin treatment in K562 cells using cDNA microarray. One of the advan-

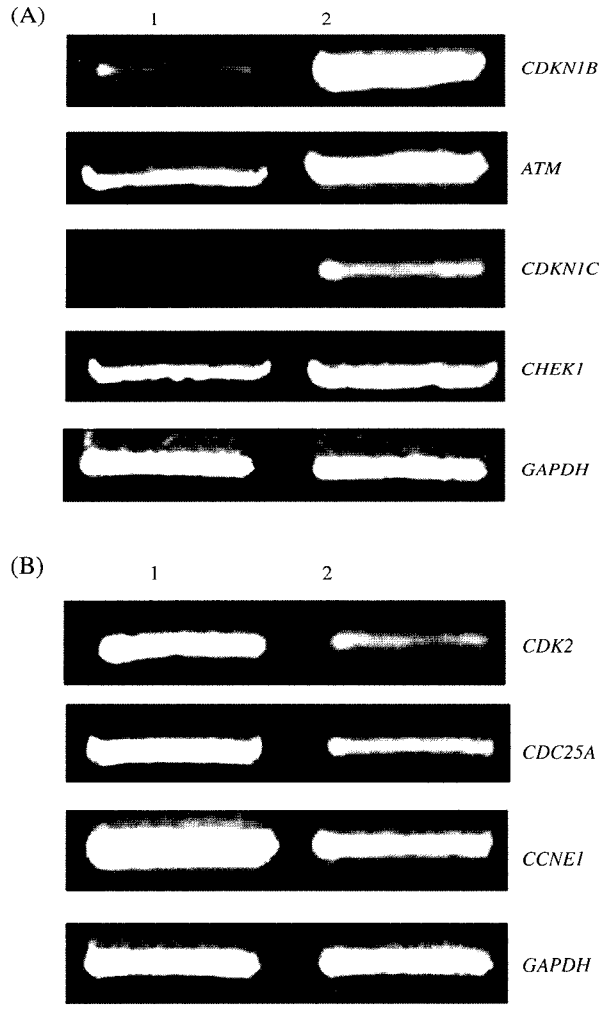

Fig. 3. Confirmation of cDNA microarray results of upregulated and downregulated genes by RT-PCR. (A) Fore genes upregulated by treatment of D-amygdalin, *cyclin-dependent kinase inhibitor 1B (p27, Kip1)* (*CDKN1B*), *ataxia telangiectasia mutated* (includes complementation groups A, C, and D) (*ATM*), *cyclin-dependent kinase inhibitor 1C (p57, Kip2)* (*CDKN1C*), and *CHK1 checkpoint homolog* (*CHEK1*, formally known as *CHK1*) and (B) three genes downregulated by treatment of D-amygdalin, *cyclin-dependent kinase 2* (*CDK2*), *cell division cycle 25A* (*CDC25A*), and *cyclin E1* (*CCNE1*) were analyzed by RT-PCR with total RNA from control and D-amygdalin (5 mg/mL, 24 h) treated human chronic myeloid leukemia cells. As an internal control, *GAPDH* was amplified.

tages of cDNA microarray is the possibility to observe the expression pattern of the whole genes and to compare with different conditions. In our data, we paid attention to genes belonging to cell cycle category of which genes showed most deferent expression between two groups. Particularly, the genes such as *CDKN1B, ATM, CDKN1C, CHEK1, CDK2, CDC25A*, and *CCNE1* which were shown the differences of remarkable expression were well known for important regulators controlled cell cycle progression.

In treatment of cancer, the arrest of cell cycle progression has been known as ideal tactic[11,12], because the cell cycle is a critical regulator of the processes of cell proliferation and growth as well as of cell division after DNA damage[13]. Progression of a cell through the cell cycle is promoted by a number of CDKs complexed with regulatory proteins called cyclins. The association of cyclin E (CCNE) with *CDK2* is active at the G1/S transition and directs entry into S phase. S phase progression is directed by the *CCNA/CDK2* complex, and the complex of *CCNA* with *CDK1* is important in G2. *CDK1/CCNB* is necessary for mitosis to occur[14]. Additionally, at an appropriate time of the cell cycle, these cyclin/CDK complexes are dephosphorylated by CDC25 and activated. *CDC25A* acting on *CCNE/CDK2* is primarily responsible for S phase progression, while *CDC25C* acting on *CCNB/CDK1* is responsible for G2/M progression[15,16]. Our study showed that expressions of *CDK2, CDC25A*, and *CCNE1* were decreased lower than 4 fold by D-amygdalin treatment. These results demonstrated that D-amygdalin would arrest S phase of cell cycle in K562 cells. Conversely, the expressions of *ATM* and *CHEK1* were increased more than 8 fold. In arresting mechanism of S phase, prior to the action of *CDC25A*, the upstream factors responsible for initiating a checkpoint response are the ATM and ATR protein kinases[17]. These two enzymes are key components of the DNA damage response that activate the *CHEK1* and *CHEK2* protein kinase. Anticancer agents are known to rapidly activated the ATM/ATR-CHEK1/2 pathway[15,18] leading to phosphorylation of *CDC25A*, and thereby resulting in the inactivation of the *CCNE/CDK2* complex[15].

In cell cycle regulation, not only CDK/cyclin complex but corresponding cell cycle inhibitory (CDK inhibitors [CDKIs]) proteins play an important role, which serve as negative regulators of the cell cycle and stop proceeding to the next phase of the cell cycle. CDKIs have been proposed to act as tumor suppresser genes, and several members have been implicated in the pathogenesis of a variety of human cancers[19-21]. Particularly, the kinase inhibitor protein (KIP) group of CDKIs, $p21^{waf1}$ (*CDKN1A*), $p27^{kip1}$ (*CDKN1B*), and $p57^{kip2}$ (*CDKN1C*), negatively regulate cyclin *E/CDK2* and cyclin *A/CDK2* complexes[22]. Our results revealed that D-amygdalin was upregulated the expressions of *CDKN1B* and *CDKN1C* more than 8 fold. Sheaff *et al.*[23] showed that expression of *CCNE1-CDK2* results

in phosphorylation of *CDKN1B*, leading to elimination of *CDKN1B* from the cell and progression of the cell cycle from G1 to S phase. *CDKN1C* is a potent tight-binding inhibitor of several G1 cyclin/CDK complexes[24]. *CDKN1C* inhibits cyclin A- and E-associated CDKs, therefore regulates G1/S transition and completion of S phase[24].

In present study, cDNA microarray revealed that D-amygdalin was regulated genes belonging to cell cycle category in K562 cells. Especially, decrease of expressions of *CDK2, CDC25A*, and *CCNE1*, and increase of levels of *CDKN1B, ATM, CDKN1C*, and *CHEK1* were remarkable, and it was confirmed by RT-PCR. Based on these results, D-amygdalin induced DNA damage and thereby triggered S phase arrest, modulated these cell cycle regulator genes. These results suggest that the treatment of D-amygdalin revealed the anticancer effect on human chronic myeloid leukemia K562 cells, and D-amygdalin might be used for anticancer drug.

Methods

Preparation of D-amygdalin

Both 500 g of Armeniacae semen hatched from the shell and 10 L of 4% citric acid solution were refluxed for 2 h. Filtered when it was still hot, the filtrate was passed through the column packed with HP-20. The substance absorbed within the column was concentrated after it had been eluted by ethanol. D-amygdalin (4.2 g; yield rate, 0.84%) was obtained by recrystallizing the extract with ethanol. The amygdalin was used after it had been determined to be over 95.0% of purity, by means of high-pressure liquid chromatography (HPLC) to measure its purity (Fig. 1).

Cell Culture

The K562 cell line was obtained from the Korean Cell Line Bank (KCLB, Seoul, Korea). Cells were cultured in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Gibco). Cultures were maintained in a humidified incubator at 37°C in an atmosphere of 5% CO_2, 95% air.

MTT Assay

Cell viability was determined by the manufacture's protocol using cell proliferation kit (MTT) (Roche, Indianapolis, IN, USA). K562 cells were treated with D-amygdalin at concentrations of 0.5, 1.0, 2.5, and 5 mg/mL for 24 h. After MTT labeling reagent was added to each group, the cells were incubated for 4 h. Then, they were further incubated with the solubilization solution for 24 h. The viability was measured with a microtiter plate reader (Bio-Tek, VT, USA) at a test wavelength of 595 nm with a reference wavelength of 690 nm. The optical density (O.D.) was calculated as the difference between the reference wavelength and the test wavelength. Percent viability was calculated as (O.D. of drug-treated sample/O.D. of untreated sample) × 100.

Microarray Hybridization, Scanning, and Data Analysis

Total RNA was extracted using RNAzol™ B (TEL-TEST, TX, USA) as per the manufacturer's protocol. The cDNA synthesis was performed with 3DNA™ Array 50™ detection method (Genisphere, PA, USA) as per the manufacturer's protocols. The cDNAs of control and D-amygdalin-treated groups (5 mg/mL, 24 h) were synthesized from total RNA. The cDNA chip of TwinChip™ Leukemia cancer 1 K (Digital Genomics) was used. The concentrated cDNA and 3DNA™ were hybridized on two identical arrays in a slide for a duplicate experiment. Hybridization, scanning, and data analysis were done at Digital Genomics. The hybridized microarray was scanned with a confocal laser scanning microscope (ScanArray 5000; Packard Inc., CT, USA) at 532 nm for Cy3 and 635 nm for Cy5. Image analysis using GenePix (Axon Inc., CA, USA) produced quantitative values for each microarray spot. Pixel intensity of the background was subtracted from those of microarray spots. Spot intensities were normalized using the intensities generated by intensity/location dependent method[25]. Normalized spot intensities were calculated into gene expression ratios between the control and treatment groups. Mean data acquired from two identical arrays in a single slide of TwinChip™ were analyzed.

RT-PCR

We selected 4 genes, *CDKN1B, ATM, CDKN1C*, and *CHEK1*, upregulated in microarray analysis by treatment of D-amygdalin. Additionally, 3 genes were selected, *CDK2, CDC25A*, and *CCNE1*, downregulated by treatment of D-amygdalin, and performed RT-PCR. Primer sequences, annealing temperatures and products size of genes were summarized in Table 3. The RT-PCR products were electrophoresed on a 1.5% agarose gel and visualized by staining with ethidium bromide.

Statistical Analysis

Results were expressed as mean ± SEM. The data were analyzed by one-way ANOVA following the Dunnett's *post-hoc* analysis, using SPSS. Differences were considered significant at $p < 0.05$.

Table 3. Sequences of the primers used in RT-PCR analysis.

Primer name	Primer sequence (sense / antisense)	Fragment length (bp)	Annealing temperature (°C)
CDKN1B	5′-GCACTCTTAAATGATCTGCC-3′ 5′-GCAGTGAGGATAGGTTTCTG-3′	474	56
ATM	5′-TTACGTAGCTTCTCCCTTTG-3′ 5′-GTGTGGTTTATTGCCATCTT-3′	524	57
CDKN1C	5′-GAGCAGCTGCCTAGTGTC-3′ 5′-AAGAAATCGGAGATCAGAGG-3′	428	58
CHEK1	5′-GTACTCCAGTTCTCAGCCAG-3′ 5′-ATCGTGTCATTCTTTTGACC-3′	200	55
CDK2	5′-CCATCTTCGACTCTGATAGC-3′ 5′-GCCTTTATAAACATTGTGGC-3′	419	58
CDC25A	5′-TCTGAAGAATGAGGAGGAGA-3′ 5′-ACTCTTTAATGAGGTTGGCA-3′	461	56
CCNE1	5′-AGCTTGTTCAGGAGATGAAA-3′ 5′-GTTGTGTGCATCTTCATCAG-3′	421	57
GAPDH	5′-ACCACAGTCCATGCCATCAC-3′ 5′-TCCACCACCCTGTTGCTGTA-3′	452	60

Acknowledgements

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