Original Article

Induction of sestrin 2 is associated with fisetin-mediated apoptosis in human head and neck cancer cell lines

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Fisetin was reported to have an anti-proliferative and apoptotic activity as a novel anti-cancer agent in various cancer cell lines. However, the possible molecular targets for the anti-cancer effect of fisetin in human head and neck cancer (HNCC) have not yet been clarified. In this study, the influence of fisetin on the growth and apoptosis of HNCCs were examined. In HSC3 cells, fisetin treatment reduced the viability and induced apoptosis. Through the results from the screening of the expression profile of apoptosis-related genes, sestrin 2 (SESN2) was functionally involved in fisetin-mediated apoptosis showing the knockdown of SESN2 by siRNA clearly restored fisetin-induced apoptosis. In addition, fisetin reduced the protein expression levels of phospho-mTOR (p-mTOR) and Mcl-1, which are the downstream molecules of SESN2. It also induced PARP cleavage by inducing an increase in the expression levels of SESN2 together with reducing mTOR and Mcl-1 proteins in other three HNCCs (MC3, Ca9.22, and HN22). Taken together, our findings suggest that the anti-cancer effect of fisetin on HNCCs is associated with SESN2/mTOR/Mcl-1 signaling axis.

***Key Words*: fisetin, sestrin 2, human head and neck cancer, apoptosis**

Sestrins (SESNs), a protein family composed of SESN1, SESN2, and SESN3 in mammals, are conserved stress-responsive proteins that reduce reactive oxygen species (ROS) and are involved in the regulation of cell survival.[1] SESN2, a homolog of p53-activated gene 26, is induced by cytotoxic events such as hypoxia, DNA damage, and oxidative stress.[2,3] SESN2 is down-regulated in cancer cells as a result of the increased production and accumulation of ROS and the redox state of tumor cells.[4,5] Similarly, a lack of SESN2 in mouse embryonic fibroblasts increases proliferation of RAS-activated tumor cells.[6] Recently, several studies have reported that SESN2 suppressed cell proliferation and was involved in apoptosis in colon cancer cells.[1,7] However, the relationship between SESN2 and oral cancer is not clear.

Fisetin (3,3',4',7-tetrahydroxyflavone) is a naturally occurring flavonoid found in numerous vegetables and fruits such as apple, onion, grape, cucumber, persimmon, and strawberry.[8,9] Anti-diabetic, cardio-protective, and neuro-protective activities of fisetin have been demonstrated by using *in vitro* and *in vivo* experimental models relevant to human diseases.[10–12] A potential against cell growth and survival of various cancer cells has been shown.[13–15] Recently, fisetin inhibited malignant proliferation in human oral squamous cell carcinoma cell lines through inhibition of Met/Src signaling pathways.[16] However, crucial molecular targets for the anticancer effect of fisetin have not been identified on human head and neck cancer cells (HNCCs).

Here, the anticancer activity and the molecular targets of fisetin in HNCCs were investigated *in vitro*. Our results indicate that fisetin induces apoptosis in HNCCs by upregulating SESN2.

Materials and Methods

Cell culture and chemical treatment. HSC3 and Ca9.22 Human oral squamous cell carcinoma cell lines were provided from Prof. Shindoh (Hokkaido University, Sapporo, Japan). MC3 mucoepidermoid carcinoma cell line was provided by prof. Wu Junzheng (Forth Military Medical University, Xi'an, China), and HN22 human head and neck squamous cell carcinoma cell line was obtained from Prof. Lee (Dankook University, Cheonan, Korea). The cells were cultured in DMEM supplemented with 10% fetal bovine serum (FBS) and antibiotics at 37°C in 5% CO_2 incubator. All experiments were prepared after the cells reached 50~60% confluence. Fisetin (Fig. 1A; Sigma-Aldrich, St. Louis, MO) was dissolved in dimethyl sulfoxide (DMSO), aliquoted, and stored at −20°C. Final concentration of DMSO did not exceed 0.1%.

Trypan blue exclusion assay. The growth inhibitory effects of fisetin were determined with trypan blue solution (Gibco, Paisley, UK). Cells were stained with trypan blue (0.4%), and viable cells were counted using a hemocytometer.

Western blotting. Whole-cell lysates were prepared with a lysis buffer and protein concentration of each sample was measured using a *DC* Protein Assay Kit (BIO-RAD Laboratories, Madison, WI). After normalization, equal amount of protein was separated by SDS-PAGE and transferred to Immuno-Blot PVDF membranes. The membranes were blocked with 5% skim milk in TBST at RT for 2 h and incubated with primary antibodies and corresponding HRP-conjugated secondary antibodies. Antibodies against cleaved PARP, cleaved caspase-3, SESN2, p-mTOR, mTOR, and Mcl-1 were purchased from Cell Signaling Technology, Inc. (Charlottesville, VA) and actin antibody was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The immunoreactive bands were visualized by ImageQuant LAS 500 (GE Healthcare Life Sciences, Piscataway, NJ).

Live/dead assay. The cytotoxicity of fisetin was determined using a Live/Dead Viability/Cytotoxicity assay kit (Life Tech-

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Table 1. Primer sequences used for real-time PCR

Gene		Sequences (5' → 3')
C1orf162	Forward:	ATCCTCCAGCCAAGCTTTC
	Reverse:	ATGGTTCTCAGCCAAGTGATT
ARRDC4	Forward:	CACATTCCTCCTTACCCTCAAC
	Reverse:	GATGTGGGTCAACCTCTGAATAA
FCER1G	Forward:	GATGTGGGTCAACCTCTGAATAA
	Reverse:	CCGTGTAAACACCATCTGATTTC
MANSC1	Forward:	AGGCAGCTTAGAAACCATACC
	Reverse:	GGAAGACTC CACATTTGACATAGA
EPHX4	Forward:	TTCTCAGCCTGGAGCATTAAG
	Reverse:	CCACAGTAGTAGTGTTGGAGTG
GBP3	Forward:	AGAGCCTAGTGCTGACCTATATC
	Reverse:	TGCGGCTGAGTTCTCTATCT
SESN2	Forward:	GCGGAACCTCAAGGTCTATATC
	Reverse:	AAGTTCACGTGGACCTTCTC
CHAC1	Forward:	GTGCTTGGTGGCTACGATAC
	Reverse:	CACATAGGCCAATGCCTTCA
LATS2	Forward:	GTAGATGAAGAAAGCCCTTGGA
	Reverse:	GTGCTCAGGATGCTTGTTATTG
ANKRD1	Forward:	GGTGAGACTGAACCGCTATAAG
	Reverse:	GGTTCCATTCTGCCAGTGTA
RAB27A	Forward:	AGGACCAGAGAGTAGTGAAAGA
	Reverse:	CGGCTTATGTTTGTCCCATTG
UACA	Forward:	CCTTTCCCAACTCACCTACAC
	Reverse:	CAGCTGTTGCTCCAGAGATT
RNF2	Forward:	CAAGTATCTGGCTGTGAGGTTAG
	Reverse:	CTGCTTCTCACTGGCTGTATC
SMC3	Forward:	GGTGGACAGAAATCCTTGGTAG
	Reverse:	ATCCAGAGCCTGGTCAATTTC
GAPDH	Forward:	GTGGTCTCCTCTGACTTCAAC
	Reverse:	CCTGTTGCTGTAGCCAAATTC

nologies, Grand Island, NY). The polyanionic dye, calcein-AM is retained within live cells, producing an intense green fluorescence through intracellular esterase activity. Ethidium homodimer-1 enters dead cells with damaged cell membranes and binds to nucleic acids, producing a bright red fluorescence. Briefly, cells were stained with 2 μM calcein-AM and 4 μM ethidium homodimer-1 and incubated for 30 min at RT. Cells were analyzed under a fluorescence microscopy (Leica DMi8, Wetzlar, Germany) with the appropriate excitation and emission filters. A total of three random photo were selected from each three independent experiments for quantification. The percentage of live cells was manually calculated by measuring the number of green fluorescence-labeled cells.

4'-6-Diamidino-2-phenylindole staining. To identify the changes in nuclear morphologies of apoptotic cells, the cells were stained with 4'-6-Diamidino-2-phenylindole (DAPI) solution (Sigma-Aldrich, Louis, MO). Briefly, cells were fixed with 100% methanol at RT for 10 min, deposited on slide glasses, and stained with DAPI solution (2 μg/ml). The morphological changes of apoptotic cells were observed under a fluorescence microscopy (Leica DMi8, Wetzlar, Germany).

Microarray. Total RNA was extracted from cells using RNeasy Mini kit (Qiagen, CA) according to the manufacturer's instructions. Two sets of samples were independently prepared and analyzed. The integrity and quantity of total RNA were assessed by Agilent 2100 Bioanalyzer and Nanodrop 1000 analyzer. For each sample, total RNA was analyzed using a Human Gene 2.0 ST Array. The GeneChip Arrays were immediately scanned with Affymetrix GeneChip Scanner 3000 7 G.

Real-Time PCR. Total RNA was extracted using easy-BLUE Total RNA Extraction Kit (INTRON, Daejeon, Korea). The isolated RNA was transcribed by AMPIGENE cDNA Synthesis Kit (Enzo Life sciences, Inc., NY) and real time PCR was performed using the StepOne Real-Time PCR System with AMPIGENE qPCR Green Mix Hi-Rox (Enzo Life sciences, Inc., Farmingdale, NY). Real-time PCR conditions for all genes were as follows: 95°C for 2 min, followed by 40 cycles of 95°C for 10 s and 62°C for 30 s. The relative expression changes of the target genes were quantified by normalizing their expression to that of GAPDH. The PCR primers of all the target genes are listed in Table 1.

siRNA transfection. siSESN2 and siControl were purchased from Bioneer (Daejeon, Korea). Briefly, HSC3 cells were transfected with 50 nM siRNA using Lipofectamine 3000 (Invitrogen, Carlsbad, CA) for 6 h followed by treatment with fisetin for 24 h. After the transfection, HSC3 cells were subjected to Western blotting.

Statistical analysis. ANOVA followed by Tukey's post hoc test were used to determine the significance between the control and treatment groups; p value of <0.05 was considered significant.

Results

Effects of fisetin on growth and apoptosis of HSC3 human head and neck cancer cells. To determine the anti-proliferative activity of fisetin on HSC3 cells, cells were treated with fisetin at various concentrations (7.5, 15, and 30 μM) for 24 h and the cell viability was measured using a trypan blue exclusion assay.

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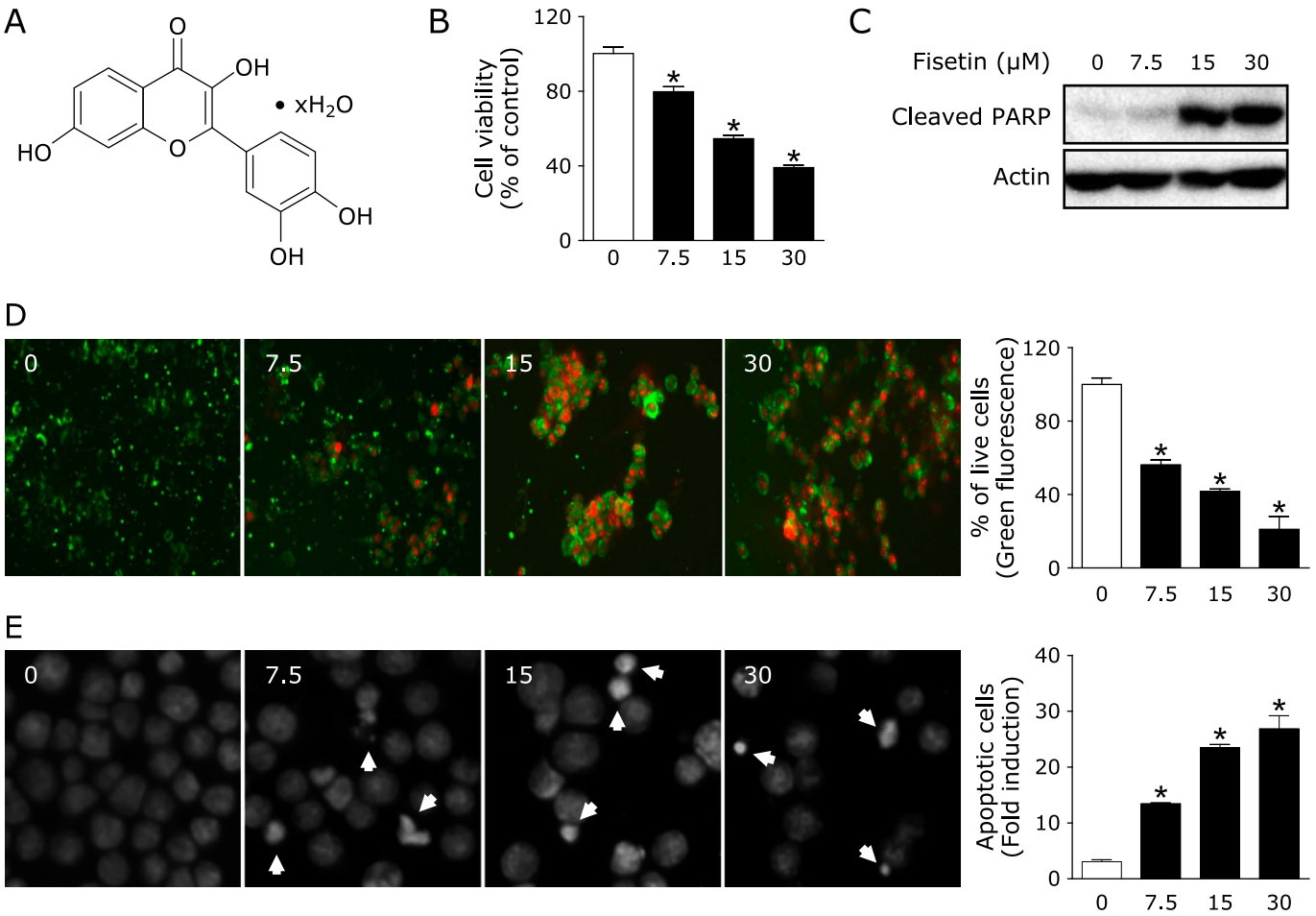

Fig. 1. A role of fisetin in growth inhibition and apoptotic induction on HSC3 cells. (A) The chemical structure of fisetin. The empirical formula of fisetin is $C_{15}H_{10}O_6 \cdot xH_2O$. (B) Cell viability was examined using the trypan blue exclusion assay. (C) The protein expression of cleaved PARP was detected using western blotting and β-actin was used as a loading control. (D) Cytotoxicity of fisetin was measured using a Live/Dead assay. Fluorescence images of HSC3 cells were observed under a fluorescence microscopy (Magnification, ×200). (E) Apoptotic cells stained with DAPI solution were observed by a fluorescence microscope (Magnification, ×400). The all graphs were expressed as the means of three independent experiments. *p<0.05.

Fisetin inhibited the viability of HSC3 cells in a concentration-dependent manner Fig. 1B). The association of the apoptosis and the growth inhibitory effects of fisetin was assessed by western blotting with an antibody capable of detecting cleaved PARP. The PARP cleavage was significantly increased in fisetin-treated groups (Fig. 1C). The fisetin-treated HSC3 cells were also stained with the live/dead assay kit or DAPI solution. The dead cells (red stained) were significantly increased after the fisetin treatment (Fig. 1D). In addition, the fisetin-treated HSC3 cells showed prominent nuclear condensation and fragmentation (Fig. 1E). These results indicate that fisetin inhibits cell growth and induces apoptosis in human head and neck cancer cells (HNCCs).

Involvement of SESN2 in fisetin-induced apoptosis in HSC3 cells. To identify the possible molecular targets for fisetin-induced apoptosis, HSC3 cells were incubated in the presence or absence of 30 μM fisetin and cDNA microarray analysis was performed using Human Gene 2.0 ST Array. The calculated upregulation or downregulation of gene expressions, as determined by the microarray, are listed in Table 2 and 3, respectively. As shown in Table 2 and 3, fisetin-treated HSC3 cells up-regulated 61 genes over 2 folds and down-regulated 81 genes over 2 folds compared with DMSO-treated cells. To verify the reliability, real-time PCR was performed with primers of three different genes, each of which increased (C1orf162, ARRDC4, and FCER1G) or decreased (MANSC1, EPHX4, and GBP3). The results from real-time PCR were similar to those of microarray (Fig. 2B) suggesting that microarray data are reliable. Among 142 genes, 7 apoptosis-associated genes were selected and SESN2 and CHAC1 (ChaC, cation transport regulator homolog 1) changed overwhelmingly compared with other 5 genes (Fig. 2C).

Next, we evaluated whether fisetin affects the expression levels of both SESN2 and CHAC1 proteins in HSC3 cells and we observed a significant increase in SESN2 protein expression in HSC3 cells treated with fisetin (Fig. 3A) while CHAC 1 protein was not affected (data not shown). To investigate the functional consequence of the increased SESN2, HSC3 cells were transfected with either siSESN2 or siCon. As shown in Fig. 3B, the knockdown of SESN2 by siSESN2 resulted in significantly less fisetin-induced apoptosis compared to the cells transfected with siCon. These data indicated that SESN2 mediates the apoptosis induced by fisetin in HSC3 cells. Since SESN2 is known to regulate mammalian target of rapamycin (mTOR) and myeloid cell leukemia-1 (Mcl-1) during apoptosis,[1,17] the effects of fisetin on mTOR/Mcl-1 was determined. The results showed that fisetin reduced the protein expression levels of p-mTOR and Mcl-1 (Fig. 3C). Taken together, these findings suggest that fisetin-induced apoptosis may be related to SESN2/mTOR/Mcl-1 signaling axis.

Table 2. Upregulation of gene expressions in fisetin-treated HSC3 cells

Gene symbol	Gene bank no.	Gene description	Relative expression
MIR100	NR_029515	microRNA 100	18.6201
SMAD9-AS1	ENST00000437983	*SMAD9 antisense RNA 1*	10.0527
MIR4742	NR_039896	*microRNA 4742*	7.0163
C1orf162	ENST00000343534	*chromosome 1 open reading frame 162*	5.1798
ARRDC4	ENST00000268042	*arrestin domain containing 4*	5.1262
FCER1G	NM_004106	*Fc fragment of IgE, high affinity I, receptor for; gamma polypeptide*	5.0681
SESN2	NM_031459	*sestrin 2*	4.7266
C14orf178	ENST00000439131	*chromosome14 open reading frame 178*	4.2350
LOC100506606	AK090590	*uncharacterized LOC100506606*	4.1875
LRP4-AS1	NR_038909	*LRP4 antisense RNA 1*	4.1462
ADAM20P1	NR_037933	*ADAM etallopeptidasedomain 20 pseudogene 1*	4.1350
IDI2-AS1	NR_027709	*IDI2 antisense RNA 1*	4.1319
LINC00641	NR_038971	*long intergenic non-protein coding RNA 641*	4.0861
CCDC62	ENST00000253079	*coiled-coil domain containing 62*	3.8391
HNRNPU-AS1	NR_026778	*HNRNPU antisense RNA 1*	3.8236
NUAK2	ENST00000367157	*NUAK family, SNF1-like kinase, 2*	3.7584
PYGM	NM_005609	*phosphorylase, glycogen, muscle*	3.7578
FBXW10	NR_051988	*F-box and WD repeat domain containing 10*	3.4988
HRH4	NM_021624	*histamine receptor H4*	3.4564
MIR3685	NR_037456	*microRNA 3685*	3.4463
GPR83	NM_016540	*G protein-coupled receptor 83*	3.3115
TMED6	NM_144676	*transmembrane emp24 protein transport domain containing 6*	3.2819
DDIT4	NM_019058	*DNA-damage-inducible transcript 4*	3.2205
NPFF	NM_003717	*neuropeptide FF-amide peptide precursor*	3.1103
RNU11	NR_004407	*RNA, U11 small nuclear*	3.0542
SERPINC1	NM_000488	*serpin peptidase inhibitor, clade C (antithrombin), member 1*	2.9955
RNU2-7P	ENST00000410794	*RNA, U2 small nuclear 7, pseudogene*	2.9560
CHAC1	ENST00000446533	*ChaC, cation transport regulator homolog 1 (E. coli)*	2.9385
SSSCA1-AS1	NR_038923	*SSSCA1 antisense RNA 1 (head to head)*	2.7727
ADAM20	NM_003814	*ADAM metallopeptidase domain 20*	2.7683
ADPRM	NM_020233	*ADP-ribose/CDP-alcohol diphosphatase, manganese-dependent*	2.7642
CLEC4A	NM_016184	*C-type lectin domain family 4, member A*	2.7375
FAM72C	ENST00000492131	*family with sequence similarity 72, member C*	2.6754
DICER1-AS1	NR_015415	*DICER1 antisense RNA 1*	2.6597
MINOS1P1	NR_051980	*mitochondrial inner membrane organizing system 1 pseudogene 1*	2.6291
LOC100287934	AK290103	*uncharacterized LOC100287934*	2.6185
C12orf66	ENST00000398055	*chromosome 12 open reading frame 66*	2.5818
RNF219	NM_024546	*ring finger protein 219*	2.5665
LRIG2	NM_014813	*leucine-rich repeats and immunoglobulin-like domains 2*	2.5337
TXNIP	NM_006472	*thioredoxin interacting protein*	2.5048
LEFTY1	NM_020997	*left-right determination factor 1*	2.4855
MIR4704	NR_039853	*microRNA 4704*	2.4683
MSH4	NM_002440	*mutS homolog 4 (E. coli)*	2.4679
LOC284513	AK096098	*uncharacterized LOC284513*	2.4190
BCO2	NM_031938	*beta-carotene oxygenase 2*	2.4055
RABGGTB	NR_073562	*Rab eranylgeranyltransferase, beta subunit*	2.3985
NEXN-AS1	ENST00000421331	*NEXN antisense RNA 1*	2.3835
OVGP1	NM_002557	*oviductal glycoprotein 1, 120kDa*	2.3758
AVPI1	ENST00000370626	*arginine vasopressin-induced 1*	2.3462
C15orf48	NM_032413	*chromosome 15 open reading frame 48*	2.2732
LOC100505616	AL360137	*uncharacterized LOC100505616*	2.2586
FAM154B	AK304339	*family with sequence similarity 154, member B*	2.2529
VHLL	NM_001004319	*von Hippel-Lindau tumor suppressor-like*	2.2319
CTSK	NM_000396	*cathepsin K*	2.2017
FRS2	NM_006654	*fibroblast growth factor receptor substrate 2*	2.1753
LOC653160	NR_037869	*uncharacterized LOC653160*	2.1697
ZBTB39	NM_014830	*zinc finger and BTB domain containing 39*	2.1601
LIG4	NM_002312	*ligase IV, DNA, ATP-dependent*	2.1438
LATS2	NM_014572	*large tumor suppressor kinase 2*	2.1389
TPTE2P3	NR_002793	*transmembrane phosphoinositide 3-phosphatase and tensin homolog 2 pseudogene 3*	2.1301
IL18BP	NM_005699	*interleukin 18 binding protein*	2.0777

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Table 3. Downregulation of gene expressions in fisetin-treated HSC3 cells

Gene symbol	Gene bank no.	Gene description	Relative expression
ANKRD1	NM_014391	*ankyrin repeat domain 1 (cardiac muscle)*	–5.7362
RAB27A	ENST00000563262	*RAB27A, member RAS oncogene family*	–3.7658
GBP3	ENST00000564037	*guanylate binding protein 3*	–3.6909
MANSC1	NM_018050	*MANSC domain containing 1*	–3.6613
SLC29A3	NM_018344	*solute carrier family 29 (nucleoside transporters), member 3*	–3.5454
EPHX4	NM_173567	*epoxide hydrolase 4*	–3.4609
SFR1	NM_145247	*SWI5-dependent recombination repair 1*	–3.2982
IL24	NM_006850	*interleukin 24*	–3.2138
CASC5	NM_170589	*cancer susceptibility candidate 5*	–3.1766
DLGAP5	NM_014750	*discs, large (Drosophila) homolog-associated protein 5*	–3.1121
HSPH1	NM_006644	*heat shock 105kDa/110kDa protein 1*	–3.0430
FAM111B	NM_198947	*family with sequence similarity 111, member B*	–3.0397
PDCD4	NM_145341	*programmed cell death 4 (neoplastic transformation inhibitor)*	–3.0233
SRGAP1	ENST00000355086	*SLIT-ROBO Rho GTPase activating protein 1*	–3.0089
ASUN	NM_018164	*asunder spermatogenesis regulator*	–2.9678
BAZ1A	NM_013448	*bromodomain adjacent to zinc finger domain, 1A*	–2.9335
GBP3	NM_018284	*guanylate binding protein 3*	–2.9132
UACA	NM_018003	*uveal autoantigen with coiled-coil domains and ankyrin repeats*	–2.9130
ASPM	NM_018136	*asp (abnormal spindle) homolog, microcephaly associated (Drosophila)*	–2.8024
FAM69A	NM_001252271	*family with sequence similarity 69, member A*	–2.7892
TAF5	NM_006951	*TAF5 RNA polymerase II, TATA box binding protein (TBP)-associated factor, 100kDa*	–2.7883
HEATR5A	NM_015473	*HEAT repeat containing 5A*	–2.7877
KIF14	NM_014875	*kinesin family member 14*	–2.7846
POLR3B	NM_018082	*polymerase (RNA) III (DNA directed) polypeptide B*	–2.7774
SH2D1B	NM_053282	*SH2 domain containing 1B*	–2.7574
NANOS1	NM_199461	*nanos homolog 1 (Drosophila)*	–2.7510
XRCC6BP1	ENST00000300145	*XRCC6 binding protein 1*	–2.7489
GLCE	NM_015554	*glucuronic acid epimerase*	–2.7447
TGFB2	NM_001135599	*transforming growth factor, beta 2*	–2.7301
ZNF488	NM_153034	*zinc finger protein 488*	–2.7141
CD58	NM_001779	*CD58 molecule*	–2.7008
HELB	ENST00000247815	*helicase (DNA) B*	–2.6975
GLMN	NM_053274	*glomulin, FKBP associated protein*	–2.6815
STAMBPL1	NM_020799	*STAM binding protein-like 1*	–2.6728
CDC14A	NM_003672	*cell division cycle 14A*	–2.6448
KIAA1107	NM_015237	*KIAA1107*	–2.6375
IFIT3	NM_001031683	*interferon-induced protein with tetratricopeptide repeats 3*	–2.6349
EXOC6	NM_001013848	*exocyst complex component 6*	–2.6189
FAM102B	ENST00000370035	*family with sequence similarity 102, member B*	–2.6185
RNF2	NM_007212	*ring finger protein 2*	–2.6137
FNBP1L	NM_001024948	*formin binding protein 1-like*	–2.5804
PIGB	NM_004855	*phosphatidylinositol glycan anchor biosynthesis, class B*	–2.5770
RPS6KA5	NM_004755	*ribosomal protein S6 kinase, 90kDa, polypeptide 5*	–2.5702
RASA3	NM_007368	*RAS p21 protein activator 3*	–2.5530
KAT6B	NM_012330	*K(lysine) acetyltransferase 6B*	–2.5422
VANGL1	NM_001172412	*VANGL planar cell polarity protein 1*	–2.5304
PWP1	NM_007062	*PWP1 homolog (S. cerevisiae)*	–2.4969
TPR	NM_003292	*translocated promoter region, nuclear basket protein*	–2.4862
CACHD1	NM_020925	*cache domain containing 1*	–2.4845
HTR1D	NM_000864	*5-hydroxytryptamine (serotonin) receptor 1D, G protein-coupled*	–2.4792
CMAS	NM_018686	*cytidine monophosphate -acetylneuraminic acid synthetase*	–2.4709
UAP1	NM_003115	*UDP-N-acteylglucosamine pyrophosphorylase 1*	–2.4536
PDS5B	NM_015032	*PDS5, regulator of cohesion maintenance, homolog B (S. cerevisiae)*	–2.4324
NR2F2	NM_001145155	*nuclear receptor subfamily 2, group F, member 2*	–2.4221
KIAA0586	NM_001244189	*KIAA0586*	–2.4081
RGCC	NM_014059	*regulator of cell cycle*	–2.3926
TENC1	ENST00000314250	*tensin like C1 domain containing phosphatase (tensin 2)*	–2.3821
AMIGO2	NM_001143668	*adhesion molecule with Ig-like domain 2*	–2.3782
SIN3A	NM_001145358	*SIN3 transcription regulator homolog A (yeast)*	–2.3752
DIO2	NM_001242502	*deiodinase, iodothyronine, type II*	–2.3311
STOX1	NM_152709	*storkhead box 1*	–2.3298
DOCK9	NM_001130048	*dedicator of cytokinesis 9*	–2.3213
DHRS3	NM_004753	*dehydrogenase/reductase (SDR family) member 3*	–2.3195
UTP20	NM_014503	*UTP20, small subunit (SSU) processome component, homolog (yeast)*	–2.3095
SMC3	NM_005445	*structural maintenance of chromosomes 3*	–2.2974
AIM2	NM_004833	*absent in melanoma 2*	–2.2717
DENND4A	NM_001144823	*DENN/MADD domain containing 4A*	–2.2705
CLEC9A	NM_207345	*C-type lectin domain family 9, member A*	–2.2468
DRAM1	NM_018370	*DNA-damage regulated autophagy modulator 1*	–2.2426
RNF19B	NM_153341	*ring finger protein 19B*	–2.2414
EPS8	NM_004447	*epidermal growth factor receptor pathway substrate 8*	–2.2327
SACS	NM_014363	*spastic ataxia of Charlevoix-Saguenay (sacsin)*	–2.2269
TARBP1	NM_005646	*TAR (HIV-1) RNA binding protein 1*	–2.1744
RAB38	NM_022337	*RAB38, member RAS oncogene family*	–2.1648
CD44	AF086543	*CD44 molecule (Indian blood group)*	–2.1532
STIL	NM_001048166	*SCL/TAL1 interrupting locus*	–2.1308
TRAPPC6B	NM_001079537	*trafficking protein particle complex 6B*	–2.1090
ALG6	NM_013339	*ALG6, alpha-1,3-glucosyltransferase*	–2.0650
ESPL1	NM_012291	*extra spindle pole bodies homolog 1 (S. cerevisiae)*	–2.0576
BLZF1	NM_003666	*basic leucine zipper nuclear factor 1*	–2.0247
PTPRJ	NM_002843	*protein tyrosine phosphatase, receptor type, J*	–2.0105

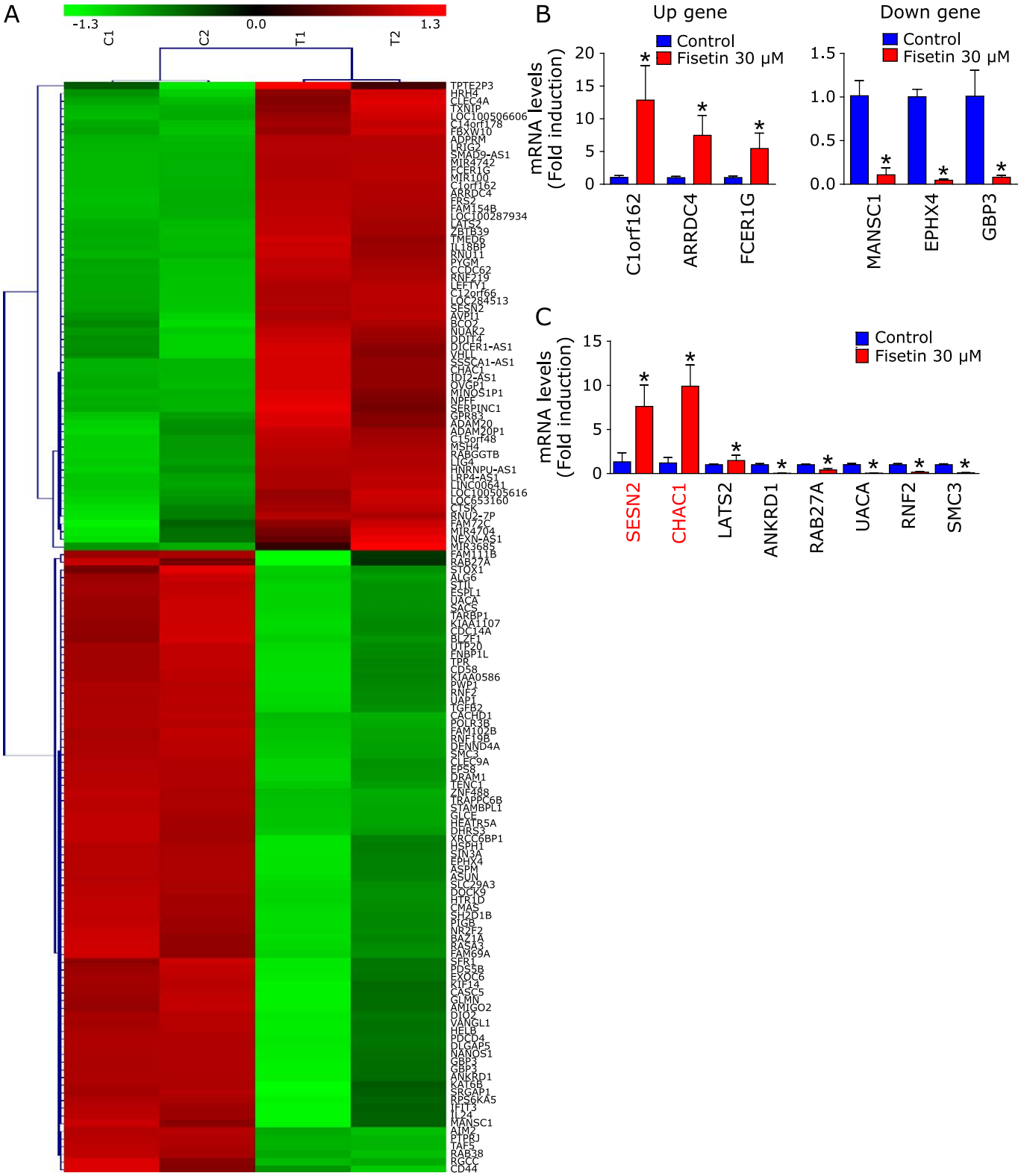

Fig. 2. Validation of molecular targets for fisetin-induced apoptosis. (A) Microarray analysis was represented as a heat map showing fold changes (>2) in the expression of regulated genes by fisetin compared with DMSO in HSC3 cells. The color gradient of heat map indicates relative up- or down-regulation than baseline. It represented two independent experiments. From left to right, the first two samples are DMSO-treated groups and the latter is two fisetin-treated samples. Up-regulated genes are shown in red and down-regulated genes are shown in green. mRNA levels of genes regulated by fisetin (B) and candidates related to fisetin-induced apoptosis (C) were measured by real-time PCR. The graphs were expressed as the means of three independent experiments. *p<0.05.

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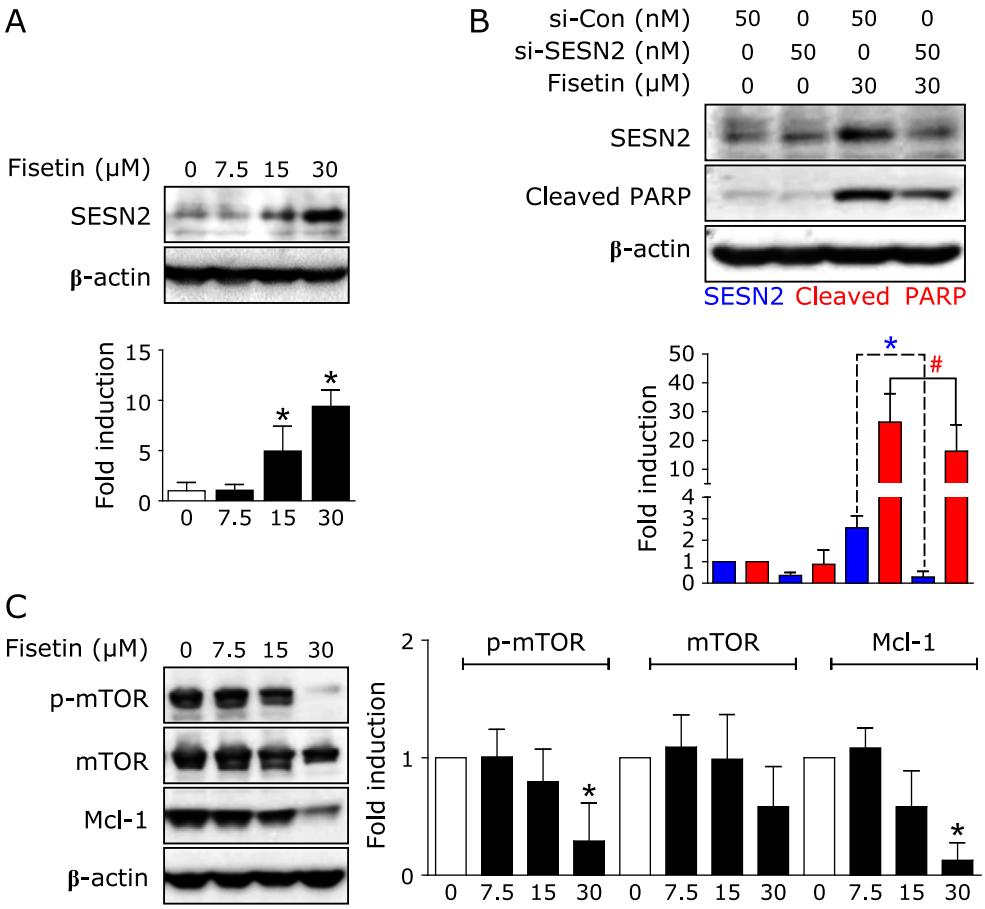

Fig. 3. Involvement of SESN2 in fisetin-induced apoptosis. (A) The expression of SESN2 was detected using western blotting. (B) HSC3 cells were transfected with siCon or siSESN2 and then SESN2 and cleaved PARP were examined by western blotting. (C) The expression levels of p-mTOR, mTOR, and Mcl-1 were detected using western blotting. β-Actin was used as a loading control. The all graphs were expressed as the means of three independent experiments. *$p<0.05$, #$p<0.05$.

Growth-inhibitory and apoptotic effects of fisetin via SESN2/mTOR/Mcl-1 in MC3, Ca9.22, and HN22 human head and neck cancer cell lines. Three other cell lines (MC3, Ca9.22, and HN22) were used to demonstrate that anti-proliferative and apoptotic efficacy of fisetin was not limited to only HSC3 cell line. The results showed that fisetin treatment significantly suppressed the viability of each cell lines (Fig. 4A) and induced PARP cleavage by inducing a dramatic increase in the expression levels of SESN2 and then reducing the expression levels of p-mTOR and Mcl-1 (Fig. 4B). These findings suggest that fisetin-induced apoptosis via SESN2 in HNCCs is a general mechanism for the anticancer effect of fisetin.

Discussion

Head and neck cancer (HNCC) is diagnosed in more than 63,000 people in the United States and is responsible for approximately 13,300 death annually in United State.[18] HNCC is a challenging clinical problem and it is recognized that there is a need to develop alternative methods for the management of this tumor. Naturally occurring compounds from plants, vegetables, and fruits have long been used in traditional medicinal systems because of their non-toxic nature in effective dosages.[20] Thus, there is an increasing interest in the efficacy analysis of inhibiting the proliferation of HNCC using phytochemicals with no toxicity. Our research group recently found naturally-derived chemicals such as silymarin, caffeic acid phenethyl ester, and oridonin have anticancer efficacies by inducing apoptosis of HNCCs.[21–23] Fisetin is also naturally derived compound that can be easily synthesized and has no toxicity to normal cells.[24] First, we demonstrated that fisetin reduced cell viability and induced apoptosis in four different HNCCs (HSC3, MC3, Ca9.22, and HN22). There are several evidences to support our present data showing that fisetin suppresses malignant proliferation and induces apoptosis in human oral squamous cell carcinoma and laryngeal carcinoma.[16,24]

As mentioned earlier, fisetin has been known to have anticancer efficacies in various types of cancers. It inhibited cell proliferation of cancer cells through modulation of multiple signaling pathways. Fisetin-induced apoptosis of human oral cancer cells was through ROS production and mitochondria-dependent signaling pathways.[25] Fisetin also induced apoptosis through p53-mediated upregulation of death receptor 5 expression in human renal carcinoma cells.[26] However, the molecular targets for fisetin-induced apoptosis in HNCCs are not yet known precisely. Here, using gene expression profiling, we found that fisetin treatment affected 142 genes (61 up-regulated genes and 81 down-regulated genes). Among them, the mRNA expression levels of SESN2 and CHAC1, which are apoptosis-associated genes, were significantly up-regulated in fisetin-treated HSC3 cells. Fisetin also increased the expression level of SESN2 protein and knockdown of SESN2 by a siRNA technique clearly recovered HSC3 cells from fisetin-induced apoptosis, suggesting that SESN2 is involved in fisetin-mediated apoptosis. Consistent with our data, it was reported that quercetin, which flavonoid and structurally similar to fisetin,

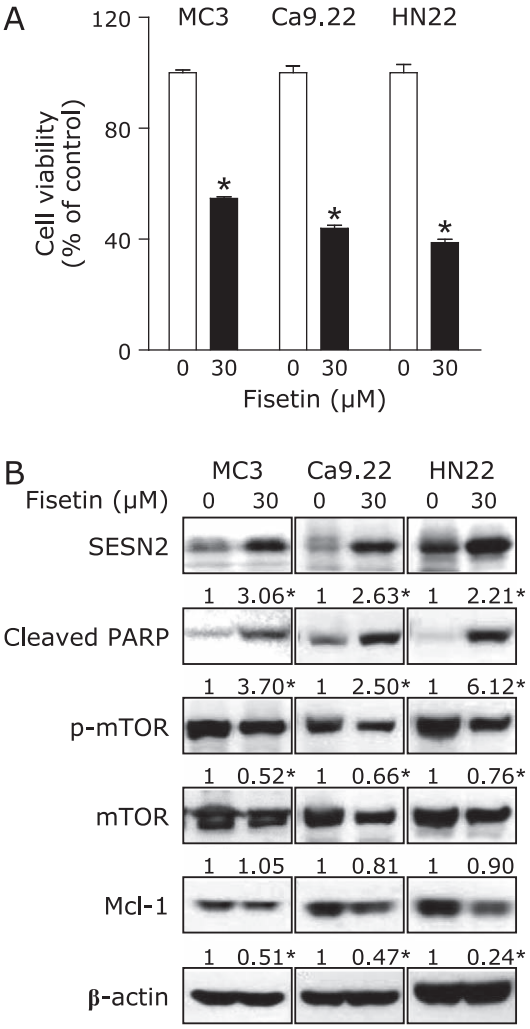

Fig. 4. Apoptotic effects of fisetin in human head and neck cancer cell lines. (A) Cell viability was examined using a trypan blue exclusion assay. The graph was expressed as the means of three independent experiments. *$p<0.05$. (B) SESN2, cleaved PARP, p-mTOR, mTOR, and Mcl-1 were detected using western blotting. β-Actin was used as a loading control.

regulates the SENS2 signaling and induces apoptosis by inducing the generation of intracellular ROS and AMPK/p38 signaling pathway.[27] It suggests the possibility of SESN2 as a therapeutic target for cancer treatment. To our best knowledge, this is the first report demonstrating that SESN2 may be a molecular target for fisetin-induced apoptosis in HNCCs. CHAC1 was identified as a component of the unfolded protein response (UPR) signaling pathway. Fisetin induced apoptosis through endoplasmic reticulum (ER) stress in oral cancer and non-small cell lung cancer.[25,28,29] However, we found that fisetin treatment did not alter the expression level of CHAC1 protein while it significantly augmented its mRNA level in the present study. These data indicate that fisetin-induced apoptosis may not be associated with ER stress in HSC3 HNCCs.

The mammalian target of rapamycin (mTOR) is a serine-threonine protein kinase that are resistant to the growth-inhibitory activities of rapamycin. mTOR comprises of two distinct multi-protein complexes; mTORC1 and mTORC2. SESN2 inhibits mTOR-dependent phosphorylation of p70S6K and 4E-BP1,[6] and knockdown of SESN2 resulted in the activation of mTOR signaling indicating that the important role of SESN2 on mTOR inhibition.[30] Our results from western blotting demonstrated that mTOR was down-regulated by fisetin treatment in HNCCs consistent with other previous studies showing tumor inhibiting potentials of fisetin through mTOR pathway.[24,31,32] These results suggest that fisetin-induced apoptotic potentials is through SESN2/mTOR signaling axis.

In conclusion, fisetin suppresses the growth of HNCCs and induces apoptotic cell death. Its functional role is attributed to SESN2/mTOR/Mcl-1 signaling axis. On the basis of our findings, we suggest that targeting SESN2 by fisetin or its derivatives could be a new strategy to overcome HNCCs.

Author Contributions

DH Won and SH Chung performed experiments and drafted the manuscript; JA Shin, KO Hong, IH Yang were responsible for the study design and data analysis; JW Yun and SD Cho designed the study and revised the manuscript. All authors reviewed and approved the final manuscript.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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