Modulation of alternative splicing of Apaf-1 and Caspase-9 by digoxin and genistein

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Abstract

Alternative splicing is an important mechanism to generate protein diversity, and regulates lots of cellular processes, such as apoptosis[1]. A large number of proteins that are involved in apoptosis are regulated by alternative splicing. Apoptosis protease activating factor 1(Apaf-1) and Caspase-9, which are factors involved in apoptosis, both have transcript variants generated by alternative splicing. Apaf-1 is an adaptor protein that can bind cytochrome C to activate caspase in mitochondria apoptosis pathway. One splice isoform, which includes exon 18, can activate procaspase-9. While the one that excludes exon 18, lose the ability to activate procaspase-9. Caspase-9, which is an initiator caspase, has two transcript variants by inclusion or exclusion of exon3 to exon6. Caspase-9a, which includes the four exons is pro-apoptotic. Caspase-9b, which excludes exon 3 to exon 6, is anti-apoptotic.

Digoxin is a plant derived compound that is used to treat congestive heart disease. Genestein is a plant-derived isoflavones. Studies have demonstrated that digoxin and genistein can modulate alternative splicing[2]. The goal of this study is to test whether digoxin and genistein treatment can modulate alternative splicing of Apaf-1 and Caspase-9. RT-PCR, qRT-PCR and western-blot shows that treatment of cells with digoxin can down-regulates the level of exon-18 containing transcript of Apaf-1 and exon 3 to exon 6-containing transcript of caspase-9.

Introduction

Sequencing of genomes of diverse species demonstrates that the number of genes encoding proteins doesn't correlate with the diversity of proteins. Drosophila has 14,000 genes, while human genome only contains 32,000 genes[3]. Number of proteins is much more than number of genes, which indicates alternative splicing plays important roles in regulating protein diversity. 35% human genes are predicted to be regulated by alternative splicing[4].

Alternative splicing of pre-mRNA is a process by which the primary transcript can be spliced in different ways to generate different mRNA transcripts. There are five patterns of alternative splicing, cassette exons, alternative 5V splice sites, and alternative 3V splice sites, mutually exclusive cassette exons and retained introns. 75% alternative splicing change coding region[5]. Alternative splicing can influence almost all aspects of protein characteristics, including enzymatic or signaling activity, localization, and stability. *Cis* regulatory elements (such as exonic splicing enhancers) and *trans* factors (SR proteins) are involved in regulating alternative splicing[3]. SR proteins are serine/arginine rich proteins that are required for regulating alternative splicing. SR proteins have RNA-recognition motif domain (RRM) and serine/arginine rich C-terminal domain. RRM domain is required for RNA binding, whereas RS domain is important for interacting with splicing apparatus[6].

Gene mutations that influence splice sites can cause hereditary disease. Mutations that influence mRNA splicing takes 15% of mutations that cause genetic diseases[6]. Alternative splicing that changes the ratio of protein isoforms can cause several central nervous system diseases that show abnormal filaments accumulations[5]. Cystic fibrosis

is also caused by mutations in factors involved in regulating alternative splicing[7].

Finding small molecules that can modulate alternative splicing provides an effective way to treat diseases that are caused by deregulation of alternative splicing. Several studies have identified small molecules that modulate splicing, including digoxin and genistein. Digoxin is a cardiac glycoside that is used for therapy of congestive heart failure. Digoxin is an inhibitor of Na/K ATPase, thus can increase intracellular calcium concentrations[8]. Genistein is a plant-derived isoflavones that functions as antioxidant and anthelmintic. Study has shown that genistein can modulate the splicing of the IKBKAP transcript in familial dysautonomia(FD)-derived cells[2].

Apoptosis or programmed cell death is a conserved cellular process that plays important role in embryotic development and homeostasis. Intracellular or extracellular stimuli, such as DNA damage, lack of nutrition and growth factor depletion intrigue extrinsic or intrinsic apoptotic pathway. Both extrinsic and intrinsic apoptotic pathway include series of proteolytic activation of caspases that finally leads to cell death. A large number of genes involved in apoptosis are alternatively spliced. Apaf-1 is an adaptor protein that involved in intrinsic apoptotic pathway. After binding to cytochrome C, in the presence of dATP, Apaf-1 undergoes conformational change that it can recruit procaspase-9. Alternative splicing can cause inclusion or exclusion of exon 18 of Apaf-1, which encodes WD-40 domain at C-terminal. Only the isoform that includes this alternatively spliced WD-40 domain can recruit and activate procaspase-9[9]. Caspase-9 is an initiator caspase, which is also regulated by alternative splicing. Exclusion or inclusion of exon 3 to exon 6 can generate anti-apoptotic caspase-9b or pro-apoptotic caspase-9a[10].

In this study we demonstrate that digoxin enhances exclusion of exon 18 of Apaf-1 and exclusion of exon 3 to exon 6 of caspase-9. Our finding suggests potential role of digoxin in regulating apoptosis.

Material and Methods

Cell culture and treatment

HEK 293 cells, A549 cells and Hela cells were purchased from the ATCC. All cells were cultured at 37 in 5% CO_2 in MEM containing 10% fetal bovine serum. Purified digoxin was purchased from Sigma-Aldrich. Genistein was purchased from Calbiochem (EMD BioSciences). Cells were seeded into 25cm^2 flask 24h before treatment. Cells were treated with 100ng/ml digoxin and 50ug/ml genistein for 48h. Cells were washed twice with PBS and harvested for RNA extraction.

RT-PCR analysis of alternative splice variants

Total cellular RNA is extracted using RNeasy Plus Mini Kits (Qiagen) according to the manufacturer's directions. 20ng of total RNA was used as template to in 20 µl RT-PCR reactions using OneStep RT-PCR Kits (Qiagen). Primers that can pick up both splice isoform are shown in Table 1[1, 9]. Primer concentration used in RT-PCR reaction is 0.5 µM. For Apaf-1, one-step RT-PCR was carried out as follows: one cycle of 50 °C x 30 min and 95 °C x 15 min, followed by 50 cycles of 94 °C x 30 s, 61°C x 30 s, and 72 °C x 30 s, and then a final extension of 72 °C x 7min. Annealing temperature used in Caspase-9 RT-PCR reaction is 55°C. 20ul RT-PCR products was mixed with 4ul loading die, total of 5ul mixture was analyzed on 1.2% agarose gels. Agarose gels were run at 160V. Gels were analyzed by Biorad UV Trans-illuminator. RT-PCR products were

purified using QIAquick Gel extraction kit according to the manufacturer's directions. 80ng of purified products were sent to sequence. Sequencing results were analyzed by Ape software.

Real-time PCR analysis of alternative splice variants

Quantitect SYBR Green RT-PCR Kits (Qiagen) were used for real-time RT-PCR (qRT-PCR) analysis. Primers used in qRT-PCR are listed in table2. ABI PRISM 7000 and 7500 Sequence Detection Systems (Applied Biosystems) was used for real-time RT-PCR. Program was set as follows: one cycle of 50 °C x 30 min and 95 °C x 15 min, followed by 40 cycles of 94 °C x 15 s, 57 °C x 30 s, and 72 °C x 30s. Primers used in real-time PCR are listed in Table2. GAPDH was used as internal control and to normalize results. Obtained results are in the form of threshold cycle, or CT. Results were then expressed as relative level of transcript in treated cells compared with untreated cells.

Western-blot analysis of alternative splice variants

Cells treated as described were washed twice with PBS and lysed in NuPAGE LDS sample buffer (Invitrogen). Equal amounts of proteins sample were analyzed on 10% NuPAGE Bis-Tris gel (Invitrogen). Proteins were then transferred onto nitrocellulose (Bio-Rad) at 100V for 1h. Nitrocellulose membrane was blocked with 5% non-fat milk for 1h at room temperature. Membrane was probed overnight with anti-caspase-9 monoclonal primary antibody (Cell Signaling Technology, Inc). Membrane was washed with PBST three times, each time for 10min. Membrane was probed with anti-mouse antibody conjugated to HRP 1h at room temperature. After three washes with PBST, results were analyzed by radioactive detection.

Table 1. Oligonucleotide primers used for RT-PCR Primers

Template	Primer	Sequence (5'-3')	Product Length
Apaf-1Exon 16	Forward	CAGCTGATGGAACCTTAAAGC	Exon 18-containing transcript 430bp
Apaf-1Exon 19	Reverse	GTCTGGTCATCAGAAGATGTC	Exon 18-lacking transcript 301bp
Casp-9 Exon 2	Forward	AGACCAGTGGACATTGGTTC	Casp-9a 690bp
Casp-9 Exon 8	Reverse	GGTCCCTCCAGGAAACAAA	Casp-9b 240bp

Table 2. Oligonucleotide primers used for qRT-PCR Primers

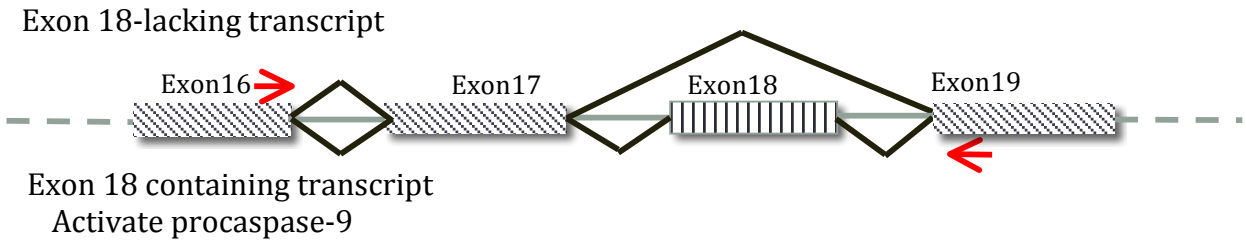
Template	Primer	Sequence (5'-3')	Product Length
APAF1 ex18	Forward	GCACCATCCAGTACTGTGAC	146bp
APAF1 ex19	Reverse	CATCACACCATGAACCCAAC	
Casp9 ex2	Forward	AGTGGACATTGGTTCTGGAG	184bp
Casp9 ex4	Reverse	CTTCTCACAGTCGATGTTGG	
GAPDH	Forward	CAGCCTCAAGATCATCAGCA	106bp
GAPDH	Reverse	TGTGGTCATGAGTCCTTCCA	

Results

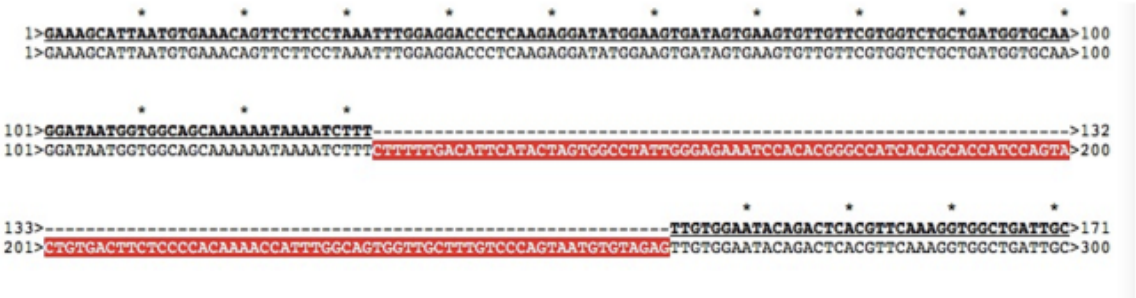
Treatment with Digoxin modulates alternative splicing of Apaf-1

Inclusion and exclusion of exon18 in Apaf-1 can generate two transcript isoforms (Fig.1A). We tested whether digoxin or genistein can modulate the ratio of these two transcripts by RT-PCR and Real-time PCR. We performed RT-PCR with RNA isolated from untreated cells and cells treated with digoxin or genistein, and primers that can pick up both splice variants. RT-PCR result shows that digoxin decreases the exon 18-containing transcript in all the cell lines we tested, including HEK293, A549 and Hela cells. But genistein doesn't impact the mRNA level of transcript of Apaf-1 in the three cell lines we tested (Fig. 1B). We purified and sequenced the products of RT-PCR. Alignment of sequencing result shows that there is a 129bp difference between the two products, which corresponds the size of exon 18 of Apaf-1 (Fig. 1C). Real-time PCR was performed with RNA isolated from HEK293 cells, and primer that is specific for exon 18-containing transcript of Apaf-1. GAPDH was used as internal control and also to normalize result. Result of real-time RT-PCR was expressed as relative level of transcript in digoxin treated cells compared with untreated cells. Real-time PCR shows that in digoxin treated HEK293 cells, relative level of exon 18-containing transcript mRNA decreases (Fig. 1D).

A


Exon 18-lacking transcript
Exon16
Exon17
Exon18
Exon19
Exon 18 containing transcript
Activate procaspase-9

B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101>GGATAATGGTGGCAGCAAAAAATAAAATCTTT--->132
101>GGATAATGGTGGCAGCAAAAAATAAAATCTTTCTTTTTGACATTCATACTAGTGGCCTATTGGGAGAAATCCACACGGGCCATCACAGCACCATCCAGTA>200
133>---TTGTGGAATACAGACTCACGTTCAAAGGTGGCTGATTGC>171
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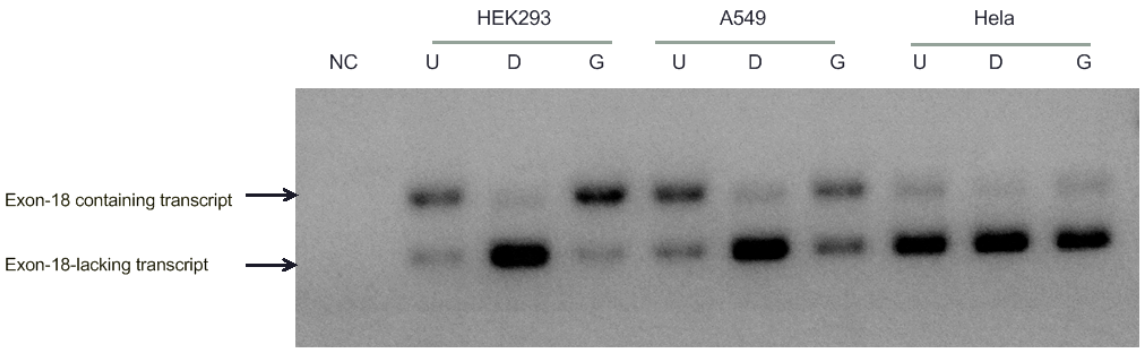
C


HEK293
A549
Hela
NC
U
D
G
U
D
G
U
D
G
Exon-18 containing transcript
Exon-18-lacking transcript

D

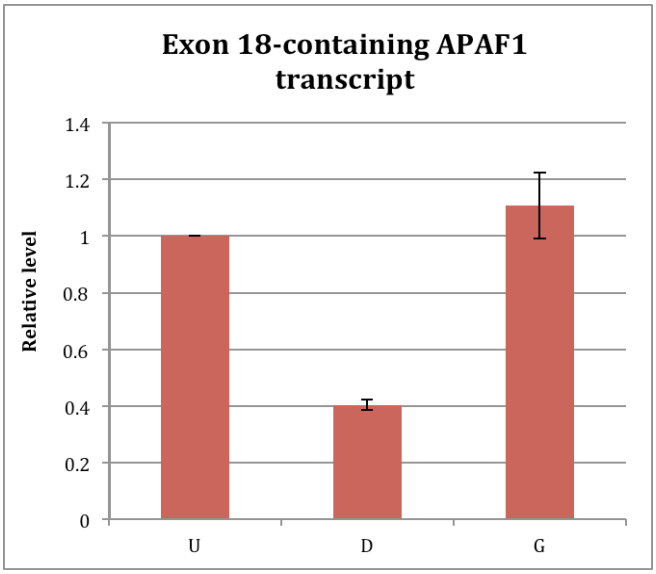
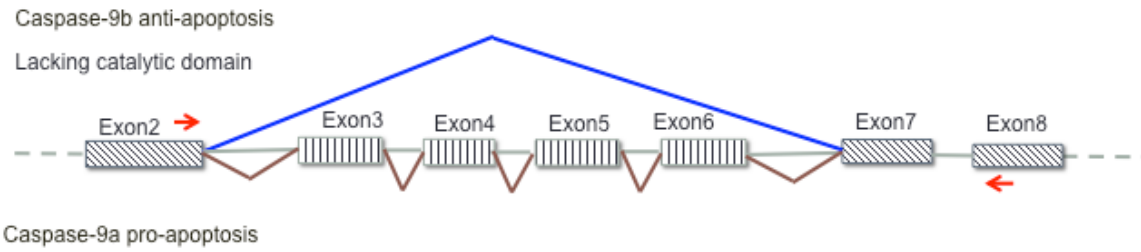


Fig1 Digoxin and Genistein modulate alternative splicing of Apaf-1. (A) Schematic representation of alternative splicing of Apaf-1. Coding exons and alternative exons are indicated as and , respectively. Primer locations are indicated by arrows. (B) HEK293 cells, A549 cells and Hela cells were treated with digoxin or genistein for 48h. RNA were extracted from untreated cells or treated cells. RT-PCR was performed with extracted RNA. Agarose gel of RT-PCR result shows the products of different Apaf-1 transcripts. (C) Alignment of sequencing results of different Apaf-1 transcripts obtained by RT-PCR. (D)qRT-PCR were performed with extracted RNA from HEK-293 cells. Y-axis represents relative amount of Apaf-1 transcripts in treated cells compared with untreated cells. NC, U, D, G represent negative control, untreated cell, digoxin treated cell and genistein treated cell, respectively.

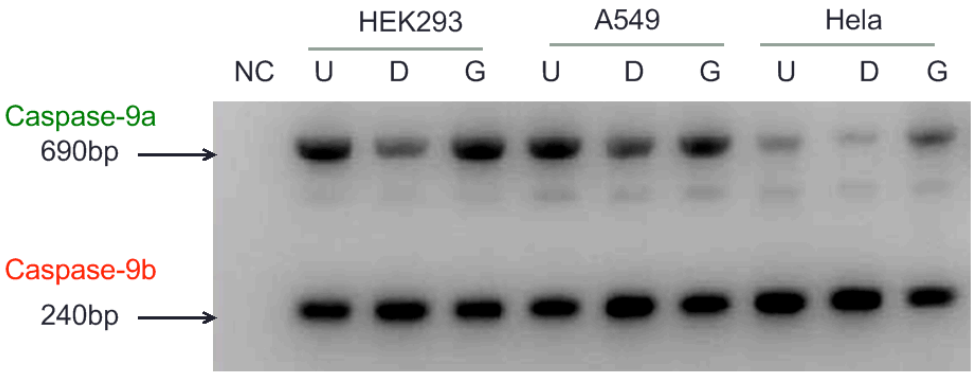
Treatment with Digoxin modulates alternative splicing of Caspase-9

Alternative splicing can create two splice variants from caspase-9 gene by inclusion or exclusion of exon3, exon4, exon5 and exon 6 cassette (Fig2. A). One variant is Caspase-9a, which is proapoptotic. The other variant is caspase-9b, which is anti-apoptotic. We tested whether treating cells with digoxin or genistein alter the ratio of the two splice variants of Caspase-9. RT-PCR result shows that digoxin decreases the amount of Caspase-9a mRNA in HEK293, A549 and Hela cells, while genistein doesn't show obvious effects (Fig. 2B). Alignment of sequencing result of RT-PCR products shows that there is a 450bp difference between the two products, which correspond the size of exon 3 to exon 6 of Caspase-9 (Fig. 2C). Real-time PCR with primers that are specific for Caspase-9a showes that in three cell lines treated with digoxin, caspase-9a mRNA decreases compared with untreated cells (Fig. 2D). Western-blot was performed with anti-Caspase-9 antibody that recognizes both Caspase-9a and Caspase-9b. Western-blot shows that protein level of Caspase-9a decreases in cells treated with digoxin compared with untreated cells (Fig. 2E). The bands of Caspase-9b were too faint to see the difference, maybe because of nonsense degradation.

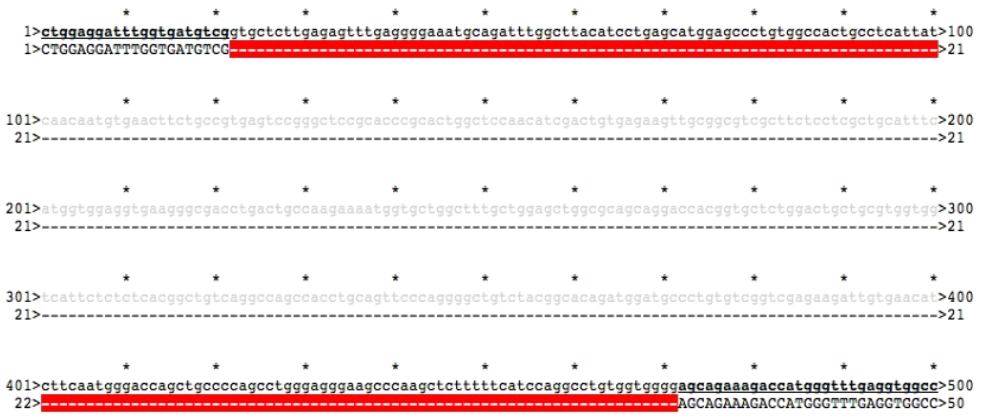
A


Caspase-9b anti-apoptosis
Lacking catalytic domain
Exon2
Exon3
Exon4
Exon5
Exon6
Exon7
Exon8
Caspase-9a pro-apoptosis

B


HEK293
A549
Hela
NC
U
D
G
U
D
G
U
D
G
Caspase-9a
690bp
Caspase-9b
240bp

C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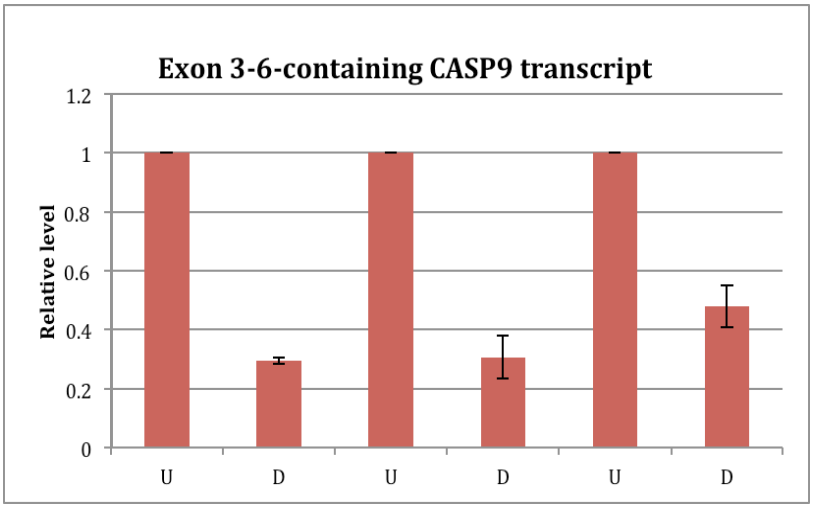
D



E

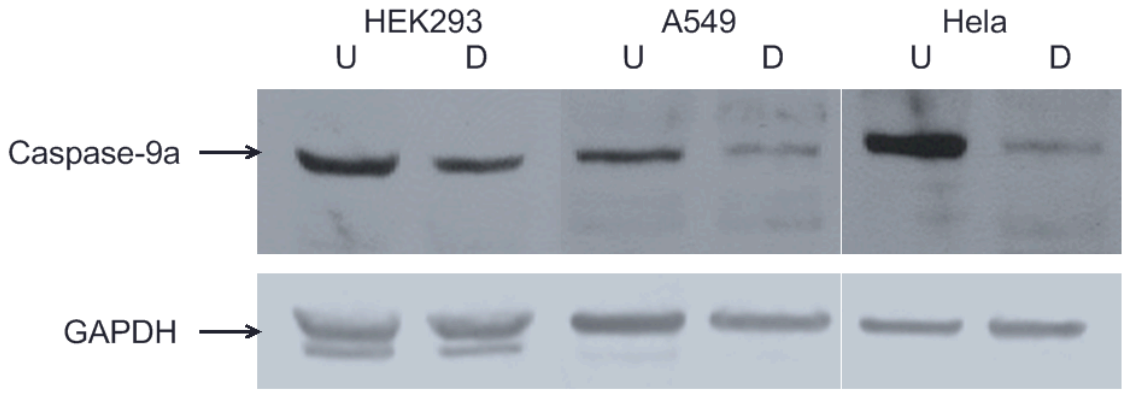


Fig2 Digoxin and Genistein modulate alternative splicing of Caspase-9. (A) Schematic representation of alternative splicing of Caspase-9. Coding exons and alternative exons are indicated as and respectively. Primer locations are indicated by arrows. (B) RT-PCR was performed with RNA obtained as the same way described in Fig1. Agarose gel of RT-PCR result shows the products of different Caspase-9 transcripts. (C)Alignment of sequencing results of different Caspase-9 transcripts obtained by RT-PCR. (D)qRT-PCR were performed with extracted RNA. Y-axis represents relative amount of Caspase-9 transcript in treated cells compared with untreated cells. NC, U, D, G represent negative control, untreated cell, digoxin treated cell and genistein treated cell, respectively. (E) Proteins were extracted from untreated cells and digoxin treated cells. Western-blot was performed with anti-Caspase-9 antibody that can recognize both Caspase-9a and Caspase-9b. GAPDH was used as loading control.

Discussion

By regulating alternative splicing of Apaf-1 and Caspase-9, digoxin increases the mRNA level of anti-apoptotic Apaf-1 and Caspase-9, which suggests a potential role of digoin to regulate apoptosis. However, some studies have shown that digoxin can trigger prostate cancer cell death by Cdk5/p25 dependent pathway. Ca^{2+} also plays important role in cytotoxic effect of digoxin [11]. So, digoxin might have dual effects in regulating apoptosis through different pathways. Studies have shown that digoxin can regulate the expression level of one of the SR proteins, which plays important role in regulating alternative splicing (Data not published). This might be the mechanism that digoxin used to enhance the skipping of exons of Apaf-1 and Caspase-9.

We also tried to test the effects of digoxin on Apaf-1 by Western Blot. We used anti-Apaf-1 antibody that can recognize both splice variants. However, the difference between the two variants is only 4kD, and the total size of Apaf-1 is about 140kD. So we can't separate the two separate the two variants by SDS-PAGE.

Genistein is a plant-derived isoflavones that has been reported to modulate alternative splicing, but it doesn't show obvious effects in modulating alternative splicing of Apaf-1 and Caspase-9 in our study.

Alternative splicing plays important role in regulating apoptosis, which is an important cellular process during embryotic development and homeostasis. We highlight the potential role of digoxin in regulation of apoptosis by modulating alternative splicing in this study.

Conclusion

Both RT-PCR and qRT-PCR show that digoxin decreases the inclusion of exon 18 of Apaf-1 in three cell lines tested. RT-PCR, qRT-PCR and Western-blot demonstrate that treatment of cells with digoxin decreases inclusion of exon 3 to exon 6 of Caspase-9.

Our finding suggests the potential roles of digoxin and genistein on regulation of apoptosis.

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References

1. Merdzhanova, G., et al., *E2F1 controls alternative splicing pattern of genes involved in apoptosis through upregulation of the splicing factor SC35.* Cell Death Differ, 2008. **15**(12): p. 1815-23.
2. Anderson, S.L., et al., *Nutraceutical-mediated restoration of wild-type levels of IKBKAP-encoded IKAP protein in familial dysautonomia-derived cells.* Mol Nutr Food Res, 2012. **56**(4): p. 570-9.
3. Modrek, B. and C. Lee, *A genomic view of alternative splicing.* Nat Genet, 2002. **30**(1): p. 13-9.
4. Hanke, J., et al., *Alternative splicing of human genes: more the rule than the exception?* Trends Genet, 1999. **15**(10): p. 389-90.
5. Stamm, S., et al., *Function of alternative splicing.* Gene, 2005. **344**: p. 1-20.
6. Caceres, J.F. and A.R. Kornblihtt, *Alternative splicing: multiple control mechanisms and involvement in human disease.* Trends Genet, 2002. **18**(4): p. 186-93.
7. Garcia-Blanco, M.A., A.P. Baraniak, and E.L. Lasda, *Alternative splicing in disease and therapy.* Nat Biotechnol, 2004. **22**(5): p. 535-46.
8. Stoilov, P., et al., *A high-throughput screening strategy identifies cardiotonic steroids as alternative splicing modulators.* Proc Natl Acad Sci U S A, 2008. **105**(32): p. 11218-23.
9. Benedict, M.A., et al., *Expression and functional analysis of Apaf-1 isoforms. Extra Wd-40 repeat is required for cytochrome c binding and regulated activation of procaspase-9.* J Biol Chem, 2000. **275**(12): p. 8461-8.
10. Massiello, A. and C.E. Chalfant, *SRp30a (ASF/SF2) regulates the alternative splicing of caspase-9 pre-mRNA and is required for ceramide-responsiveness.* J Lipid Res, 2006. **47**(5): p. 892-7.
11. Lin, H., J.L. Juang, and P.S. Wang, *Involvement of Cdk5/p25 in digoxin-triggered prostate cancer cell apoptosis.* J Biol Chem, 2004. **279**(28): p. 29302-7.