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Caffeine induces tumor cytotoxicity via the regulation of alternative splicing in subsets of cancer-associated genes

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ABSTRACT

Caffeine causes a diverse range of pharmacological effects that are time- and concentration-dependent and reversible. The detailed mechanisms of caffeine in tumor suppression via tumor suppressor protein p53 remain unclear. The isoforms of p53 are physiological proteins that are expressed in normal cells and generated via alternative promoters, splicing sites and/or translational initiation sites. In this study, we investigated how caffeine modulated cell cycle arrest and apoptosis via the expression of various alternatively spliced p53 isoforms. Caffeine reduced p53α expression and induced the expression of p53β, which contains an alternatively spliced p53 C-terminus. In HeLa cells, the expression levels of many serine/arginine-rich splicing factors, including serine/arginine-rich splicing factors 2 and 3, were altered by caffeine. Serine/arginine-rich splicing factor 3 was a promising candidate for the serine/arginine-rich splicing factors responsible for the alternative splicing of p53 in response to caffeine treatment. In addition to p53-dependent functions, multiple target genes of serine/arginine-rich splicing factor 3 suggest that caffeine can regulate epithelial-mesenchymal-transition and hypoxic conditions to inhibit the survival of tumor cells. In summary, our data provide a new pathway of caffeine-modulated tumor suppression via the alternative splicing of the target genes of serine/arginine-rich splicing factor 3.

1. Introduction

Tea and coffee have been associated with both positive and negative risks for cardiovascular disease (Bohn et al., 2012; Di Castelnuovo et al., 2012). The phytochemical compounds in tea and coffee and their metabolites, including caffeine, have been suggested to influence protective endogenous pathways via the modulation of gene expression. Caffeine causes a diverse range of pharmacological effects that are time- and concentration-dependent and reversible (Ferre et al., 2008; Ferre, 2008; Bode and Dong, 2007). In addition to its various effects on the cell cycle and growth arrest, caffeine affects tumor cells via various pathways, including caspase-3 and p53 (Essmann et al., 2004) pathways and has therefore been suggested to be useful for the treatment of malignant tumors. The detailed mechanisms of caffeine in tumor suppression via p53 remain unclear.

A recent study demonstrated that the caffeine-induced serine/arginine-rich (SR) splicing factor SC35 (or SRSF2) is required for the alternative splicing of the tumor suppressor gene *Krüppel-like factor 6*, *KLF6* (Shi et al., 2008). Most human pre-mRNA transcripts are alternatively spliced by specific trans-factors, including SR splicing factors (SRSFs), but the role and regulatory mechanism of alternative splicing in different biological processes is not well studied (Erkelenz et al., 2013; Busch and Hertel, 2012; Graveley, 2000). SR proteins are a group of essential splicing factors required at the different steps of spliceosome assembly (Graveley, 2000; Lou et al., 1998; Anko et al., 2012). To date, nine classical SR splicing factors have been identified, including SF2/ASF (splicing factor 2/alternative splicing factor), SRp20 (or SRSF3), SC35 (or SRSF2), SRp40, SRp30c, SRp55, SRp75, SRp54 and 9G8, in addition to a number of non-classical proteins, e.g., SRp38 and Tra2β (transformer-2 protein beta). SRSF3 has been shown to regulate

Abbreviations: SR, serine/arginine-rich; ACTN, alpha actinin; KLF6, Krüppel-like factor 6; EMT, epithelial-mesenchymal-transition; HIF-1α, hypoxia inducible factor 1α; L-OHP, L-oxaliplatin; VEGF, vascular endothelial growth factor; SREBP1c, sterol regulatory element-binding protein 1c; Glut1, glucose transporter 1; COX-2, cyclooxygenase-2; FASN, fatty acid synthase; EGFR, epidermal growth factor receptor.

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the splicing of *fibronectin* (de la Mata and Kornblihtt, 2006), itself (Jumaa and Nielsen, 1997), *pyruvate kinase M* (Christofk et al., 2008) and *p53* (Tang et al., 2013). Knockout studies indicated that SRSF3 is essential for mouse development, hepatocyte differentiation and metabolic function, as well as tumor cell proliferation and maintenance (Sen et al., 2013; He et al., 2011; Jia et al., 2010; Jumaa et al., 1999). However, an understanding of the regulatory mechanisms of SRSF3 function is necessary to decipher the different effects caused by different modulators, such as caffeine.

The isoforms of p53 are physiological proteins that are expressed in normal cells and generated from the *p53* gene via alternative promoters, splicing sites and/or translational initiation sites (Bourdon et al., 2005; Marcel et al., 2011). Tang et al. demonstrated that SRSF3 regulates cellular senescence via *p53* alternative splicing (Tang et al., 2013). The downregulation of SRSF3 in normal human fibroblasts undergoing replicative senescence is associated with the upregulation of p53β, which is an alternatively spliced isoform of *p53* that promotes p53-mediated senescence. In this work, we found that caffeine altered the expression level of the p53α and p53β isoforms that were mediated by the downregulation of the *SRSF3* gene and protein. In addition to the p53 isoforms, other SRSF3 target genes were also alternatively spliced in response to caffeine treatment. The effects of caffeine on cells depended on the alternative expression of target genes, such as senescence associated with the upregulation of p53β or epithelial-mesenchymal-transition (EMT) associated with the downregulation of *Hypoxia inducible factor 1α* (*HIF-1α*). We believe that SRSF3 is not the only SRSF protein affected by caffeine because of the increased expression of SC35 observed in this study. However, this work might provide a new direction to understand the roles of caffeine in the alternative splicing mechanism for antitumor functions.

2. Materials and methods

2.1. Cell culture, plasmids, and transfection

HeLa (human cervical carcinoma), H9c2 (rat cardiomyocyte) and WI-38 (normal human fetal lung fibroblast) cells were cultured in Dulbecco's modified Eagle's medium, A253 cells (human submandibular duct cell) were cultured in McCoy's medium, and Reh cells (human, African, peripheral blood, leukemia, pre-B cell) were cultured in RPMI medium supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA, USA). The *p53α* and *β* isoforms and *SRSF3* (or *SC35*) were constructed by inserting the appropriate PCR fragments into the pSG5.HA vectors via the *BamH*I-*Xho*I and *Eco*RI-*Xho*I restriction sites, respectively. The cells were transfected in 24-well plates with jetPEI (PolyPlus-transfection, France) according to the manufacturer's protocol.

2.2. Cell viability assay

The cells were seeded in 96-well culture plates and allowed to grow for 1 day. The cells were then exposed to caffeine in fresh DMEM for the indicated periods of time. MTT (3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) solution (0.5 mg/ml in PBS) was added to each well, and the plate was incubated for 2 h at 37 °C. Dimethylsulfoxide (DMSO) (150 μl) was then added as a solubilizing agent, and the absorbance at 540 nm was measured using an ELISA plate reader (Multiskan EX, Thermo, USA). As a control, cells treated with media containing no compound were defined as 100% cell survival.

2.3. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was isolated using the TRIsure (BIOLINE, London, UK) reagent according to the manufacturer's instructions. One microgram of total RNA was subjected to reverse transcription using MMLV reverse transcriptase for 60 min at 37 °C (Epicentre Biotechnologies, USA), and PCR reactions were run on a GeneAmp PCR system 9700 (Applied Biosystems, NJ, USA). All PCR primer sequences are shown in Table 1.

Table 1
PCR primers used in this study.

Gene name	Primer sequence (5′ → 3′)
p53α	Forward: 5′-GATGAAGCTCCCAGAATGCCAGAG-3′ Reverse: 5′-GAGTTCCAAGGCCTCATTCAGCTC-3′
p53β	Forward: 5′-ATGGAGGAGCCGCAGTCAGAT-3′ Reverse: 5′-TTTGAAAGCTGGTCTGGTC-3′
Δ 133 p53α	Forward: 5′-CTGAGGTGTAGACGCCAACTCTCTCTAG-3′ Reverse: 5′-AATGTCAGTCTGAGTCAGGCCCTTCTGTC-3′
p53α/β	Forward: 5′-CTCTGACTGTACCACCATCCACTA-3′ Reverse: 5′-GAGTTCCAAGGCCTCATTCAGCTC-3′
GAPDH	Forward: 5′-CTTCATTGACCTCAACTAC-3′ Reverse: 5′-GCCATCCACAGTCTTCTG-3′
SC35	Forward: 5′-CGAGAAGTACGGGCGCGTCG-3′ Reverse: 5′-GGCTGCGAGACCTGGAACGG-3′
SRSF3	Forward: 5′-ATGCATCGTGATTCCTGTCCATTG-3′ Reverse: 5′-CTATTTCCTTTCATTTGACCTAGATC-3′
PK M2	Forward: 5′-GGTGGCTCGTGGTGATCTAGG-3′ Reverse: 5′-CGATTATGGCCCCACTGCAGC-3′
PK M1	Forward: 5′-GCGAGCCTCAAGTCACTCCAC-3′ Reverse: 5′-TCACGGCACAGGAACAACACG-3′
KLF6	Forward: 5′-ATGGACGTGCTCCCCATGTGC-3′ Reverse: 5′-GCTCAGTTCCGGAGAAGATGG-3′
Snail	Forward: 5′-ATGCCGCGCTCTTTCCTCGTCAGG-3′ Reverse: 5′-TCAGCGGGGACATCCTGAGCAGCC-3′
Slug	Forward: 5′-ATGCCGCGCTCCTTCCTGGTCAAG-3′ Reverse: 5′-TCAGTGTGCTACACAGCAGCCAGAT-3′
Vimentin	Forward: 5′-ATGTCCACCAGGTCCGTGTCCTCG-3′ Reverse: 5′-TTATTCAAGGTCATCGTGATGCTGAGAA-3′
E-cadherin	Forward: 5′-CCTGGGACTCCACCTACAGA-3 Reverse: 5′-GGATGACACAGCGTGAGAGA-3′
HIF-1α	Forward: 5′-GAACCTGATGCTTTAACT-3′ Reverse: 5′-CAACTGATCGAAGGAACG-3′
VEGF	Forward: 5′-GGACATCTTCCAGGAGTACC-3′ Reverse: 5′-GTTCCCGAAACCCTGAGGG-3′
SREBP1c	Forward: 5′-GGAGCCATGGATTGCACTTTC-3′ Reverse: 5′-CAGGGGTGGAGCTGAACTGC-3′
Glut1	Forward: 5′-CCATGGAGCCCAGCAGCAAG-3′ Reverse: 5′-GCAGTACACACCGATGATGA-3′
HIF-2α	Forward: 5′-TGCATCATGTGTGTCAACTACG-3′ Reverse: 5′-TGAAATCCGTCTGGGTACTGC-3′
FASN	Forward: 5′-TGAGCCTCATGCGCCTGGAC-3′ Reverse: 5′-CGCACCTCCTTGGCAAACAC-3′
EGFR	Forward: 5′-GCTTTGGTGCCACCTGCGTG-3′ Reverse: 5′-CTCCATCACTTATCTCCTTGAG-3′
Ki-67	Forward: 5′-CGAGACGCCTGGTTACTATC-3′ Reverse: 5′-CTTGGAAATTCAGTTGACTTCC-3′

2.4. Western blot analysis

HeLa cell lysates were prepared in lysis buffer (100 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.1% SDS and 1% Triton X-100) at 4 °C. The transfected cell extracts were separated by SDS-PAGE, transferred onto a polyvinylidine difluoride membrane (Millipore, USA) and detected using antibodies against PARP, α-actinin (ACTN), caspase-3, p53, p21, cyclin D1, SRSF3, vimentin, snail,