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Interaction between DNA-PKcs and AMP-activated Protein Kinase (AMPK)

조선대학교 대학원

생물신소재학과

파르메수어 나르얀 아마티야

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DNA-PKcs 와 AMPK 의 상호작용연구

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ABSTRACT

Interaction between DNA dependent Protein Kinase (DNA-PKcs) and AMP-activated Protein Kinase (AMPK)

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I: AMPK activated Protein Kinase (AMPK) knockdown by small interfering RNA induces apoptosis under UV stress condition in Human Colorectal Carcinoma cell line.

Two systems are essential in humans for genome integrity, DNA repair and apoptosis. Cells that are defective in DNA repair tend to accumulate excess DNA damage. Cells defective in apoptosis tend to survive with excess DNA damage and thus allow DNA replication pass DNA damages, causing mutations leading to carcinogenesis. Human DNA-dependent protein kinase (DNA-PK) is a nuclear localized serine /threonine protein kinase that is composed of a large catalytic subunit, DNA-PKcs (approximately 470kDa) and a heterodimeric protein Ku. It is involved in the repair of DNA double strand break repair by non homologous end joining pathway. Besides that it also acts as a scaffold protein to aid the localization of other DNA repair proteins to the site of damage. It also plays a role in the stability of telomere and the prevention of chromosomal end fusion. We

found that DNA-PKcs interacts with AMP activated Protein Kinase, gamma 1 subunit. This interaction is prominent in UV stress condition. This interaction is confirmed by yeast two hybrid assay and co-immunoprecipitation test. Under UV stress condition, these two proteins co-localize, which is confirmed by confocal microscopy. Cells become more apoptotic when AMPKγ-1 is knockdown by siRNA as confirmed by cell death assays. These results give clue in the development of anticancer drugs targeting AMPK.

II: Human glioma cell line proficient in DNA-dependent Protein Kinase (DNA-PKcs) is sensitive to cell death under glucose depleted conditions.

M059K and M059J are human glioma cell lines. The former one is DNA dependent protein kinase catalytic subunit (DNA-PKcs) proficient, while the latter one is DNA-PKcs deficient. We checked the sensitivity of these two cell lines under low glucose condition. Under low glucose condition, AMP activated protein kinase (AMPK) is activated. AMPK is a serine/threonine protein kinase. It is the energy sensor in the eukaryotic cell. It regulates energy balance by monitoring changes in the cellular concentrations of the nucleotides AMP and ATP. An increase in AMP concentration, indicating an energy deficit, activates AMPK and initiates ATP generating pathways and suppresses those involved in ATP consumption. Conversely, high ATP concentration, indicating energy sufficiency prevents activation of AMPK. Under glucose depleted condition, in the glioma cell line, there is considerable cell death. Human glioma cell line, M059K is more sensitive than M059J as shown by cell death assays. Under glucose depleted condition, AMPK is activated and phosporylated at Threonine 172 of AMPK alpha in a time dependent manner. This phosphorylation effect is more prominent in M059K cell than M059J. This shows that DNA-PKcs, a DNA repair protein, is involved in the activation of AMPK under glucose depleted condition and causes the cell death.

INTRODUCTION

Two systems are essential in humans for genome integrity, DNA repair and apoptosis. Cells that are defective in DNA repair tend to accumulate excess DNA damage. Cells defective in apoptosis tend to survive with excess DNA damage and thus allow DNA replication pass DNA damages, causing mutations leading to carcinogenesis [1]. It has recently become apparent that key proteins which contribute to cellular survival by acting in DNA repair become executioners in the face of excess DNA damage. Five major DNA repair pathways are homologous recombinational repair (HRR), non-homologous end joining (NHEJ), nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR).

DNA double strand breaks (DSB) are the most serious form of DNA damage. If not repaired prior to DNA replication or mitosis, they can lead to cell death. If misrepaired, DSBs contribute to chromosomal aberrations, genomic instability and cancer predisposition [8, 9].

Double strand breaks (DSBs) are caused by ionizing radiation, chemicals like bleomycin, neocarzinostatin, sodium arsenite, and anticancer drugs like etoposide. It also occurs normally during the process of DNA replication, meiotic exchange and V(D)J recombination. Two distinct mechanisms for double strand break repair are the error-free homologous recombination repair (HRR) pathway and the error-prone nonhomologous end joining (NHEJ) pathway. HRR functions primarily in repairing both DSBs that arise at DNA replication forks and DSBs arising in S or G2 phase chromatid regions that have replicated. NHEJ functions at all stages of the cell cycle, but plays the predominant role in both the G1phase and in S-phase regions of DNA that have not yet replicated. Proteins known to be required for NHEJ include the DNA-dependent protein kinase (DNA-PK), XRCC4, DNA ligase IV and Artemis etc [10].

DNA-dependent Protein Kinase (DNA-PK)

Human DNA-dependent protein kinase (DNA-PK) is a nuclear localized serine /threonine protein kinase that is composed of a large catalytic subunit, DNA-PKcs (approximately 470kDa encoded by the gene PRKDC) and a heterodimeric protein Ku which has high affinity for the ends of double-stranded DNA [2]. The Ku heterodimer is composed of a 73kDa subunit (Ku70) and an 86kDa subunit (ku80). Apart from its large size, the most noticeable feature of DNA-PKcs is a carboxy-terminal catalytic domain which bears amino acid similarity to the catalytic domain of the phosphoinositide-3,4-kinase (PI3,4-kinase) family of lipid kinases. The presence of this conserved region classifies DNA-PKcs as a member of the phosphatidylinositol-3 kinase related protein kinases (PIKKs). The PIKK family also includes ATM (ataxia-telangiectasia mutated), ATM and ATR (ATM-and Rad3-related), mTOR (mammalian target of rapamycin), TRRAP and ATX/hSMG-1. DNA-PKcs, ATM, ATR and mTOR have a similar structural organization, such that in addition to a carboxy-terminal PI3, 4-kinase domain, they have a large amino-terminal domain, as well as FAT and FATC domains that flank the kinase domain [11].

The similarities between DNA-PKcs, ATM and ATR suggest that they share similar structural characteristics. In the case of ATM, the FAT and FATC domains may interact to stabilize the catalytic domain [15], and it is possible that the FAT/FATC domains serve a similar function in DNA-PKcs. The large N terminal domains in ATM and ATR are predicted to contain multiple HEAT (huntingtin, elongation factor 3, A subunit of protein phosphatase 2A and TOR1) repeats that are arranged to form a large, highly helical surface that could provide multiple sites for protein–protein interaction, and it is likely that the amino-terminal domain of DNA-PKcs adopts a similar structure [16]. The structure of the DNA-PKcs polypeptide has been solved to approximately 20 Å and predicts that DNA-PKcs

contains a globular head or crown region and a tail or base [17–19]. Interestingly, the molecule contains cavities or channels that are large enough to accommodate dsDNA. The overall shape of the DNA-PKcs molecule as determined in one of these studies [19] is similar to that determined for ATM [20]. However, at approximately 20 Å resolution, the structure of both proteins gives only the overall shape and dimensions of the molecule; atomic detail and the location and structure of the various domains awaits the generation of a higher resolution structure.

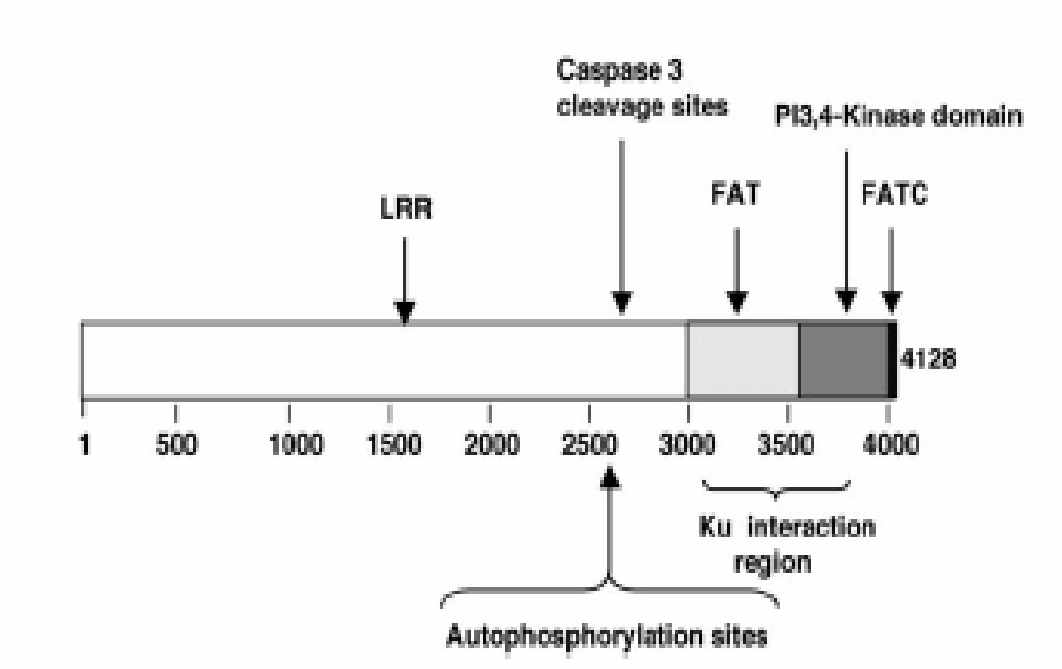


Fig.1. Schematic of the structural organization of the human DNA-PKcs polypeptide.

-Biochimie 85 (2003) 1161–1173

DNA-PK plays a critical role in DNA double stand break (DSB) repair and in V(D)J recombination. It also plays a role in triggering apoptosis in response to severe DNA damage or critically shortened telomeres [3]. DNA-PK has important roles in the maintenance of telomeres and in prevention of chromosomal end fusions. Cells

defective in DNA-PK are radio sensitive, defective in DNA double strand break repair and immune deficient [10].

When a double strand break occurs due to various stresses in a cell, first, the Ku70/80 heterodimer binds to the ends of dsDNA at the site of DSB. The catalytic subunit of the DNA-dependent protein kinase (DNA-PKcs) is then recruited to DNA-bound Ku to form the DNA-PK holoenzyme. The ends of the break are brought together as two molecules of DNA-PK (one at each end of the break) are joined in a synaptic complex. Other factors, such as polynucleotide kinase (PNK), Artemis, the MRE complex, Human tyrosyl DNA Phosphodiesterase (hTdp1) or the Werner Syndrome protein (WRN) may be required for processing of the DNA ends prior to end rejoining, but exactly when processing takes place is not known. Following the formation of the synaptic complex, the XRCC4/DNA ligase IV complex is recruited. Prior to end rejoining, protein factors must be removed from the DNA. This may involve DNA-PK autophosphorylation [21, 33, and 35]. After removal of the repair factors, the DNA ends are ligated and the DNA is repaired. Both Mg-ATP and the protein kinase activity of DNA-PKcs are required for NHEJ [38, 39], probably through phosphorylation of DNA-PKcs and/or other proteins. In addition, inositol hexaphosphate (IP6) stimulates end joining in vitro [72] and binds to Ku, but its precise role in NHEJ is unknown.

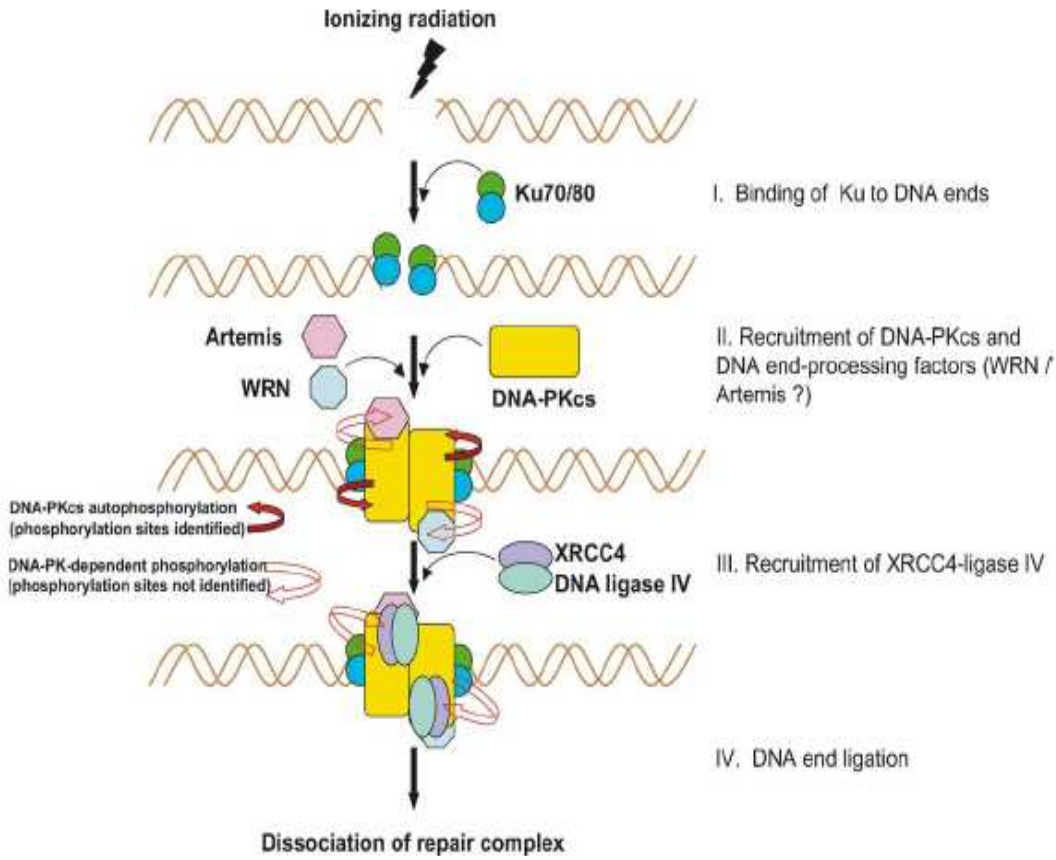


Fig.2.The repair of double stands breaks by Non homologous end joining

-DNA Repair 3 (2004) 909–918

Stresses that activate DNA-PK

Treatment	Used cell line	Reference
Bleomycin (50ug/ml)	HeLa, normal human skin fibroblasts (HSF), human glioma cell lines M059K (wild type) and M059J (DNA-PKcs-defective) and Chinese hamster ovary (CHO) cell lines AA8 (wild type) and V3 (defective in both DSB repair and DNA-PKcs expression)	The Journal of Biological Chemistry, Vol 280, No. 15 April 15, 14709-14715; 2005
Ectoposide (30ug/ml)	Same as above	Same as above.
Ionizing Radiation (10-50Gy);30 min recovery time.	Same as above	Same as above.
Sodium arsenite	-Chinese hamster ovary (CHO-K1), xrs-5 (Ku80 mutant) and irs-20 (DNA-PKcs mutant) cells	Elsevier; DNA Repair 2 (2003) 309-314
Nitric Oxide	Human fetal kidney cell line EcR293 transfected with pIND vector containing cDNA encoding human inducible nitric oxide synthase (iNOS)	Nature Cell Biology; VOL 2; June 2000

AMP activated Protein Kinase (AMPK)

AMPK is a serine threonine protein kinase, which serve as an energy sensor in all eukaryotic cell types [42]. AMPK is heterotrimer consisting of an alpha catalytic subunit and non catalytic beta and gamma subunits. In mammals, there are two α-subunits, three γ-subunits, and two β-subunits (α1, α2, γ1, γ2, γ3, β1 and β2) [44]. The γ-subunits each contain four tandem cystathione β-synthase (CBS) domains that together bind two molecules of AMP and allow the kinase complex to sense adenine nucleotide levels. The β-subunits have been reported to serve a scaffolding function for the α-and γ-subunits as well as target the complex to intracellular sites such as the cell membrane through myristoylation of its N-terminus[51,52] and to intracellular glycogen through a glycogen binding domain[53]. Binding of AMP to AMPK leads to phosphorylation of the α-subunit at Thr-172 by a family of upstream kinases. At least two kinases can phosphorylate AMPK: tumor suppressor LKB1, which transduces signals that are generated by changes in the cellular energy status[54,55] and Ca2+ calmodulin dependent protein kinase kinase β (CaMKKβ) which senses changes in intracellular calcium [54, 56-60].

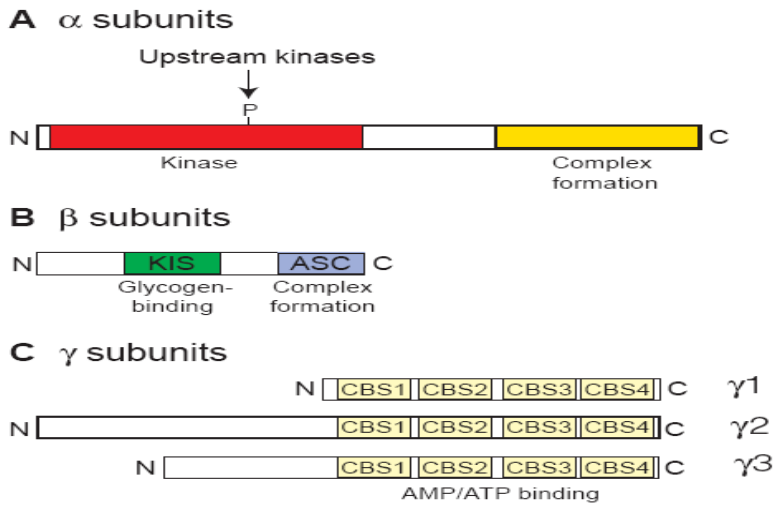


Fig.3. Conserved domains in AMPK subunits.

-Journal of Cell Science 117; 5479-5487; 2004

Stresses that activate AMP activated Protein Kinase (AMPK)

Type of Treatment	Treatment	Reference
Respiratory chain inhibitors	Antimycin A	Witters et al.,1991
	Azide	Witters et al.,1991
	Nitric Oxide	Almeida et al.,2004
ATP synthase inhibitor	Oligomycin	Marsin et al.,2000
Mitochondrial uncouplers	Dinitrophenol	Witters et al.,1991
	Over-expression of UCP1	Matejkova et al.,2004
	Over-expression of UCP3	Schrauwen et al., 2004
TCA cycle inhibitor	Sodium arsenite	Corton et al.,1994
Environmental stresses	Heat Shock	Corton et al.,1994
	Oxidative stress	Choi et al.,2001
	Osmotic stress	Fryer et al.,2002
Metabolic stresses	Ischaemia	Kudo et al.,1995
	Hypoxia	Marsin et al.,2000
	Low glucose	Salt et al., 1998
	Exercise (muscle)	Winder and Hardie,1996
	Contraction (muscle)	Hutaber et al., 1997
		Vavvas et al.,1997
Biguanide drugs	Metformin	Zhou et al., 2003
	Phenformin	Hawley et al.,2003
Thiazolidinedione drugs	Rosiglitazone	Fryer et al.,2002
	Pioglitazone	Saha et al., 2004

Activators of AMPK

Treatment	Used Cell Line	Reference
Oxidative Stress -Hydrogen Peroxide	Mus musculus Fibroblast NIH-3T3 cells	Biochemical and Biophysical Research Communications 287, 92-97 (2001)
Metabolic stress -Hypoxia and glucose deprivation	Pancreatic cancer cell lines PANC-1, AsPC-1, KP-3, PSN-1and MiaPaCa-2; hepatic cancer cell lines HLE and HepG2;and normal colonic fibroblasts HF.	Oncogene (2002) 21, 6082 – 6090
Anti-diabetic Drugs Rosiglitazone and Metformin	H-2Kb cells -derived from skeletal muscle of heterozygous H-2Kb tsA58 transgenic mice	The Journal of Biological Chemistry Vol. 277, No. 28, July 12, pp. 25226–25232, 2002
Arsenite and Heat shock	Tissue-Hepatocytes	Current Biology,1994;Vol 4, 315-324,
AICA riboside (1mM), Oligomycin (0.5uM)	CHO, FTO-2B, HEK-293 and COS-7	Current Biology 2000, VOL 10:1247–1255
Nitric Oxide	Cortical astrocytes; HEK293 T Cells transfected with pcDNA5 vector encoding human inducible nitric oxide synthase (iNOS)	NATURE CELL BIOLOGY VOLUME 6 | NUMBER 1 | JANUARY 2004

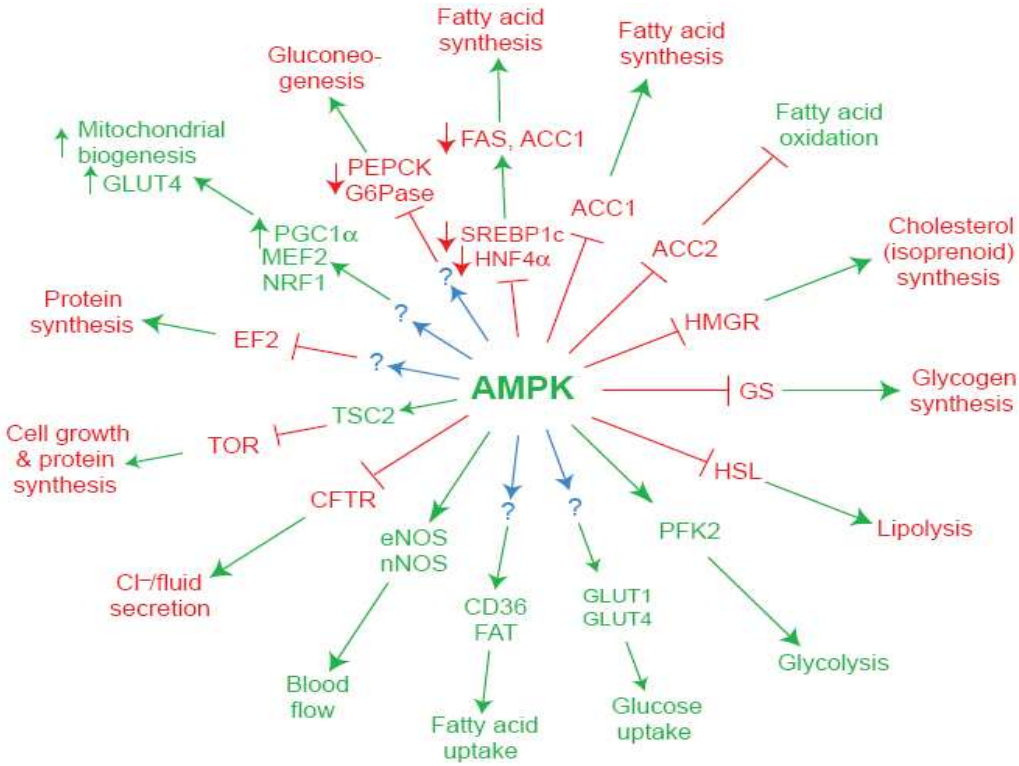


Fig.4. Targets of AMPK. Target proteins and processes activated by AMPK activation are shown in green, and those inhibited by AMPK activation are shown in red.

-Journal of Cell Science 117; 5479-5487; 2004

The above figure shows some of the downstream targets of AMPK. AMPK is activated by increases in the cellular AMP-to-ATP ratio as the result of ischemia/hypoxia, nutritional deprivation, oxidative stress etc. In general, activation of AMPK down regulates biosynthetic pathways such as fatty acid and cholesterol biosynthesis, yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation, glucose uptake and glycolysis. It achieves this not only through direct phosphorylation of metabolic enzymes, but also through effects on gene expression, such as upregulation of

the glucose transporter GLUT4, mitochondrial genes in muscle, down regulation of genes encoding enzymes of fatty acid synthesis and gluconeogenesis in liver. Although in no single case do we completely understand the detailed mechanisms by which AMPK activation regulates the expression of a particular gene, it has many effects on individual transcription factors and coactivators. For example, it upregulates the expression of the co-activator PGC1α[65], which may be involved in the increased expression of mitochondrial genes in muscle[71], yet it downregulates the transcription factors SREBP-1c [70]. and HNF4a[64]. , which may be involved in decreased expression of genes involved in lipogenesis, glucose uptake and glycolysis in liver. In muscle, it causes increased DNA binding by the transcription factors NRF1 [62] and MEF2 [69]. which may be involved in regulation of mitochondrial genes and GLUT4, respectively. Finally, it phosphorylates the ubiquitous co-activator p300 at a specific site (Ser89), reducing its ability to bind to nuclear hormone receptors and thus activate their target genes [68].

Another mechanism by which AMPK activation affects the expression of proteins is through effects on mRNA stability. Through an undefined mechanism, AMPK activation reduces the cytoplasmic level of the RNA-binding protein HuR, which stabilizes specific mRNAs in the cytoplasm by binding to their3'-untranslated regions [66].Target mRNAs for HuR include proteins that regulate the cell cycle, such as cyclinA, cyclin B1 and p21. The same group showed that activation of AMPK by an elevated AMP: ATP ratio in senescent fibroblasts in culture can explain many of the features of the senescent phenotype [67]. Indeed, LKB1–/– MEF cells, in which AMPK cannot be activated by agents that elevate cellular AMP levels (phenformin) or that mimic the effects of AMP (AICA riboside) [63]., are resistant to passage-induced senescence [61].Another key biosynthetic pathway that is downregulated by AMPK is translation, which can account for around 20% of energy turnover in growing cells and is particularly sensitive to decreases in ATP synthesis. Inhibition occurs by at least two mechanisms: (1) phosphorylation and activation of elongation factor-2 kinase which switches off the elongation step in

translation; and (2) inhibition of the target of rapamycin (TOR) pathway, which is a major positive stimulus for protein synthesis, cell growth and cell size. The TOR pathway is activated by growth factors and amino acids, and is thought to stimulate translation, and hence cell growth, by two mechanisms: (1) activation of ribosomal protein S6 kinase (S6K1); and (2) increased phosphorylation of elongation factor-4E binding protein 1 (4E-BP1), which stimulates the initiation step of translation. Recent studies suggest that inhibition of the TOR pathway by AMPK might occur through phosphorylation of TSC2. TSC1 and TSC2 (also known as hamartin and tuberin) are partners that form a stable complex in the cell, and mutations in either leads to the human disease tuberous sclerosis complex. AMPK phosphorylates TSC2 which increase the ability of the TSC complex to inhibit the TOR pathway. The LKB1→AMPK→TSC2 pathway negatively regulates the target of rapamycin and this appears to be responsible for limiting protein synthesis and cell growth and protecting against apoptosis, during cellular stresses such as glucose starvation.

AMPK also regulates cell cycle which is mediated by up-regulation of the p53–p21 axis as well as regulation of TSC2–mTOR (mammalian target of rapamycin) pathway. The AMPK signaling network contains a number of tumor suppressor genes including LKB1, p53, TSC1 and TSC2, and overcomes growth factor signaling from a variety of stimuli (via growth factors and by abnormal regulation of cellular proto-oncogenes including PI3K, Akt and ERK). These observations suggest that AMPK activation is a logical therapeutic target for diseases rooted in cellular proliferation, including atherosclerosis and cancer. AMPK activation strongly suppresses cell proliferation in non malignant as well as in tumor cells by regulation of cell cycle and inhibition of protein synthesis, de novo fatty acid synthesis [42].

Yeast Two Hybrid

It is a sensitive in vivo assay for the detection of specific protein-protein interaction. The two hybrid system utilizes the yeast GAL4 transcriptional activator which is required for the expression of genes encoding proteins involved in galactose metabolism. GAL4 transcriptional activator has a distinct domain for specific DNA binding and one or more additional domains required for activation or interaction with other regulatory proteins. The gene of interest is expressed as a fusion to the DNA binding domain (BD) of GAL4 while cDNA library insert is expressed as a fusion of to the GAL4 activation Domain (AD).

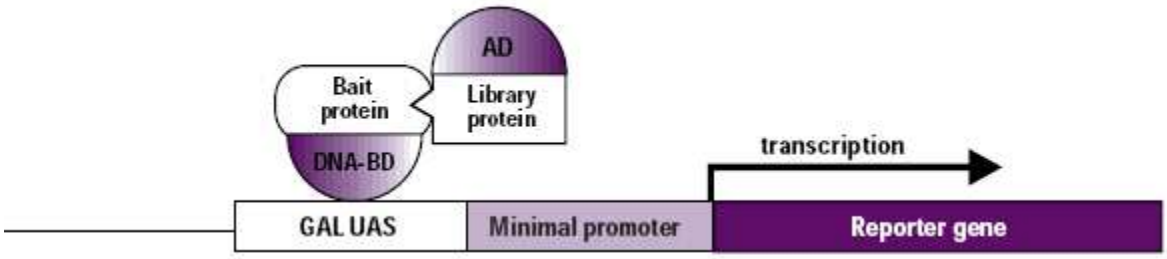


Fig.5. Schematic representation of yeast two hybrid.

When the protein of interest and library fusion proteins interact, the GAL4 DNA-BD and AD are brought into close proximity, activating the transcription of the reporter gene i.e. galactosidase gene.

MATERIALS AND METHODS

1. Maintenance of Cell Lines

Human Glioma Cell Lines-M059J and MO59K were purchased from American Type Culture Collection. M059J (ATCC number:CRL-2366) and M059K (ATCC number CRL-2365) were maintained in a medium containing a 1:1 mixture of Dulbecco's Modified Eagle's Medium and Ham's F12 medium with 2.5mM L-glutamine. It was adjusted to contain 15mM HEPES, 0.5mM Sodium pyruvate and 1.2g/L sodium bicarbonate supplemented with 0.05mM non-essential amino acids, 10% fetal bovine serum, 100 units of penicillin/ml and 100ug of streptomycin/ml (Invitrogen, Carlsbad, CA). Cells were maintained in 5%CO_2, 95% air at 37°C in a humidified incubator.

Human Colorectal carcinoma cell line HCT116 p53+/+ cells were obtained from Korean Cell line Bank (KCLB number 10247). It was maintained in an IMDM medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units of penicillin/ml and 100ug of streptomycin/ml. Cells were maintained in 5%CO_2, 95% air at 37°C in a humidified incubator.

2. RNA extraction

First, draw the media from culture dish by suction pump. Add 1000µl of Trizol reagent (Invitrogen). Spread carefully by rotating the plate and by repetitive pipetting of reagent. This process was carried out till the reagent becomes nonviscous. The homogenate was transferred to an e-tube and allowed to stand for 5 min at room temperature. Add 200µl of chloroform per 1 ml of Trizol reagent. Shake tubes vigorously by hand for 15 seconds and stand at room temperature for 5-10mins. Centrifuge at 12,000X g for 15 minutes at 4°C. The homogenate separates into two layers- a lower red phenol chloroform layer and the transparent upper layer. RNA remains exclusively in the upper layer whereas

DNA and proteins remain in interferes and organic phase. Draw about 500µl of upper layer and transfer into e-tube containing 500µl of isopropanol. Stand at room temperature for 10-15 mins. Mix gently by inversion and centrifuge at 12,000 X g for 10 minutes at 4°C. Discard supernatant by taking care of pellet. Place e-tube in ice and add 1000µl of 75% ethanol (in DEPC-treated water) and centrifuge at 7500X g for 5 minutes at 4°C Discard supernatant and add 500µl of 100% ethanol and store at –70°C. Purification of RNA-Take out the RNA sample from -70°C and centrifuge at 7500X g for 5mins at 4°C. Discard the supernatant and add 1000ul of 80%ethanol. Centrifuge at 7500X g for 5mins at 4°C. Discard the supernatant. Remove all the ethanol by means of pipette. Dissolve the RNA in 0.01% DEPC water and measure the concentration.

3. Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

First, prepare RNA by following Trizol reagent protocol. Measure OD. In an e-tube, take total RNA (~5µg) in 10µl volume (Volume taken can be adjusted with DW depending upon concentration), 10mMdNTP in 1µl, Oligo dT (10pmole/µl) in 1µl. The total volume is 12 µl/ sample. Incubate at 65°C for 5 minutes. Immediately keep on ice for 2 minutes. Add buffer mix containing 5X First strand buffer-4µl, 0.1MDTT-2µl and distilled water-1µl. Mix gently by pipetting and incubate at 37°C for 2 minutes. Add 1µl of M-MLV Reverse Transcriptase (Invitrogen) and incubate at 37°C for 1hr 30 mins. Again incubate at 70°C for 15minutes. The product i.e. cDNA obtained is stored at -20°C.

4. Polymerase Chain Reaction (PCR)

Dilute the cDNA obtained after reverse transcription (10µl sample and 390µl of DW). Take 2µl of diluted sample as a template for PCR. Adjust the following condition in PCR machine.a) Denaturation at 94°C for 3mins b) Denaturation at 94°C for 30secs c) Annealing at 60°C for 1mins (variable) d) Polymerization at 72°C for1mins (variable) e) Repeat the cycle from step (b) to (d) for 32 times. f) Final polymerization temperature

72°C-10mins. PCR cycles can be varied upon one's interest. For GAPDH gene, adjust the PCR cycle to 20. Add necessary primers, buffers and Taq DNA polymerase enzyme in the PCR tube and run the PCR. The primers used for PCR are as follows:

PRKAG1BamHI F1 5'- GGA TCC GTA TGG AGA CGG TCA TTT CTT CAG-3'

PRKAG1 XhoI R 996 5'-CTC GAG TCA GGG CTT CTT CTC TCC ACC TGT-3';

GAPDH forward, 5'-CCA TGG AGA AGG CTG GGG-3'; and

GAPDH reverse 5'-CAA AGT TGT CAT GGA TGA CC-3'

After PCR, confirm the result by gel electrophoresis.

5. Yeast Two Hybrid

In this experiment, bait Protein is DNA-PKcs (8722-10614). It is cloned in pGBT9 vector while the prey Protein is the prostrate cDNA library insert. It is cloned in PACT2 vector. (Clontech).

First transformation: Streak competent yeast culture, *Saccharomyces cerevisiae*, AH109 into YPD agar plate. Incubate at 30°C for 3-5days. Streak a single colony from above YPD agar into new YPD agar plate and incubate at 30°C for 3-5days. For storage at -70°C, prepare suspension in 500µl YPD and add 50% glycerol. Prepare 1ml Step buffer which consists of 1M DTT -100µl, 10X Lithium acetate (LiAc)-200µl (pH 7.50), 50%Polyethylene glycol-700µl (Mol. Wt.-3350).

Prepare suspension of yeast culture by inoculating one or two loopful of culture into 1ml of step buffer. Vortex it to mix well. Place 1.5µl of Carrier DNA (Herring sperm DNA) 10mg/ml into an e-tube. Add 200ng of DNA (pGBT9 vector carrying gene of interest) i.e. about 1.6µl. Mix 100µl of suspension of yeast culture to the sample prepared above. Mix gently by tipping. Spin down and keep on ice for 5mins. Place in water bath at 42°C for 40 minutes. Place it on ice for 5 mins. Again gently spin down (5-6 seconds).

Draw the supernatant and discard it. Add 70-100µl of distilled water to suspend pellet. Mix by pipetting. Inoculate 100µl of above suspension into –Trp/SD agar. Spread the culture and incubate at 30°C for 3-5days. Select some transformants and subculture in –Trp/SD agar and incubate at 30°C for 3-5days. Store sub cultured plates at 4°C.

Second Transformation: Prepare required set of 50ml and 300ml of YPD media. Inoculate one or two loopful of first transformant culture into 50 ml of YPD media. Incubate at 30°C for 16-18hrs with shaking (180 rpm). Inoculate 50 ml of above broth culture into 300 ml of YPD media. Mix and measure the Optical Density at 600 nm (OD=0.2-0.3). Incubate at 30°C for 3 hours with shaking (230-270rpm). Measure the optical density and it will be 0.5±0.1. Place the broth culture in six 50ml tubes and centrifuge at 1000X g (3000rpm) for 5 minutes at room temperature or 4°C Discard the supernatant and resuspend cell pellets by vortexing in 5 ml of sterile 1XTE. Place all the suspension in one tube and centrifuge at 1000X g for 5minutes at room temperature. Decant the supernatant. Add 25 ml of 1X TE and recentrifuge. Resuspend the cell pellet in 1.5 ml of freshly prepared sterile 1XTE/LiAc.

Prepare required quantity of PEG/LiAc/ TE (10 ml per sample). For the large scale, in labeled 50ml tube add a) Herring sperm DNA - 2 mg (200µl) b) Activation Domain/library –10 to 50µg (50µl) For the small scale, in 15 ml tube, add a) Herring sperm DNA-0.1mg (10µl) b) Activation Domain-100ng (1µl) Add 1 ml of yeast competent cell (obtained in step 7) in large scale but 100µl in small scale and mix well by vortexing.

Add 6 ml of PEG/LiAc/ TE solution in large scale but 600µl in small scale and mix well by vortexing. Incubate at 30°C for 30 minutes with shaking (200rpm). Add 700µl of Dimethyl sulfoxide(DMSO) in large scale but 70µl in small scale. Mix well by gentle inversion or swirling. Do not vortex. Heat shock for 15 min in a 42°C water bath and swirl

occasionally to mix. Place on ice for 1-2 minutes. Centrifuge cells at 1000X g for 5minutes at room temperature in large scale but centrifuge at 14,000rpm for 5 seconds in small scale. Discard the supernatant. Resuspend the cells in 5000µl of 1X TE in large scale but 500µl in small scale. Spread 250µl of above suspension in twenty -Leu/-Trp/-His/SD plate (150mm diameter) in large scale but in case of small scale, spread 200µl in two -Leu/-Trp/-His/SD plate (100mm diameter). Incubate at 30°C for 3-5 days.

6. Transient transfection of pcDNA3.1 hygro PRKAG1 in HCT116p53+/+ cell line.

Human PRKAG1 was amplified from prostrate cDNA library (Clontech) by using following primers PRKAG1BamHI F1 5'- GGA TCC GTA TGG AGA CGG TCA TTT CTT CAG-3'; PRKAG1 XhoI R 996 5'-CTC GAG TCA GGG CTT CTT CTC TCC ACC TGT-3'; The amplified PRKAG1 was inserted into pGEM-T easy vector (Promega) and transformed into DH5α *E. coli*. (Promega). Later, it was sub-cloned into mammalian expression vector pcDNA3.1 hygro PRKAG1 (Invitrogen). Plasmid, pcDNA3.1 hygro PRKAG1 was purified by using plasmid DNA Purification Kit (QIAGEN) and OD was measured by using Nanodrop Spectrophotometer. After confirming the sequence and frame of PRKAG1, it was transfected into HCT116p53+/+ by using Lipofectamine 2000 reagent (Invitrogen). After 24-48 hrs, cells were harvested to check over expression of PRKAG1 (AMPKγ1).

7. Extraction of Proteins from adherent mammalian cells.

First, scrape the culture plate containing the medium. Repeat the process 4 or 5 times by rotating the plate. Transfer the contents into a 15ml tube and centrifuge at 2000rpm for 3 mins at 4°C. Discard the supernatant and resuspend the pellet in 4ml of PBS (1X). Centrifuge at 2,000rpm for 3 mins. Discard the supernatant and add 200 to 300µl of mammalian protein extraction reagent, M-PER (PIERCE) containing proteinase inhibitor

depending upon the size of pellet and confluence of growth. If amount of protein is small, add less than 100µl of M-PER. Mix by pipetting and keep on ice for 5 mins. Run the sample on micro tube mixer for 5mins and then keep on ice for 5mins. Centrifuge at 13,000rpm for 15mins at 4°C. Draw the supernatant and transfer into the new tube. Measure the protein concentration by using Bio-Rad Protein Assay Solution. Add the 5X Protein sample buffer to make final concentration of 1X. Mix the sample and boil the sample for 5 mins and then keep it on ice for 5 mins. Store the sample at -70°C.

8. Immunoprecipitation-

First, scrape the culture plate containing the medium. Repeat the process 4 or 5 times by rotating the plate. Transfer the contents into a 15ml tube and centrifuge at 2,000rpm for 3 mins at 4°C. Discard the supernatant and resuspend the pellet in 4ml of PBS (1X). Centrifuge at 2000rpm for 3 mins. Discard the supernatant and add 100 to 200µl of NP-40 cell lysis buffer (50mM Tris-HCL pH8.0, 150mM Nacl and 1%NP-40) or Pierce Co-IP buffer (25mM Tris, pH 7.2, 150mM NaCl, 5mM $MgCl_2$, 0.5%NP-40, 1mM DTT, and 5% glycerol and protease inhibitors) containing proteinase inhibitor depending upon the size of pellet. Mix by pipetting and transfer into an e-tube and keep on ice for 5 mins. Run the sample on micro tube mixer for 5 mins and then keep on ice for 5mins.

Centrifuge at 13,000rpm for 15mins at 4°C. Immediately transfer the supernatant into the new tube and discard the pellet. If the supernatant is turbid, centrifuge again at 13,000rpm for 5mins and transfer the supernatant. Measure the protein concentration by using Bio-Rad Protein Assay Solution. To prepare Protein A/G agarose, or any other agarose beads, wash the beads twice with PBS and restore to 50% slurry with PBS. Cut the pipette tip when manipulating agarose beads to avoid disruption of the beads. For washing the bead, centrifuge the define volume (for example 500ul) of agarose beads at 2000rpm for 2mins. Discard the supernatant and add 500ul of DPBS and centrifuge at 2000rpm for

2mins. Repeat the process twice. After washing, suspend the bead in 500ul of DPBS. Pre-clear cell lysate by adding 20ul of bead slurry per 300-500ul of cell lysate and incubate at 4°C for 30 mins on a rocker. Remove the agarose beads by centrifugation at 2000 rpm for 2min at 4°C. Transfer the supernatant to a new tube. Add 2ug of primary antibody. Gently rock the cell lysate/antibody mixture overnight at 4°C on a rocker. Capture the immunocomplex by adding 30ul-50ul of bead slurry and gently rock on a rocker for overnight at 4°C. Collect the agarose beads by centrifugation at 6000rpm for 5mins at 4°C. Discard supernatant and wash the beads 3times with 800ul of ice cold PBS by centrifugation at 2000rpm for 2mins at 4°C. Resuspend the agarose beads in 30ul of 5X sample buffer and mix gently. Boil the agarose beads for 5minutes to dissociate the immunocomplexes from the beads. Keep it on ice for 5mins.Centrifuge at 6000rpm for 5mins at 4°C. Transfer the supernatant into a new tube and load into the SDS-PAGE gel. Confirm the immunoprecipitation by Western Blot.

9. Western Blot-

First, prepare required percentage of SDS-PAGE gel. Pour required quantity of 1X running buffer. Pre-run the gel for 30 minutes at 80 volts. Clean the wells by injecting buffer. Load protein marker (5µl) and calculated sample volume. Run at 80 volts for about 30mins and then to 120volts for 1hr 30mins to 2 hrs depending upon interest protein size. Remove stacking gel. Cut a small portion of a bottom of a separating gel. Wash the separating gel in transfer buffer for 20-30 minutes. Cut the nitrocellulose membrane or PVDF membrane of required size (8.3cmX4.3cm) and soak in methanol till color changes into off white and then in D/W and lastly in (1X) transfer buffer. Pour (1X) transfer buffer in a gel box. Arrange following things in a cassette starting from white part of cassette. Absorbent pad- 1 wet 3M paper (7cmX10cm)-Membrane-developed gel-1wet 3M paper-absorbent pad. Place the arranged cassette in the gel box. Pour transfer buffer. Keep the ice pack and magnetic stirrer. Apply 90 volts. Perform at 4°C for 2hours depending upon

protein size. Change the ice pack every 30 minutes. After 2hours, take out the membrane and wash it in 1X TBST (5-10mins).Stain with Ponceau S to confirm transfer of proteins. Wash with 10 ml of 1XTBST for three times (15mins, 5mins, and 5mins).Add 10ml of blocking solution for small membrane (20-30 ml for normal size membrane). Shake at RT for 3hrs. Keep at 4°C for overnight with shaking. Next day (second day), shake for 1 hour at room temperature. Wash with 10 ml of 1X TBST for 1hour with 8-10minutes interval. Prepare required concentration of primary antibody. In 10ml of primary antibody solution, add 200ul of 0.5M Sodium Azide. Add primary antibody to the membrane and keep at 4°C for overnight. Next day (third day), remove primary antibody. Wash with 1XTBST for hour with 8-10minutes interval. Add secondary antibody (mouse-1/4000, rabbit-1/4000 and goat-1/5000). Keep it at 4°C for 3 to 4 hours. Wash with 1XTBST for 1hour with 8-10 minutes interval. Mix west-zol solution A and B (400ul each). Spread over membrane and stand for 1min. Place X-ray film over the membrane and develop film.

10. Confocal Microscopy.

Select the desired culture dish i.e. 35mm dish. Sterilize the 12mm cover slip by dipping in 70% ethanol and allow it to dry by flaming. Place it in the well of the culture dish. Place 90-100ul of poly-D-lysine on the cover slip. Wait for 30minutes to 1hour. Wash with water for 3-4 times. Expose UV for 1hour to sterilize the plate. Seed the cells to get 60-70% confluence. After 24 hours of seeding, transfect siRNA and incubate for 48hours.

After 48hrs, treat the cells with required dose of UV and harvest at different time period. For harvest, suck out the media. Wash with DPBS twice. Fix with 1 or 2ml of 4% paraformaldehyde or 98% methanol for 10mins at -20°C. Wash with DPBS for 3 times. Transfer the cover slip into 6well plate. Wash with DPBS for 10mins by shaking Repeat the process thrice. Add 1000ul of 0.3%Triton X-100. Shake gently for 10mins. Wash with DPBS for 10mins by shaking. Repeat the process thrice. Add 2ml of 0.1% BSA and keep it

at RT for 2hr or 4°C for overnight in a rocker. Transfer the cover slip into 24 well plate. Prepare primary antibodies in DPBS. (For example, AMPK-gamma 1 (1:50) and DNA-PKcs (1:20). In 2500ul of DPBS, take 50ul of AMPK-gamma 1 and 125ul of DNA-PKcs antibody). Transfer 200ul of antibody mixture into each well. Keep it at 4°C for overnight in a rocker. Transfer the cover slip into the 6well plate and wash with DPBS for 30minutes. Repeat the process six times. Alternatively washing can be done at 4°C for overnight. Add secondary antibody (1:1000). Shake it at RT for 2hrs or 4°C for overnight. Wash with DPBS for 30minutes for 6times and observe the prepared slide on confocal microscope.

11. Cytotoxicity Assay by MTT

The cell cytotoxicity was assessed using a 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay. Cells were seeded on 24well plate. After 24 hrs, cells were treated with UV (10J) for 24 hours. MTT (10mg/ml) was incubated with cells in a plate for 4 h at 37℃. The medium containing MTT was removed, and dimethyl sulfoxide (DMSO) was added. Cells were incubated for 20mins at room temperature with gentle shaking. The absorbance was read on a scanning Elisa reader (BIO-TEK INSTRUMENTS, INC.) using a 570nm filter. Cell viability was calculated from relative dye intensity compared with untreated samples.

12. Transient transfection with PRKAG1 siRNA and Cytotoxicity Assay by MTT

The PRKAG1 siRNA (smart Pool) was purchased from Dharmacon. HCT116p53+/+ cells were transfected with this siRNAs (10pmol) by the Lipofectamine RNAiMAX method according to the manufacturer's protocol (Invitrogen). After 48hrs, Cells were treated with different doses of UV for 24 hours. The cytotoxicity was determined using a 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT)-based colorimetric assay.

13. DNA-PK kinase activity test.

For the DNA-PK kinase assay, nuclear extract was prepared. Nuclear extract was sonicated and incubated with DNA-PK Biotinylated Peptide Substrate, 5X reaction buffer, [γ-32P] ATP. All the procedure of DNA-PK kinase assay was followed as per the SignaTECT DNA-Dependent Protein Kinase Assay System (Promega).

RESULTS

1. Interaction of DNA-PKcs and AMPKɣ1 in yeast strain, AH109.

To study about the DNA-PKcs interacting gene, we first cloned DNA-PKcs (8722-10614) in pGBT9 vector. We then cloned pACT2AD vector containing AMPK ɣ1. Interaction of these proteins in results in the formation of colonies in –leu/-trp/-His plate. Interaction is confirmed by the formation of blue colonies (Fig 6) in X-alpha gal/–leu/-trp/-His plate after 3-5days of incubation period at 30˚C.

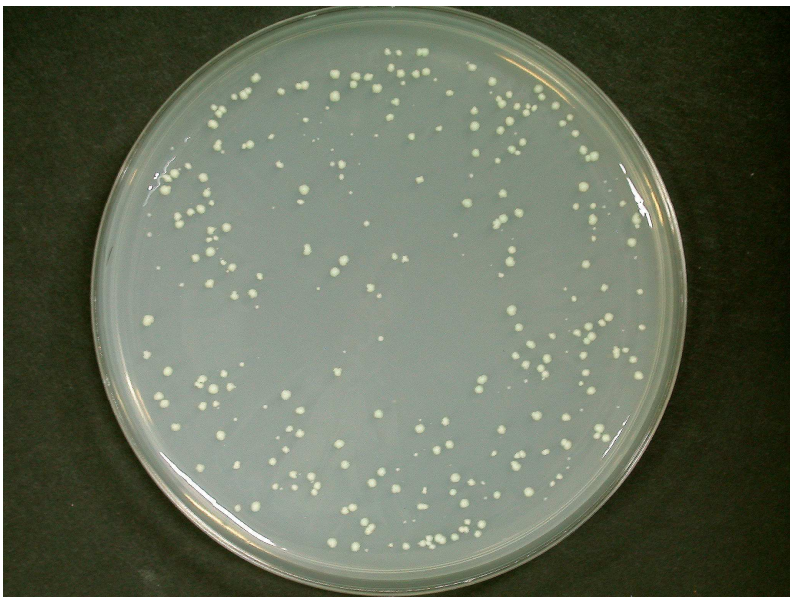


Figure 6 Yeast cells in X-alpha gal/–leu/-trp/-His plate. Reconfirmation of yeast two hybrid experiment showing the growth of yeast culture AH109. In this experiment, Bait Protein is DNA-PKcs (8722-10614) and Prey Protein is AMPK gamma 1.

2. Interaction of DNA-PKcs and AMPKγ1 in mammalian cell line.

In order to confirm the interaction of DNA-PKcs and AMPKγ1, we used human colorectal carcinoma cell line with wild type p53, HCT116p53+/+ and human glioma cell line. Figure 7 shows the binding of these two proteins under UV stress condition in HCT116 p53+/+. While Figure 15 shows interaction in glioma cell line after hydrogen peroxide treatment. Figure 16 shows that the expression level of AMPKγ1 is comparatively higher in M059K cell than M059J cells as confirmed by Western Blot (Fig 16A) and RT-PCR (Fig16B)

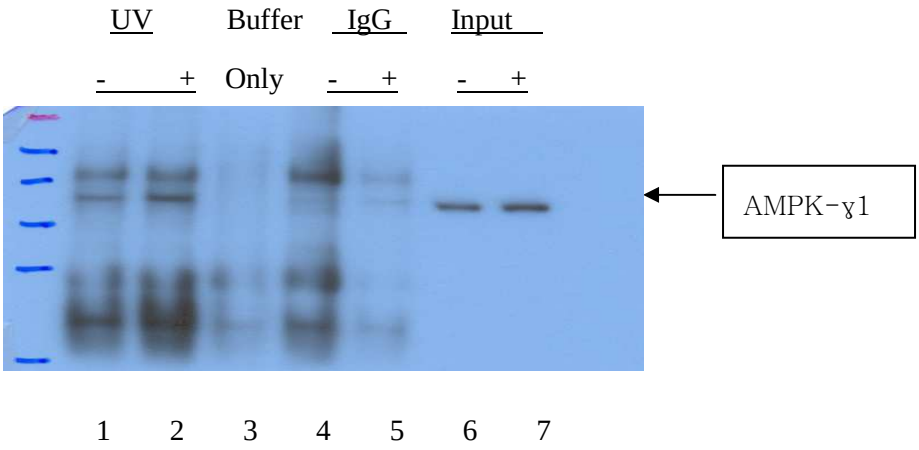


Figure 7. Immunoprecipitation (IP) test in HCT116p53+/+ cell line under UV stress condition. Cells are transiently transfected with pcDNA3.1 hygro PRKAG1 and treated with 10J of UV and proteins were harvested after 6hrs. IP was performed with DNA-PKcs and WB with AMPK gamma1.

3. Colocalization of DNA-PKcs and AMPKɣ1 under UV stress condition.

To study about the co-localization of two proteins, 10X10^4 cells were seeded in 35mm culture dish containing sterile cover slip. All other procedures were followed as mentioned in materials and methods. Co-localization is maximum in 6hrs time period and gradually decreased after that time period (Fig 8). Results are same in the sicontrol transfected cell line. (Fig 9) while there is no co-localization once AMPKɣ1 is silenced (Fig 10).

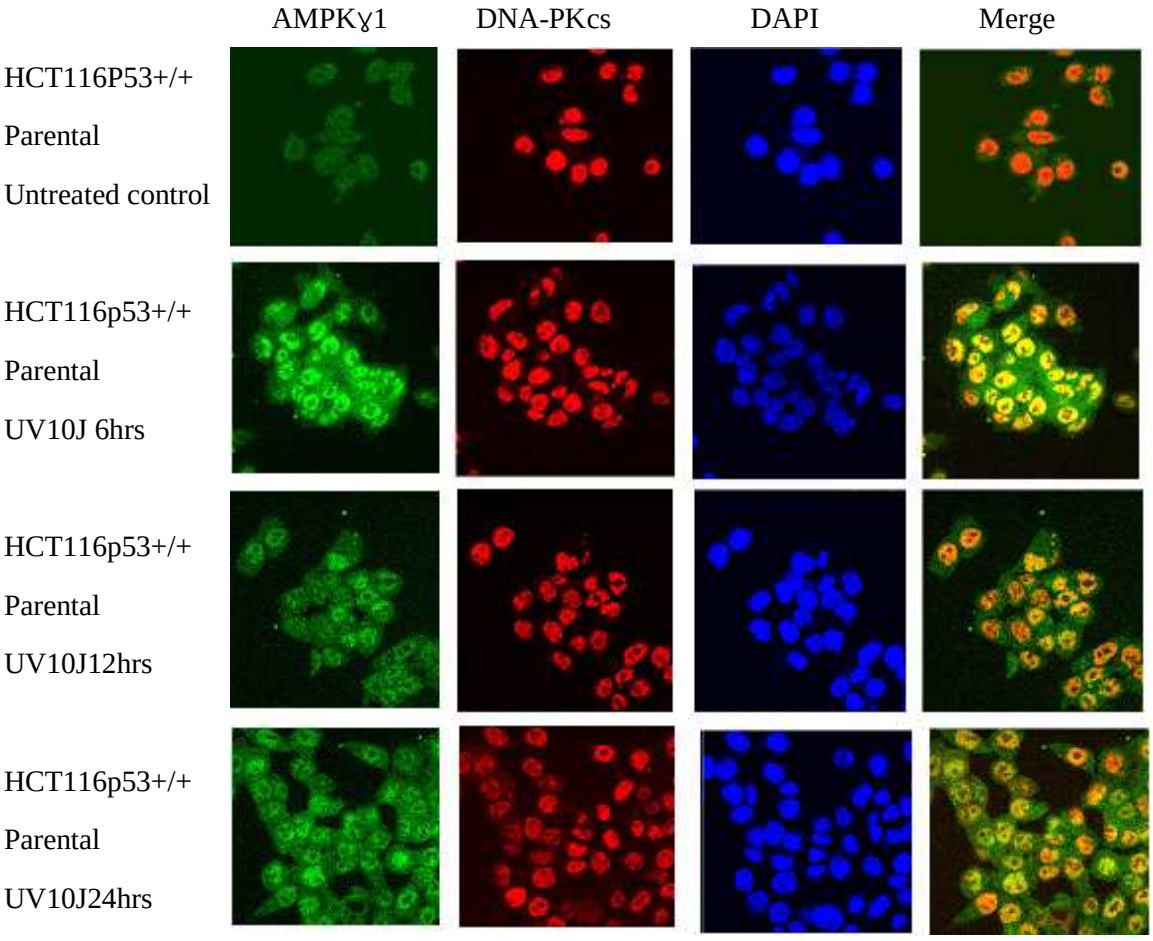


Figure 8 Co-localization of AMPKɣ1 and DNA-PKcs in parental HCT 116p53+/+ cell line.

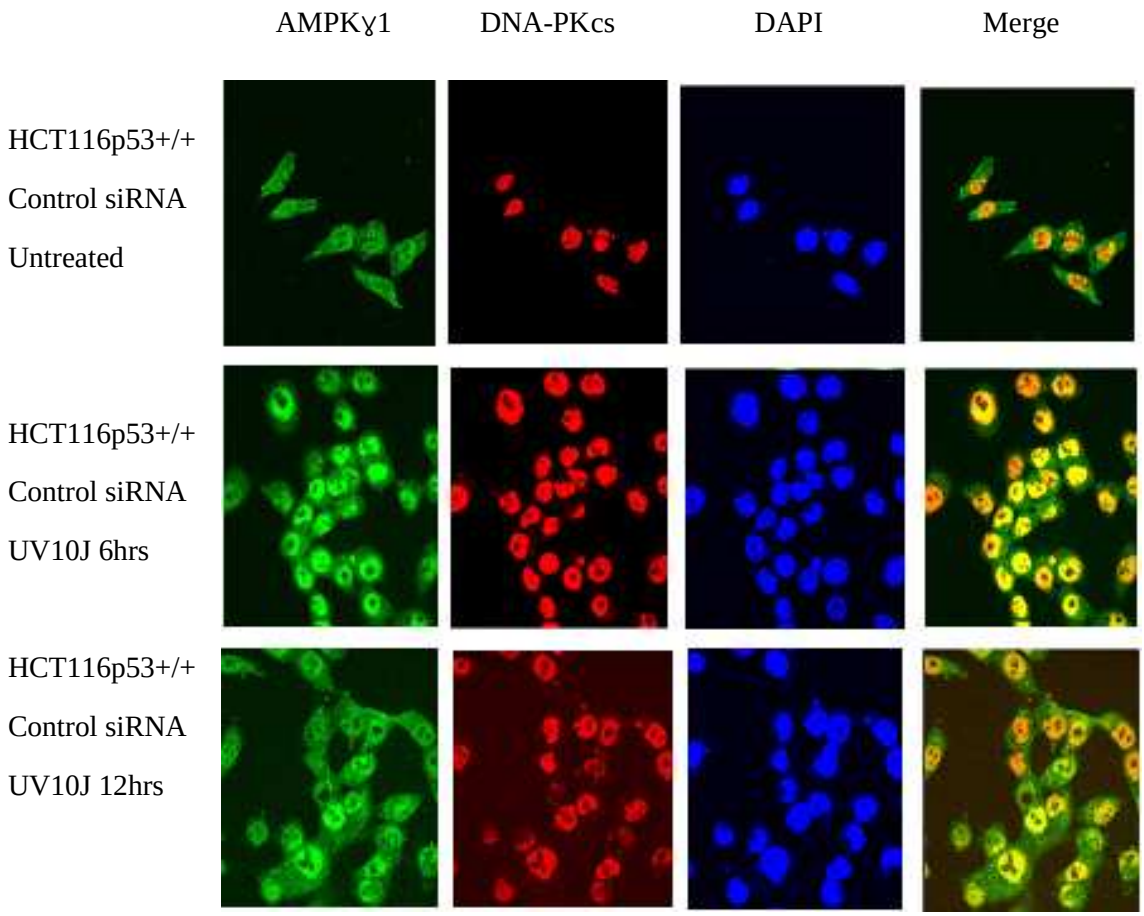


Figure 9. Co-localization of AMPKγ1 and DNA-PKcs in control siRNA transfect ed HCT116p53+/+ cell line. 48hrs after siRNA transfection, cells were immunostained with AMPKγ1 and DNA-PKcs antibody.

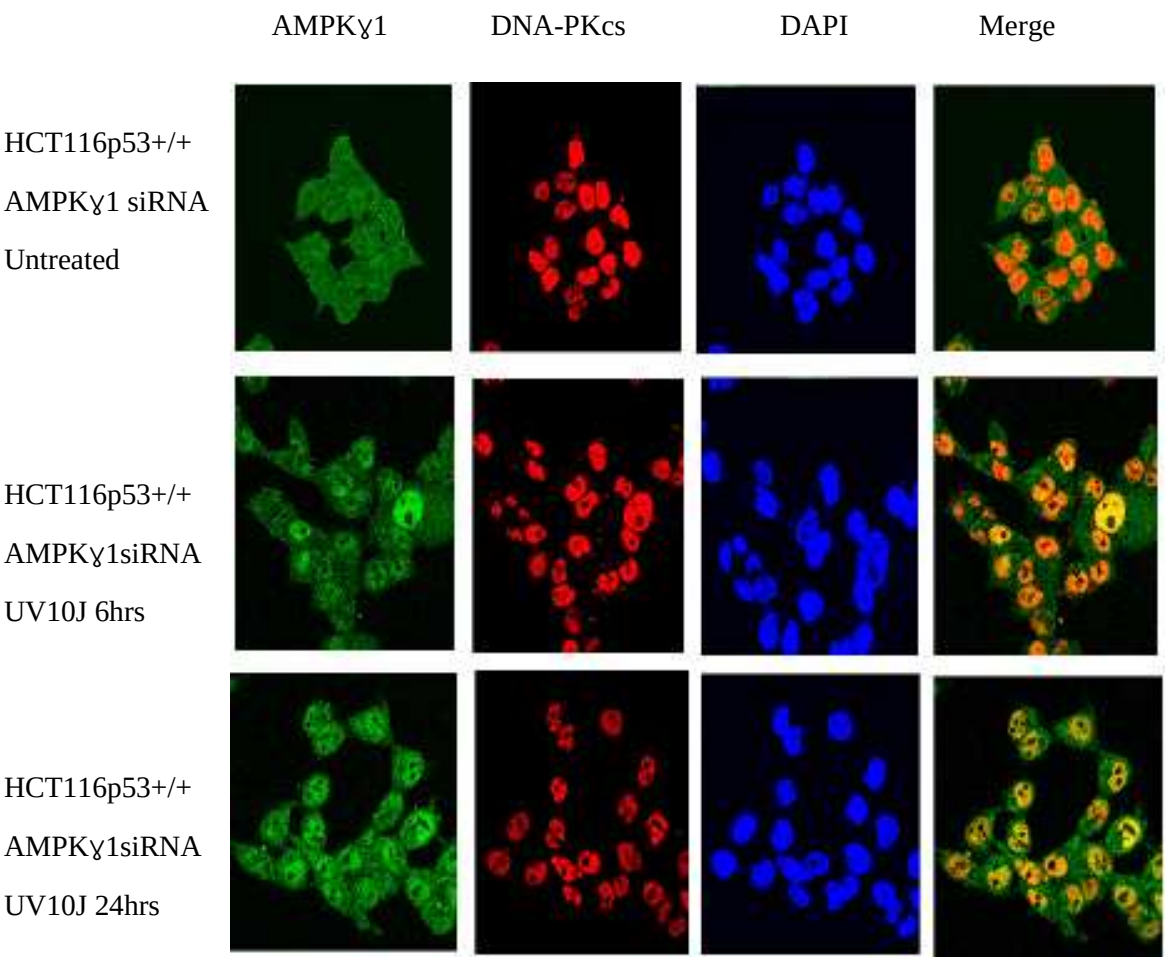


Figure 10 Effect on the co-localization of AMPKɣ1 and DNA-PKcs after transient transfection of AMPKɣ1 siRNA in HCT 116 p53+/+ cell line. 48hrs after siRNA transfection, cells were immunostained with AMPKɣ1 and DNA-PKcs antibody.

4. Expression of Gamma H2AX is comparatively higher in AMPKɣ1 silenced cell line.

Under UV stress (Fig 11) and in Bleomycin treated condition (Fig12), phosphorylation of H2AX is comparatively higher in AMPKɣ1 silenced cell line compared to control.

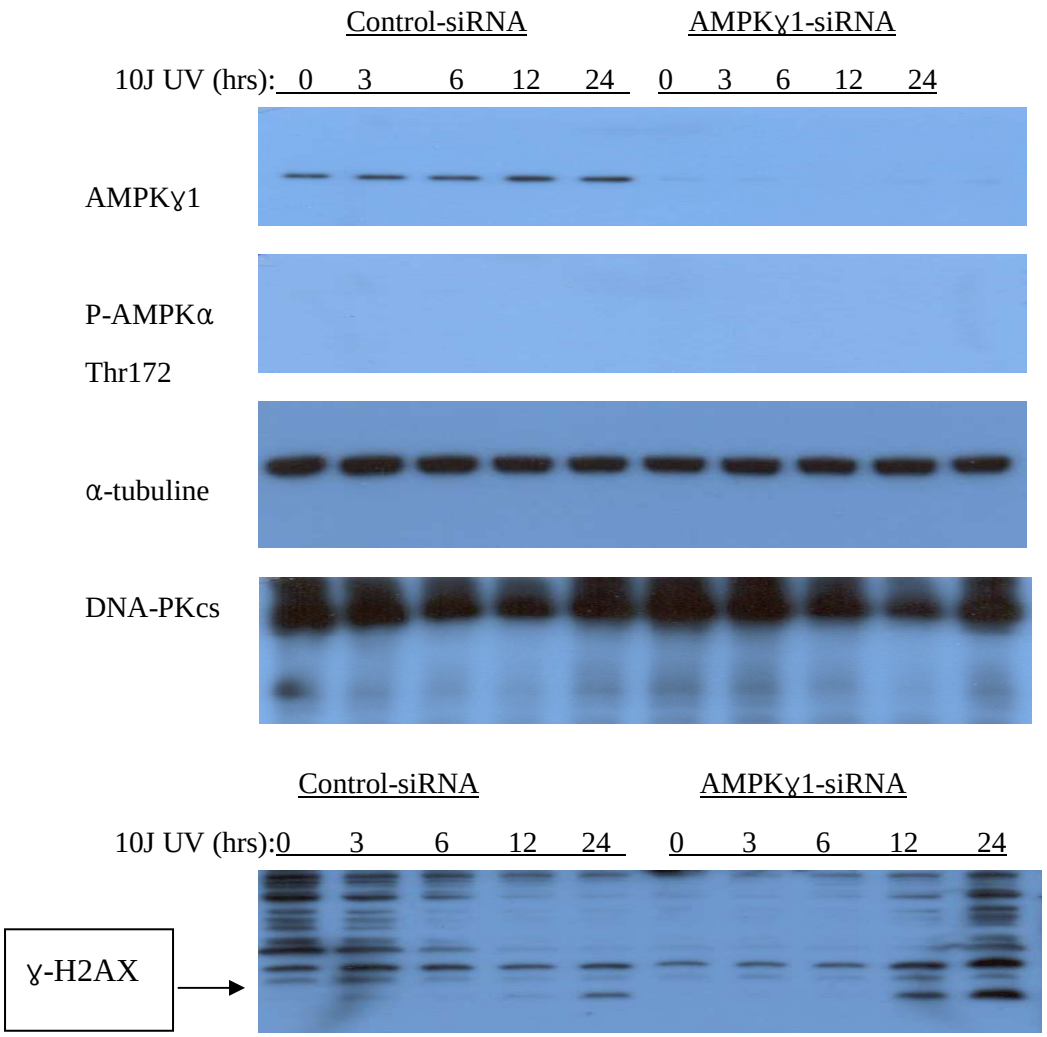


Figure 11. Effect of UV in HCT116 p53+/+ cell line.

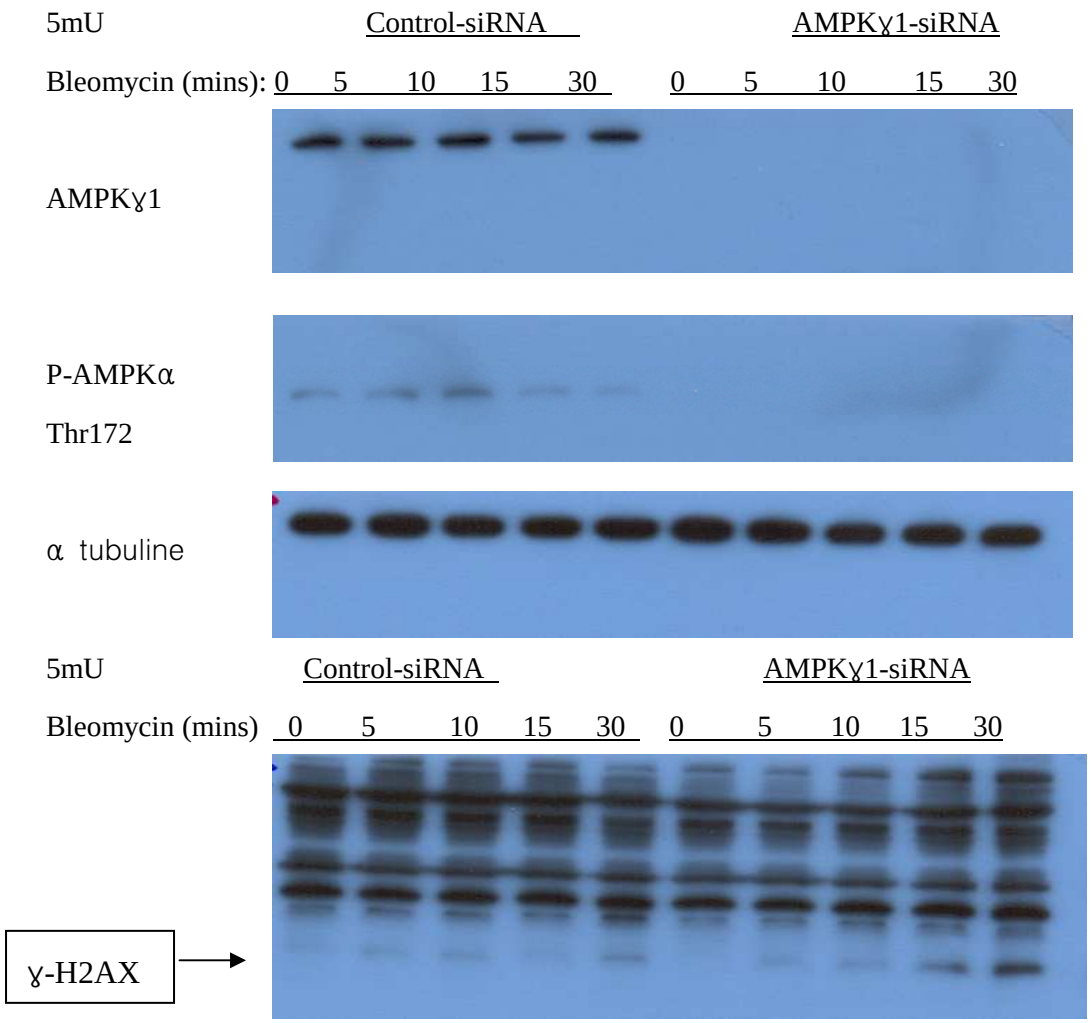


Figure 12. Effect of Bleomycin (5mU) in HCT116p53+/+ cell line.

5. HCT116p5.+/+ cell line becomes more sensitive to UV when AMPKɣ1 is silenced.

Before treating with UV, we first silenced the AMPKɣ1 in HCT116p53+/+ cell line. 48hours after siRNA treatment, we treated UV to cells and checked the cell viability after 24hrs. It was found that AMPKɣ1 silenced cells were found to be more sensitive to UV stress (Fig 13). And the DNA-PK activity is reduced in AMPKɣ1 silenced cell lines (Fig 14).

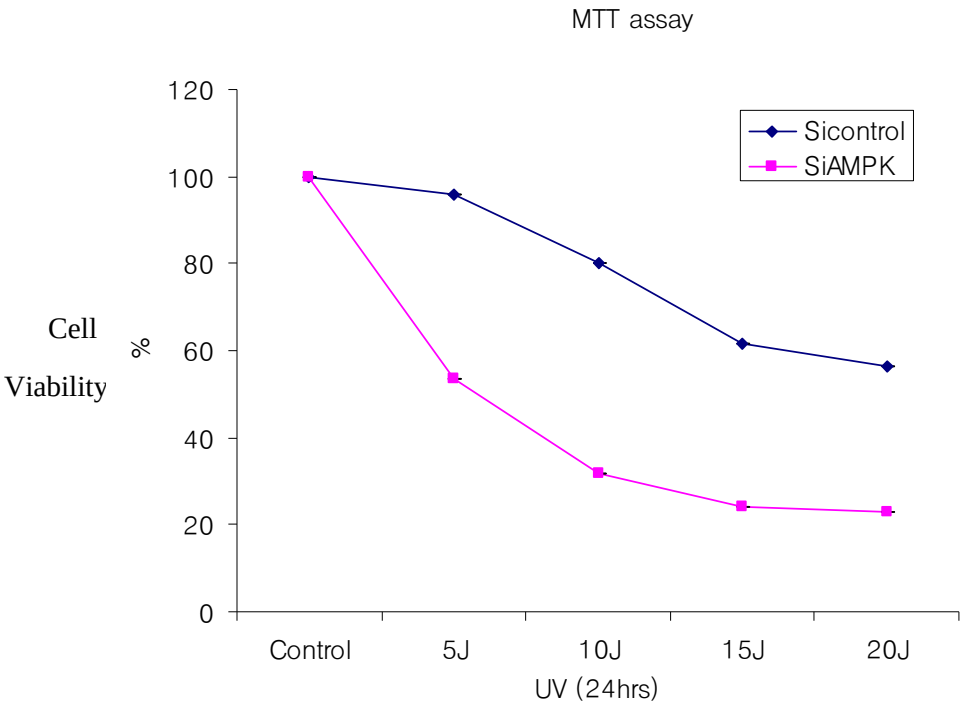
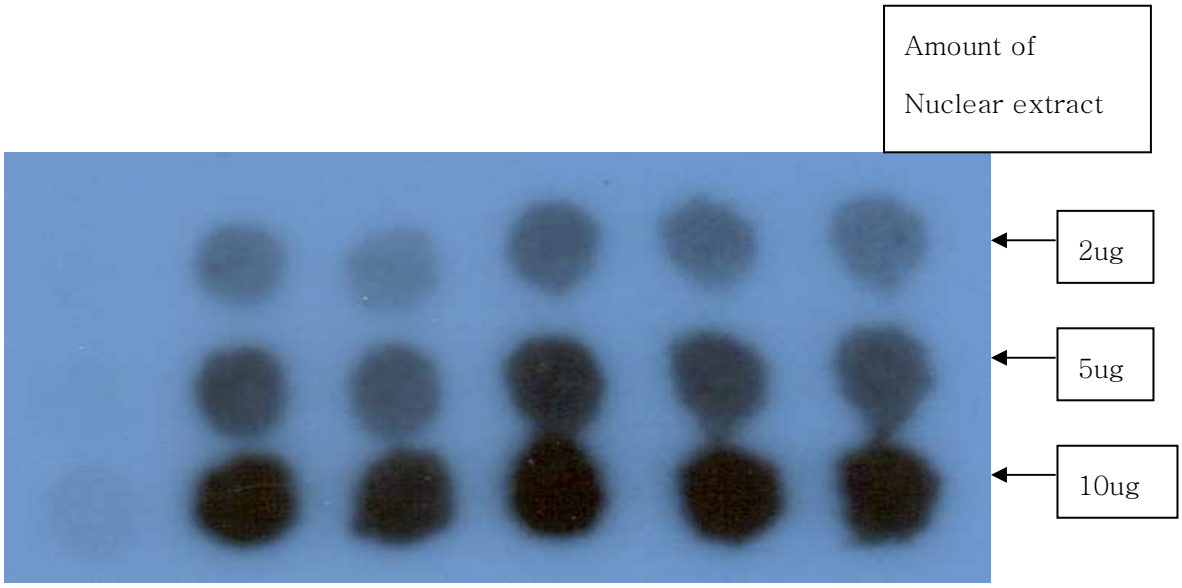


Figure 13. Effect of UV on cell viability in AMPKɣ-1 silenced HCT116p53+/+ cell line.



M059J	sicontrol siAMPKɣ1	sicontrol siAMPKɣ1	Hela S3
Negative	M059K	HCT116p53+/+	Positive
Control			control

Figure 14 DNA-PK kinase activity. This test is performed by using SignaTECT DNA-Dependent Protein Kinase Assay System.

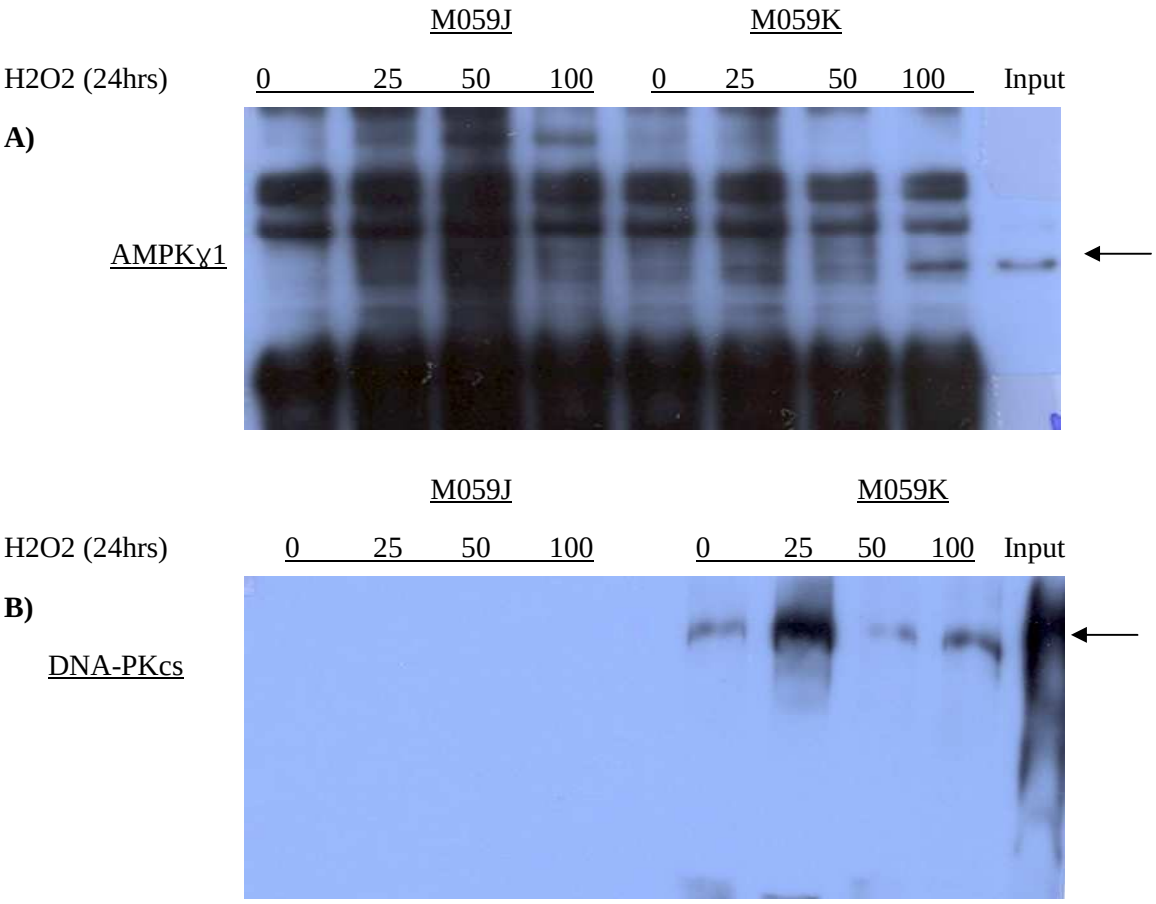


Figure 15. Immunoprecipitation (IP) test in human glioma cell line after treatment with hydrogen peroxide. In figure 15A, IP was performed with DNA-PKcs and WB with AMPK gamma1. In figure 15B, IP was performed with AMPK gamma -1 and WB with DNA-PKcs antibody.

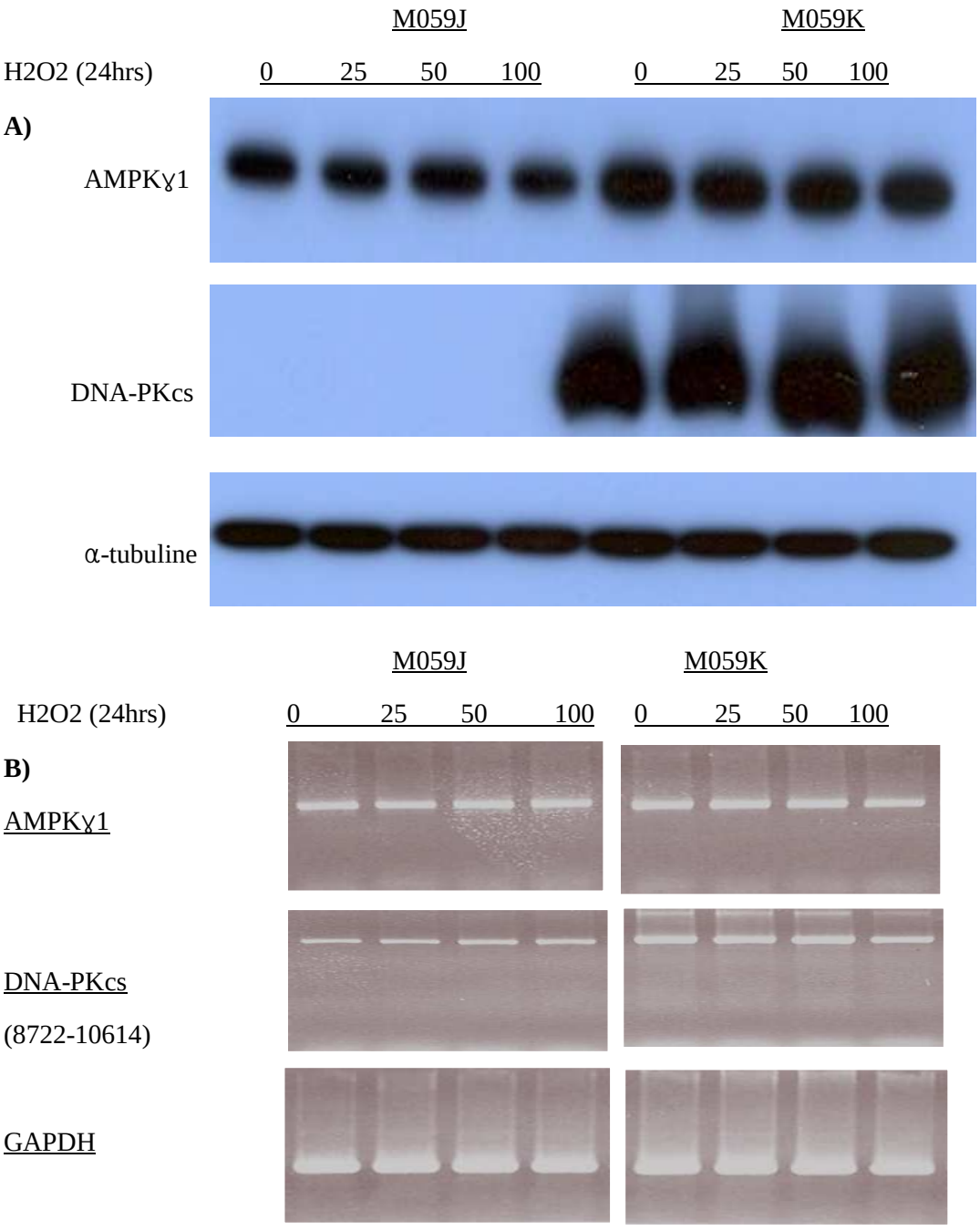


Figure 16 Effect of Hydrogen peroxide in human glioma cell line. M059J and M059K cells were treated with different doses of hydrogen peroxide and proteins were harvested after 24 hours. Expression level of AMPK gamma 1 is higher in M059J cells as compared to M059K cells as shown by WB in Fig 16A and RT-PCR in Fig 16B.

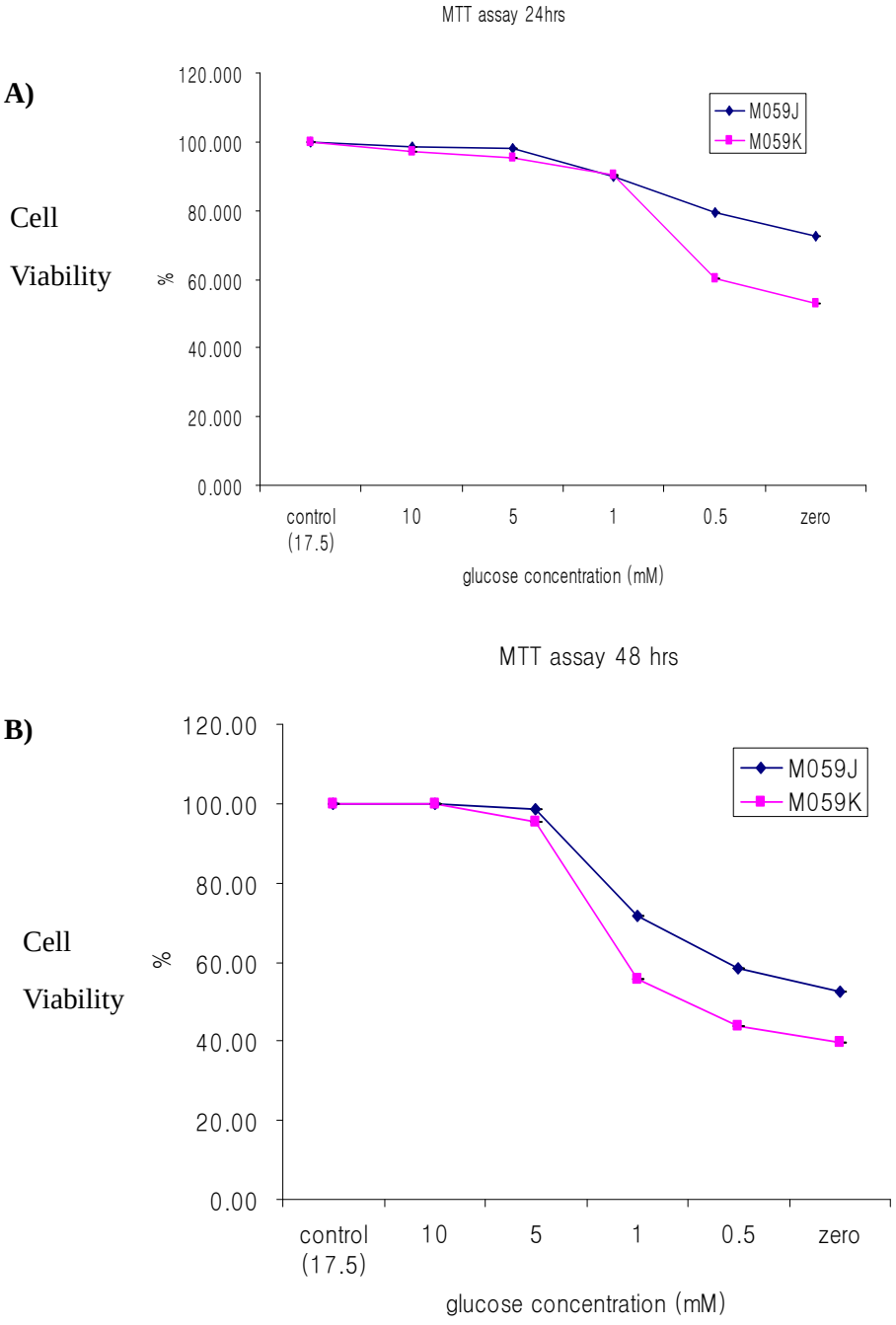


Figure 17 Sensitivity tests of glioma cell lines to low glucose concentration.

A) After 24hrs B) After 48hrs.

6. DNA-PKcs proficient cells are more sensitive and activated under low glucose condition.

M059K cells are more sensitive than M059J cells in low glucose conditions in both 24hrs and 48hrs (figure 17A and 17B). Under energy stress condition M059K is more phosphorylated than M059J cells (figure18).

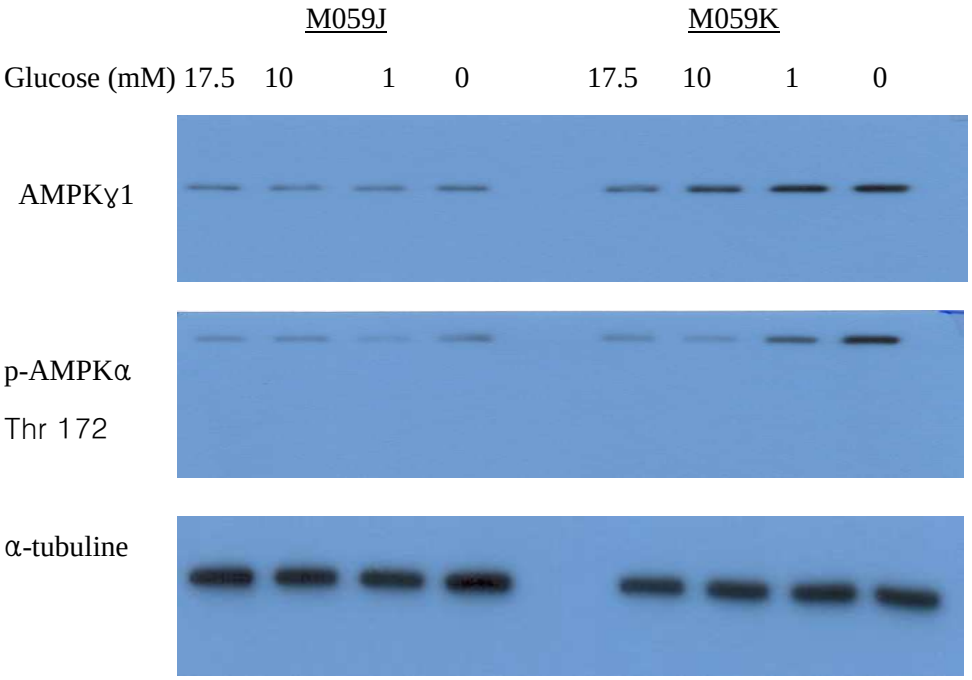


Figure 18 Phosphorylation of AMPK in glioma cell lines in response to low glucose.

DISCUSSION

Inside the cell, during normal or stress condition, different proteins interact with each other to carry out various cellular functions. In this study, we checked the interaction of DNA dependent protein kinase catalytic subunit (DNA-PKcs) and AMP activated Protein Kinase gamma 1 non-catalytic subunit (AMPKɣ1). This type of interaction study is novel and till date there has been no publication regarding its interaction and possible consequences. Our study showed that DNA-PKcs and AMPKɣ1 interact with each other under UV stress as well hydrogen peroxide treated condition. In this research work, we have given much priority on the effect of binding under UV stress. Further studies will be carried out in detail in future for the publication of our results.

I: AMPK activated Protein Kinase (AMPK) knockdown by small interfering RNA induces apoptosis under UV stress condition in Human Colorectal Carcinoma cell line.

First, our Yeast two hybrid result showed that these two proteins interact with each other. Based on this result, we started our experiment in mammalian cells. In Human Colorectal Carcinoma cell line, HCT 116p53+/+, the level of AMPK is low. The expression level of DNA-PKcs is comparatively higher than AMPK. Due to that we have transiently transfected pcDNA3.1 AMPKɣ1 into HCT116p53+/+ cell line. In the immunoprecipitation experiments, we used that transiently transfected cell line. Our result showed that DNA-PKcs and AMPKɣ1 binds in the normal as well as UV stress condition. The binding of these two proteins is increased under UV stress condition. These two proteins also co-localize under UV stress condition. The intensity of co-localization is higher in 6hr time period as compared to other time period.

Later, we knocked down the AMPK γ1 by using smart pool of AMPK γ1siRNA. We found that cells are found to be more sensitive to UV stress, once the AMPK γ1 is silenced. This is one of the interesting finding of our research. We then tried to find the UV induced formation of foci. Western Blot result showed that induction of gamma H2AX is higher in AMPK γ1 silenced cell line as compared to siRNA control. We got the same result upon the treatment of bleomycin in the cell.

Our DNA-PK kinase assay showed that DNA-PK activity is reduced once the AMPKγ1 is knocked down. This gives an additional proof that these two proteins interact with each other and has effect on either's activity.

II: Human glioma cell line proficient in DNA-dependent Protein Kinase (DNA-PKcs) is sensitive to cell death under glucose depleted conditions.

Glucose is the major source of energy in the cell. Once the energy is depleted in the cell, AMPK is activated. Once activated, AMPK shuts down ATP-consuming pathways, whereas AMPK facilitates the activation of ATP producing pathways in order to protect the cells. In this study, we used two different cell line-M059J and M059K to check the cell viability. Surprisingly, it was found that M059K, DNA-PKcs proficient cell line was found to be more sensitive to low glucose condition as compared to M059J, a DNA-PKcs deficient cell line.

Till now, there are only two known kinases i.e. LKBK and CaMKKβ, to be involved in the phosphorylation of AMPK [60]. We got some interesting result in the phosphorylation of AMPK under low glucose condition in human glioma cell line. Under glucose depleted condition, AMPK is activated and phosporylates Threonie 172 of AMPK alpha in a time dependent manner. This phosphorylation effect is more prominent in M059K cell than M059J. This shows that DNA-PKcs, a DNA repair protein, is also involved in the activation of AMPK under glucose depleted condition.

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저작물 이용 허락서

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논문제목	한글: DNA-PKcs 와 AMPK 의 상호작용연구 영문: Interaction between DNA-PKcs and AMP-activated Protein Kinase (AMPK)				

본인이 저작한 위의 저작물에 대하여 다음과 같은 조건아래 조선대학교가 저작물을 이용할 수 있도록 허락하고 동의합니다.

- 다 음 -

1. 저작물의 DB구축 및 인터넷을 포함한 정보통신망에의 공개를 위한 저작물의 복제, 기억장치에의 저장, 전송 등을 허락함
2. 위의 목적을 위하여 필요한 범위 내에서의 편집·형식상의 변경을 허락함. 다만, 저작물의 내용변경은 금지함.
3. 배포·전송된 저작물의 영리적 목적을 위한 복제, 저장, 전송 등은 금지함.
4. 저작물에 대한 이용기간은 5년으로 하고, 기간종료 3개월 이내에 별도의 의사표시가 없을 경우에는 저작물의 이용기간을 계속 연장함.
5. 해당 저작물의 저작권을 타인에게 양도하거나 또는 출판을 허락을 하였을 경우에는 1개월 이내에 대학에 이를 통보함.
6. 조선대학교는 저작물의 이용허락 이후 해당 저작물로 인하여 발생하는 타인에 의한 권리 침해에 대하여 일체의 법적 책임을 지지 않음
7. 소속대학의 협정기관에 저작물의 제공 및 인터넷 등 정보통신망을 이용한 저작물의 전송·출력을 허락함.

2007 년 6 월 29 일

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