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Cellular Effects of Quercetin on YD15 Tongue Carcinoma Cells

조선대학교 대학원

치의생명공학과

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YD15 설암 세포에 대한 quercetin의 작용기전 연구

지도교수 유훈

이 논문을 치의생명공학 박사학위신청 논문으로 제출함

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ABSTRACT

Cellular Effects of Quercetin on YD15 Tongue Carcinoma Cells

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Quercetin, one of flavonol compounds with anti-oxidant and anti-inflammatory properties, has been shown anti-carcinogenic activities on various cancer cell types. In this study, we investigated the cellular effects of quercetin on YD15 tongue carcinoma cells. In an MTT data quercetin was cytotoxic on YD15 cells and quercetin strongly inhibited the proliferation of YD15 cells. The inhibition of cell proliferation was accompanied by cell cycle arrest at S phase and/or G2/M phase and apoptosis with increased cell population at sub-G1, depending on time and dose of quercetin. Quercetin dramatically inhibited the expression of several cell cycle proteins such as cyclin D1, cycline B1 and cdk4 at high dose and slightly inhibited the expression of cyclin E1, cdk1. Quercetin-induced p21 expression was maximized at 10 μg/ml and reduced at higher dose (> 10 μg/ml). Quercetin decreased procaspase 3 with the concomitant activation of caspase 7 and cleaved PARP. The level of cleaved caspase 7 and PARP was maximized at 10 μg/ml of quercetin. Interestingly, the expression of Bcl-2 family proteins was not affected by quercetin treatment. Instead, aberrant multipolar mitotic spindle was induced at 10 μg/ml. This was accompanied by centrosome supernumerary / declustering

and the increased frequency of multinucleated cells. The multipolar mitotic spindles with supernumerary / declustered centrosomes, induced by 10 μg/ml quercetin, were disappeared with the formation of pseudo-bipolar mitotic spindle (with pseudo-two centrosomes) when quercetin was removed supporting that quercetin induced multipolar mitosis leading to cell cycle arrest, and mitotic catastrophe causing cell death by apoptosis. Quercetin also induced senescence. The positive staining of senescence-associated beta galactosidase (SA-βgal) and the suppression of Lamin B1 expression at both mRNA and protein confirmed that quercetin induced senescence on YD15 cells. Finally, quercetin suppressed AKT phosphorylation while increasing ERK phosphorylation. Reduced AKT phosphorylation might be involved in the inhibition of cell proliferation. Taken together, our data supports that quercetin suppressed the proliferation of YD15 cells through the mitotic catastrophe and the induction of senescence.

초 록

YD15 설암 세포에 대한 quercetin의 작용기전 연구

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플라보노이드 화합물 중 하나인 Quercetin은 항산화, 항염증 및 항암작용을 갖는 것으로 보고되었다. 본 연구에서는 YD15 혀 암세포에 대한 Quercetin의 항암작용을 조사하였다. Quercetin은 YD15 세포에 강한 세포독성을 보였고 세포증식을 억제했다. 즉 시간과 농도에 따라 S 및 G2 / M 세포주기를 억제하거나 세포사멸을 유도하였다. 특히 고농도 처리조건에서 cyclin D1, cycline B1 및 cdk4와 같은 세포주기 단백의 발현을 강력히 억제하였고 cyclin E1, cdk1의 발현도 억제했다. Quercetin은 낮은 농도(10 μg/ml) 조건에서 p21의 과발현을 유도하지만 높은 농도 조건(> 10 μg / ml)에서는 오히려 감소시켰으며 이러한 p21 발현의 변화는 p53에 무관하게 진행되었다. Quercetin은 procaspase 3의 감소를 유도하였고 caspase 7과 cleaved PARP를 활성화시켜 세포사멸을 유도하였다. 또한 Quercetin은 비정상적인 다극성 유사분열 스핀들 (multipolar mitotic spindles) 형성을 유도하였고, 중심체 증폭(supernumarery centrosome) / 군집 제거(declustering) 및 다중소핵 (multinucleated) 세포의형성을 유도하였다. 즉 Quercetin이 양극성 유

사분열 스핀들(pseudo-bipolar spindles)에 영향을 주어 세포주기를 정지시키는 다극성 유사분열을 일으켰고 결과적으로 세포사멸을 유도했다. 고농도 조건(30 μg/ml)에서 Quercetin은 lamin B1 유전자 (LMB1)의 전사 및 lamin B1 단백의 발현을 억제하였다. 또한 노화의 마커인 senescence associated beta-galactosidase (SA-βgal)의 발현을 유도하였다. Quercetin은 AKT 인산화(phosphorylation)를 억제시키며 ERK 인산화를 강하게 증가시켰는데 AKT 인산화억제소가 Quercetin에 의한 세포증식 억제에 기여하였다. 그러므로 Quercetin은 유사분열 억제 및 노화유도에 의해 YD15 세포의 증식을 억제하는 것으로 판단된다.

I. INTRODUCTION

Head and neck cancers (HNC) are one of the top ten global malignancies [1] and the sixth most common carcinoma worldwide [2]. Oral cancers are one of the leading cancer incidence in many countries and a major cause of morbidity and mortality [1]. Oral cancers are predominant in India, Pakistan and other Southeast Asian countries while oropharyngeal and tongue cancers are common in the Western world [1]. In Japan, incidence of cancer has increased in the tongue, oropharynx and hypopharynx regions in males, while cancer in the oropharynx region has increased in females [3]. Regional differences in oral cancer may be related to the prevalent habits in the respective region [4]. HNC was generally diagnosed as squamous cell carcinoma (HNSCC), affected more than 40,000 people each year in the U.S., and at least 13,000 people each year died of this disease. Chronic use of tobacco as well as alcohol consumption and infection by human papillomavirus (HPV) are considered as main risk factors [5-7]. Surgery, radiation therapy, chemotherapy, immunotherapy, gene therapy or combinations of these are the treatment options for head and neck cancer. Despite the improved understanding in diagnosis and therapeutics [8, 9] as well as genetic alternations [10] of HNSCC, 5-year survival rate of patients still remains low at 50 % or so [11, 12] and the mortality rates was not improved in the past 40 years [13]. The main reason for the treatment failure is due to the late identification of lesions at advanced stages or the recurrences and/or metastasis after treatment of the primary tumor. The success of chemotherapy depends on the specificity of the drug. Under prolonged treatments, drug-resistance seriously reduces the efficacy of the treatment. Thus, identify new molecular targets and novel drugs could be a good strategic goal for improving head and neck cancer treatment.

Apoptotic cell death is a critical element in cellular defense against unrestrained cell proliferation [14-16]. Apoptosis are extremely complicate [17] and the pathway is characterized

by the activation of a family of cell death proteases called the caspases [18]. Caspases are effector molecules of apoptosis in mammals, playing a key role in the initiation and execution of apoptosis, necrosis and inflammation [19-21]. They are synthesized as inactive precursors or zymogens (procaspases) and then proteolytically processed to become active, cleave another caspase to amplify the apoptotic signal. Caspase-9 is the initiator of the mitochondrial pathway and caspase-8 is regarded as the originator of the death receptor-mediated apoptotic pathway. Caspase-3 is the most important protease in the caspase-dependent apoptosis pathway since it is required for chromatin condensation and fragmentation [22, 23], and activated by the initiator caspases (caspase-8, caspase-9, or caspase-10) [24]. Poly-ADP ribose polymerase (PARP-1) is a major target of caspase-3. Cleavage-mediated inactivation of PARP-1 preserves cellular ATP that is required for apoptosis [25].

There are two types of apoptotic pathways, intrinsic and extrinsic (or death receptor) [26]. The extrinsic pathway is initiated by the cell surface death receptors [27-29] through the formation of the death-inducing signaling complex (DISC) [30], leadings to an activation of caspases (caspase-8, caspase-3). The intrinsic pathway is triggered by the response to DNA damage and associated with mitochondrial depolarization and the release of cytochrome c from mitochondrial inter-membrane space into the cytoplasm. Cytochrome c, apoptotic protease-activating factor 1 (APAF- 1) and procaspase-9 then form a complex termed “apoptosome” [17] to activate caspase-9, promotes activation of caspase-3, -6 and -7 [31]. Bcl-2 family members play important roles in regulating mitochondrial integrity and mitochondria-initiated caspase activation [32]. Both intrinsic and extrinsic pathways of apoptosis are associated with each other [33] through the cleavage of BID (BH3 interacting-domain death agonist). These pathways of apoptosis come together putting cell death by the activation of cas-pase-3/-7, cell shrinkage, chromatin condensation and fragmentation of chromosomal DNA, degradation of nuclear and cytoskeleton

proteins [29]. p53 induced by DNA damage is an essential molecular in apoptosis regulation [34, 35]. p53 is implicated in both apoptotic signaling pathways [36]. When cells are under the stress condition, p53 is drastically altered and activated. The activation of p53 induces cell growth inhibition, senescence or apoptosis [37]. Bcl-2 family proteins are involved in cell death by p53-dependently and -independently, at least in part, by releasing cytochrome c from the mitochondria into cytosol [38-40]. p53 inhibit Bcl-2 function by transactivation of cdc42 [41], up-regulates Bax expression [42] as well as facilitates the release of the apoptosis-inducing factor and cytochrome *c* from the mitochondria, thus activating the caspase cascade [43]. Almost 40 - 60 % of head and neck cancer patients has p53 gene mutations. The mutation in this gene is frequently associated with cancer progression from premalignant to invasive form. Patients with p53 gene mutations are at an increased risk of early recurrence of cancer [44]. Detecting apoptotic cells or monitoring cell progression to apoptosis is an essential process for the development of anti-cancer drugss. Various assays were developed to detect or quantitate apoptotic cells such as an assay of caspase enzyme activity, DAPI staining and DNA ladder band detection.

Cell death also occurs in the absence of apoptosis by non-apoptotic pathways, autophagy and necrosis [45, 46]. Autophagy is a lysosomal mechanism of self-digesting cytosolic components, which are characterized by the conversion of the protein marker LC3-I to LC3-II [47, 48]. Non-apoptotic cell death is occasionally named necrosis to indicate that cell death is uncontrolled [49] and mediated by DNA degradation, membrane distortion and cellular swelling [46].

Cellular senescence, irreversible arrest of cell growth, has often been compared with apoptosis as an intrinsic tumor-suppressive mechanism. As senescent cells are permanently arrested, it was often assumed that senescence is functionally similar to apoptotic cell death in terms of its effects on tumor suppression and tumor therapy [50]. Senescence was considered as an essential early line of defense against tumor development by preventing cell proliferation with damaged

DNA [51-53]. Senescent cells develop the formation of unique morphological changes such as increased size of cell and nuclear, flattened cytoplasm, irregular nuclear envelope, changes in chromosome condensation and distribution, heterochromatic foci and larger chromosomes migrating toward the nuclear periphery [54-57] and increased senescence-associated beta-galactosidase (SA-βgal) activity [58]. Cellular senescence is triggered by a number of factors such as aging, DNA damage, oncogene activation, oxidative stress and therapy-induced genotoxic stress [59]. Molecular mechanism of senescence typically involves in p53 and retinoblastoma (Rb) pathways and telomere shortening [60-62]. Activation of either p53 or Rb tumor suppressor pathway was sufficient to induce lamin B1 loss [63]. Lamin B1 loss is a robust hallmark of senescence and serves as a detectable biomarker of the senescence in cell culture and in vivo [63].

The inner surface of the nuclear envelope is lined by a lamina, a network composed of intermediate filaments and membrane associate proteins. Lamina contributes to the size, shape, and stability of the nucleus [64, 65]. The important structural proteins of the lamina are nuclear lamins, which are subdivided into A type (lamin A, C) or B type (lamin B1, B2) based on their sequence homologies, structure features and isoelectric points [66]. The B-type lamins encoded by two different genes, *LMNB1* and *LMNB2,* respectively [67, 68]. One or the other lamin type B may be sufficient for individual cell survival [69, 70]. However, other reports suggest that embryonic stem cells can self-renew and retain their pluripotency without lamins [71]. In cultured cells, B-type lamins play a significant role in the formation of the mitotic spindle [72-76]. It has been implied that lamin B1 has a role in the organization of replicating chromatin during late S phase in mouse 3T3 cells [77] suggesting that lamin B1 play a role in DNA replication. Lamin B1 also play a role in transcription because silencing lamin B1 leads to a significant inhibition of RNA polymerase II transcription in HeLa cells [78]. In addition, lamin B1 was required for maintaining chromatin condensation in interphase nuclei. Loss of lamin B1 results in prolongation of S phase and de-

condensation of chromosome territories [79]. Veronika et al. reported that reducing lamin B1 expression to approximately 50% of normal levels by short hairpin RNA in human osteosarcoma cell line (U-2-OS) or human colon cancer cell line (HCT116) delayed cell cycle and accumulated cells in early S phase, suggesting that the maintenance of lamin B1 levels is required for DNA replication and DNA repair by the regulation of key factors such as BRCA1 and RAD51 [80]. Thus, lamin B1 plays important roles in maintaining the nuclear structure and function.

Any forms of DNA damage kill cells through caspase-dependent apoptosis or mitotic catastrophe [45, 81, 82]. In respond to DNA damage, cell cycle arrest at G1/S or G2/M phase transition is activated, allowing DNA repair machinery time to recover from the damage. If the damage is beyond repair, cells undergo apoptotic cell death or senescence, often through p53-dependent mechanism [83]. The G2 checkpoint is crucial for preventing mitotic cell death. When it is aborted, mitotic catastrophe (a consequence of a mitotic delay) is potentiated, driving cells to irreversible fate such as apoptosis, necrosis or senescence [84] by aberrant mitosis [85, 86]. Mitotic catastrophe also occurs as a result of centrosome over-duplication and consequent entry into mitosis with multiple spindle poles [87]. Mitotic catastrophe is often characterized by the formation of giant micro-nucleated cells, which reflects the abnormal segregation of chromosome. The major cell cycle checkpoint ensuring the correct segregation of chromosomes between daughter cells is the spindle assembly checkpoint (SAC), which prevents metaphase–anaphase transition until a correct bipolar spindle is established [88]. This checkpoint function is activated by chromosomes unattached or inadequately attached to the spindles as well as improper spindle tension exerted on kinetochores [89, 90]. Defects in chromosome segregation during mitosis are caused by deficiencies in SAC or by microtubule mis-attachments or aberrant mitotic division [91], triggering chromosome instability or aneuploidy. Mitotic aberrations associated with the abnormalities of the function and amount of centrosome (for example, generation of misaligned or fragmented

chromosomes, failure of spindle attachment or abnormal spindle tension at kinetochores) trigger spindle checkpoint responses, leading to mitotic catastrophe and cell death [87]. Over-duplication of centrosome which leads to multipolar mitosis [92] or duplication failure of centrosome which leads to incomplete in chromosome segregation [93] may also induce mitotic catastrophe. Thus, the function as well as abnormalities of centrosomes destabilize chromosomes and drive tumor progression with the function of compromised spindle checkpoint [87]. In mammalian cells, the chromosome missegragation induced by the complete inactivation of mitotic spindle checkpoint leads to cell death [94, 95]. Prolonged SAC activation leads to mitotic arrest and often to mitotic catastrophe [96]. However, in cancer cells, genes coding for components of SAC are often altered or mutated [97-99] and these cells can escape from cell death reaching to the aneuploidy or chromosome instability stage [100, 101] and drive tumor progression [102] by avoiding mitotic catastrophe. Cancer cells are often more sensitive to “mitotic catastrophe” than normal cells [103]. Thus, activating mitotic catastrophe might be a promising therapeutic anti-cancer strategy.

Centrosomes are organelles that function as microtubule-organizing centers in most animal cells. Centrosomes are composed of two centrioles. Each centriole is made up by nine microtubule triplets. The older centriole, maternal centriole, carries distal as well as subdistal appendages where microtubule (MT) anchorage and nucleation take place. Both centrioles of a centrosome are embedded in an amorphous protein mass termed pericentriolar material (PCM) and are tethered together in most cell cycle phases (Fig. 1) [104]. Besides controlling microtubule-associated processes such as cell shape and intracellular transport, centrosomes are of crucial importance for the assembly of the bipolar mitotic spindle which ensures the accurate segregation of chromosomes during cell division. Therefore, centrosome duplication is precisely regulated to ensure that centrosomes are duplicated only once per cell cycle.

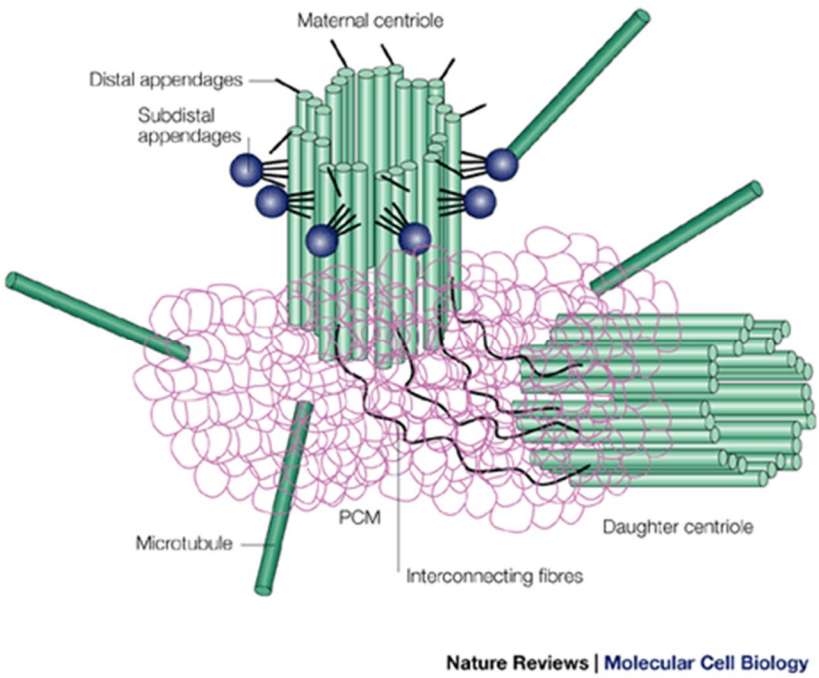

Figure 1. Centrosome structure.

Recently, centrosomes have attracted considerable attention primarily due to their potential importance in carcinogenesis [105]. Abnormal amplification of centrosomes was observed in response to DNA damage [92, 106] and in cells carrying mutations in DNA repair or checkpoint genes [107-109] or expressing mutant forms of telomerase [110]. Centrosome abnormality (numerical amplification as well as hypertrophy of centrosomes) is common in human cancer [111, 112]. In most cases, tripolar spindles undergoing cytokinesis and some viable daughter cells suffering severe aneuploidy [87]. Cells that have spindles with > 3 poles fail to undergo cytokinesis [87]. Failure to undergo cytokinesis triggers a p53-dependent checkpoint response leading to cell-cycle arrest and eventually cell death through a mechanism poorly understood [113]. However, in the absence of p53, cells that fail cytokinesis continue to cycle and become large multi-nucleated polyploid cells [87], eventually undergoing cell-cycle arrest and/or death, although some cells might be able to survive by resuming cytokinesis, probably through the formation of pseudo-bipolar spindles [87]. Cancer cells minimize the lethal impact of multipolar mitosis by clustering the amplified centrosomes to form "pseudo-bipolar" spindle poles [114-116] positioning clustered centrosome on a bipolar axis (centrosome clustering) [116] for survival. Thus, inhibiting centrosome clustering might offer an opportunity for nontoxic chemotherapy.

There are several types of functional defects of the centrosome that interfere with the formation of bipolar spindles. When the duplicated centrosomes fail to separate in late G2, aberrant mitotic spindles with a single spindle pole was formed. Also, cells failed to undergo a proper centrosome maturation process during S and G2 phases were unable to form proper bipolar mitotic spindles. In mitosis, centrosomes are subject to strong pulling forces exerted by the spindles attached to chromosomes and therefore they need to be fortified before mitosis. Failure to do this causes centrosome fragmentation and the acentriolar centrosomal fragments, leading to the formation of multipolar spindles.

The mammalian cell cycle is divided into four distinct phases (G1, S, G2, and M). Entry into each phase of cell-cycle is regulated by receptor collectives termed cell-cycle checkpoints. The progression of cells through the cell cycle is promoted by cyclin-dependent kinases (CDKs), which are positively and negatively regulated by cyclins (A, B, D, and E) and cyclin-dependent kinase inhibitors (CDKIs), respectively [117, 118]. The retinoblastoma tumor suppressor protein, Rb, acts as a critical regulator for the G1–S phase progression of the cell cycle by trapping E2F1, an essential transcriptional factor required for the expression of cell proliferation-associated genes. Hypophosphorylated Rb binds to and sequesters the transcription factor E2F1, resulting in cell cycle arrest at the G1 phase [119]. Cyclin D isoforms (cyclin D1–D3) interact with CDK4 and CDK6 to drive the progression of a cell through G1 phase. The association of cyclin E with CDK2 is active at the G1-S transition and directs the entry into S-phase. Complexes formed by cyclin D and CDK4 or CDK6 and cyclin E and CDK2 phosphorylate Rb and enable the G1–S transition. S-phase progression is directed by the cyclin A/CDK2 complex, and the complex of cyclin A with Cdc2 (also known as cdk1) is important in G2 phase. Cdk1/cyclin B is necessary for the entry into mitosis. Progression from G2 to M phase is driven by the activation of the Cdk1/cyclin B1 complex, whose activity must be sustained from prophase to metaphase. Subsequent entry into the anaphase critically relies on the sudden destruction of the Cdk1/cyclin B1 activity by the anaphase-promoting complex (APC) [120, 121]. At the end of the metaphase, the APC (also called “cyclosome”) destroy cyclin B1 to allow mitosis to proceed [122]. The Cdk1/cyclin B1 heterodimer induces mitosis by phosphorylating and activating enzymes regulating chromatin condensation, nuclear membrane breakdown, mitosis-specific microtubule reorganization and the actin cytoskeleton allowing for mitotic rounding up of the cell [120]. There are multiple steps to cell division. Chromosomes first condense (prophase), then matching pairs of chromosomes line up along the middle of the cell (metaphase). Chromosomes are then attached to

microtubules which form a spindle-like network at either end of the cell. Microtubules now pull the chromosomes a part (anaphase). The cell then splits into two with each new cell containing an identical set of genes (telophase). Cdk inhibitors fall into two families: INK4 inhibitors and Cip/Kip inhibitors. Both families of Cdk inhibitors play regulatory roles during the G1/S cell cycle checkpoint [123]. p21 Waf1/Cip1, a p53 downstream target, is a member of Cip/kip family of Cdk inhibitors, bind and inhibit the indicated Cdk or cyclin/Cdk complexes, effectively block the progression of cells from G1 into S-phase [124].

Microtubules are key components of the cytoskeleton and play crucial roles in cellular metabolism and intracellular transport. They help maintain cell shape, intracellular organization and are instrumental in interphase homeostasis as well as mitosis and cell division [125]. At the onset of mitosis, the entire microtubule network undergoes rearrangement from the interphase microtubule array to specialized highly dynamic mitotic spindles nucleated from the centrosomes or the kinetochores. These specialized microtubule structures are responsible for guiding the sister chromatids toward the poles of the new daughter cells during mitosis, ensuring a complete genetic content for each. Mitotic spindles are highly susceptible to the actions of microtubule disrupting compounds such as microtubule stabilizers, and these effects can be used for identification and characterization of anti-mitotic drugs [126, 127]. Microtubule targeting agents are often referred to mitotic poisons (anti-mitotic drugs) due to their ability to interrupt the formation of bipolar spindle, thus leading to cell death by mitotic failure.

Quercetin (3,3', 4', 5,7‑pentahydroxyflavone) is a flavonoid found in a wide variety of fruits, vegetables, nuts and red wine in human diet. Quercetin is well-known for its biological functions in anti-oxidant, anti-carcinogenic, anti-inflammatory and cardioprotective properties [128, 129]. Its anticancer effects have also been reported in various cancer cells including leukemia, breast, ovarian, colon, cervical, prostate, lymphoma and head and neck [130-134]. The use of

quercetin in various food has been increased due to its potential anti-oxidant and other beneficial properties. Quercetin has a function in reversing drug resistance and re-sensitizing cancer cells to some chemotherapeutic agents [135-138]. A diet rich in quercetin lowered pancreatic cancer risk in smokers [139]. Different molecular mechanisms underlying antitumor activity of quercetin have been reported such as upregulation of cell cycle inhibitors, downregulation of oncogene expression and the inhibition of glycolysis [130, 140-142]. Quercetin-induced cell cycle arrest at G1/S or G2/M phase has been reported in various types of cancer cells [143-149]. Furthermore, quercetin-induced apoptotic cell death was investigated in several human cancer cell lines including glioma, liver, lung, prostate and colon cancer cells by focusing on the Bcl-2 family members and/or caspases [150-153]. Quercetin treatment suppressed cell growth by inducing G2 arrest and apoptosis in EGFR-overexpressing HSC-3 and TW206 oral cancer cells [154]. Quercetin also suppressed cellular migration and invasion in human head and neck squamous cell carcinoma (HNSCC) [134].

The precise target for quercetin action and its mechanisms remains to be elucidated. Studies indicated that quercetin inhibited cells proliferation via perturbation of microtubule polymerization in bovine aortic endothelial cells [155] and prostate cancer cells [156] or by perturbed microtubule functions through tubulin binding [157]. Within the M-phase of the cell cycle, highly dynamic mitotic microtubules are essential for the proper orchestration of chromosomal segregation prior to cytokinesis [155]. Treating cells of amplified centrosomes with drugs that attenuate microtubule dynamicity (for example griseofulvin [158] or bromonoscapine [159] induces declustering of supernumerary centrosomes.

In this study, we investigate the cellular effects of querccetin on human tongue carcinoma YD15 cells to understand the molecular mechanism underlying the growth inhibition and cell death.

We demonstrate that quercetin suppresses the proliferation of YD15 cells by inducing mitotic catastrophe and senescence.

II. MATERIALS AND METHODS

2.1. Chemicals and antibodies

Quercetin was purchased from Jena Bioscience (Germany) and was dissolved in dimethyl sulfoxide (DMSO) at stock concentration = 30 mg/ml.

Antibodies for cdk1, cdk2, cdk4, Cyclin B1, Cyclin A, cyclin E1, p27, FADD and caspase 3 were purchased from Santa Cruz Biotechnology Inc. (USA) and antibodies for pAKT, pERK1/2, pJNK, p21, cleaved caspase 7 and cyclin D1 were from Cell signaling Technology Inc. (USA). PARP-p85 was purchased from Epitomics, Burlingame, CA. Retinoblastoma protein was purchased from Abfrontier (Korea). All antibodies for immunofluorescence were purchased from Abcam.

2.2. Cell line and cell culture

YD15 tongue carcinoma cell, YD38 gingival carcinoma cells, Fadu hypopharyngeal carcinoma cells were purchased from Korean Cell Line Bank (Seoul, Korea) and were grown in RPMI-1640 or MEM media which was supplemented with 10 % FBS and 1 % antibiotics. The cells were maintained in a humidified and 5 % CO_2 controlled incubator at 37 ^{o}C.

2.3. Cell viability by MTT assay

Cell proliferation was detected by 3-(4-,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) assay. Cells were seeded on a 96-well plate (2 x 10^4 cells/200 μl/ well) for 24 hours and the cells were treated with 0 – 30 μg/ml of quercetin for 24 hours. After removing the medium, 200 μl MTT working solution (0.5 mg/ml) was added to each well and incubated for 4 hours at 37 ^{o}C. The medium was removed, and then 200 μl of lysis buffer (10 % sodium dodecylsulfate, 0.1 N HCl) was added to each well and continued to incubate at 37 ^{o}C for 2 hours to dissolve the formazan crystals. The absorbance was read at 540 nm analyzed using Microsoft

Excel. The percentage of viable cells was calculated based on the following formula: The percent of cell viability = (Absorbance of quercetin-treated group / absorbance of control group) × 100. All results were averaged after triplicated experiments at a given concentration.

2.4. Cell proliferation assay

YD15 cells were seeded in 60 mm plates and treated with different concentration of quercetin (0, 7.5, 10, 30 μg/ml) for 24, 60, and 72 hours. After treatment, cells attached were collected for counting by using cell counter nucleocassette (Chemometec, Denmark). The data was analyzed using Microsoft Excel. All results were averaged after triplicated experiments at each concentration.

2.5. Observation of cell morphology

YD15 cells were seeded in 96-well plate with 1 x 10^5 cells / ml for 24 hours and then treated with different concentrations of quercetin for 24 or 48 hours. Images of cell morphology were captured by using an optical contrast phase microscopy at magnification of x 200.

2.6. Nuclear staining

YD15 cells were seeded in a 8-chamber slide with 1 x 10^5 cells / ml for 24 hours and then treated with different concentrations of quercetin for 24 or 48 hours. After treatment, the medium were removed, washed with PBS and fixed in 4 % paraformaldehyde for 30 min at room temperature. The fixed cells were washed with PBS and stained with DAPI (0.5 μg/ml) at room temperature in the dark for 30 minutes. After staining, cells were washed two times by PBS. The nuclear morphology of the cell was captured on a fluorescent microscopy (Zeiss, Germany).

2.7. Cell cycle analysis by flow cytometry

Cells were seeded into a 100 mm dish (1 x 10^5 cells/ml) for 24 hours before treated with an indicated concentration of quercetin (0, 10, 20, 30 μg/ml). After incubating for 24 or 48 hours, both

floating and adherent cells were collected, washed with PBS, fixed in 70 % ethanol, washed again by PBS, rehydrated in PBS, treated with RNase (200 μg/ml) and stained with propidium iodide (50 μg/ml) for flow cytometry. DNA content of stained cells was analyzed by using a FACS Calibur flow cytometer (BD Biosciences). Data from 10,000 cells per each sample was analyzed with CellQuest software (BD Biosciences).

2.8. Western blot analysis

The effects of quercetin on cell cycle proteins, apoptosis related proteins, MAPKs family and Bcl2 family were investigated by western blotting. YD15 cells were seeded on 60 mm dishes (1 x 10^5 cells/ml) for 24 hours and then treated with indicated concentration of quercetin (0, 10, 20, 30 μg/ml) for 24 or 48 hours. Cell lysates were extracted by using cell lysis buffer (Cell Signaling, MA) and protein concentration was determined by BCA Kit (PIERCE, IL). 20 μg of total proteins were loaded on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred into PVDF membranes (Millipore, MA). The membranes were blocked with 5 % skim milk in Tris-buffered saline buffer (20 mM Tris-HCl, 136 mM NaCl, pH 7.4) for 2 hours, and then incubated overnight with primary antibody (1:1000 dilution) in blocking agent at 4 ^{0}C. After washing with Tris-buffered saline or Tris-buffered saline plus 0.1 % Tween buffer (x 3, 10 minutes each), the membrane was incubated with secondary antibody (1:1000 or 1:2000 dilution) for 2 hours at room temperature. Antibody-bound protein bands on membrane were exposed with Western blotting luminol reagent (ECL) and captured by using Kodak image station system (USA).

2.9. Immunofluorescence microscopic images of microtubules, centrosome and lamin B1

YD15 cells were seeded in 8-chamber slide (1 x 10^5 cells / ml) for 24 hours and then treated with different concentrations of quercetin for 24 hours or 48 hours. After treatment, the medium were

removed and the cells were washed with PBS and fixed in 4 % paraformaldehyde for 30 min at room temperature. The fixed cells were washed with PBS and permeabilized with 0.1 % Triton-X-100 for 10 minutes at room temperature. After permeabilized, the cells were washed with PBS and blocked with 1 % BSA for 1 hour at room temperature. Then, the cells were incubated with the following primary antibodies: anti-tubulin antibody ab6160 at 1/1000 dilution for microtubule staining, anti-pericentrin antibody (centrosome marker) ab4448 at 1/1000 dilution for centrosome staining and anti-lamin B1 (nuclear envelope marker) at 1/500 dilution for lamin B1 staining. The chamber slide with primary antibodies was incubated at 4 °C for overnight, and then washed three times with Tris-buffered saline or Tris-buffered saline plus 0.1 % Tween buffer. The secondary antibodies, Goat Anti-Rat IgG H&L (Alexa Fluor 488) Ab150165 and Goat Anti-Rabbit IgG H&L (Alexa Fluor 594) Ab150080 were used at 1/1000 dilution by incubating for 1 hour at room temperature. DAPI (0.5 μg/ml) for DNA staining was also added to each chamber and incubated for 20 minutes at room temperature. Cells were washed again three times in TBS or TBST buffer. The immunofluorescence images were captured by using a fluorescence microscopy (Zeiss, Germany).

2.10. Senescence-associated (SA)-β gal staining

YD15 cells were seeded in 24-well plate (1 x 10^5 cells/ml) for 24 hours and then treated with different concentrations of quercetin (0, 10, 20, 30 μg/ml) for 24 hours or 48 hours. SA-β gal staining was performed as described in Koji Itahana et al. [160]. After treatment, cells were washed twice with PBS and then fixed by neutral buffered 4 % formaldehyde for 3 minutes at room temperature. The cells were washed twice more with PBS, exposed with SA-β gal staining solution (300 μl/well) and incubated at 37 °C in a dry incubator (no CO_2) for 16-18 hours. Cells were

washed twice with PBS, and added PBS (300 μl/well) and observed under a microscope (200x total magnification).

2.11. RNA extraction and RT-PCR

YD15 cells were seeded into 60 mm dishes (1 x 10^5 cells/ml) for 24 hours before treated with indicated concentration of quercetin (0, 10, 20, 30 μg/ml) for 24 hours or 48 hours. Total RNA was isolated by using RNeasy Mini Kit (QIAGEN, USA) by following the manufacturer protocol. Contaminated genomic DNA in total RNA was removed by RNase-free DNase I digestion. The reverse transcription was performed in a total volume 50 μl (3 μg of total RNA, Oligo$(dT)_{12-18}$ Primer and SuperScript™ II Reverse Transcriptase (Invitrogen, USA)) by following the supplied protocol. The obtained cDNA was used to determine the amount of lamin B1 mRNA (LMNB1) by using a Thermal cycler 2720 PCR (Thermo Fisher Scientific). Human Glyceraldehyde 3-phosphate dehydrogenase (h-GADPH) gene was used as an internal control. Each reaction was run in triplicate with the reaction volume of 20 μl by using PCR premix (Bioneer) which contains 2 μl of cDNA template along with primers and water. Program of RT-PCR were as follow: 2 minutes for primary denature at 95 °C; 32 cycles: denature at 95 °C, 20 seconds; primers annealing at 55 °C, 15 seconds; elongation at 72 °C, 20 seconds; final elongation at 72 °C in 7 minutes and kept at 4 °C. RT-PCR products were electrophoresis on 2.5 % agarose gel in TBE buffer, stained with ethidium bromide and checked under UV-lamp (302 nm). The primers used for the amplification of h-GADPH and LMNB1 transcripts are as follows: h-GAPDH (200 bp) forward, 5'-CTCTGACTTCAACAGCGACA-3', h-GAPDH reverse, 5'-TCTCTCTCTTCCTCTTGTGC-3'; LMNB1 (64 bp) forward, 5'–AAGCAGCTGGAGTGGTTGTT–3', LMNB1 reverse 5'-TTGGATGCTCTTGGGGTTC-3'. Primer sequences of LMNB1 gene were based on Adam

Freund et al. (reference 54). The LMNB1 forward and LMNB1 reverse primer pairs bind to cDNA of LMNB1 gene sequence to produce amplified PCR product (64 bp fragment in length).

III. RESULTS

3.1. Cytotoxicity of quercetin on head and neck cancer cell lines

The cytotoxicity of quercetin on several different head and neck cancer cell lines was investigated by an MTT assay. Cancer cell lines were seeded in 96-well plates (1 x 10^5 cell/ml) for 24 hours and then treated with different concentrations of quercetin (0, 10, 20, 30 μg/ml) for 24 hours. As shown in Fig. 2, quercetin inhibited cell viability of all three tested cell lines in a dose-dependent manner. However, the relative inhibition activity was different. YD15 cells were more sensitive with quercetin than YD38 and Fadu cells which we chose YD15 cells for further investigation on the cellular effects of quercetin.

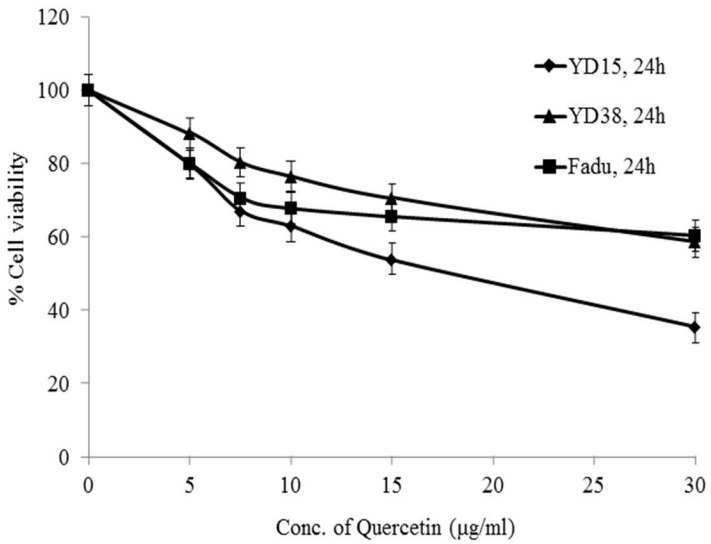

Figure 2. Cytotoxicity of quercetin on several different head and neck cancer cell lines.

Cells were treated with indicated concentration of quercetin for 24 hours. The percent cell viability was determined by an MTT assay. Quercetin inhibited the growth of all tested cancer cell lines with the most sensitive inhibition on YD15 cells. Vertical bars indicate means and standard errors (n=3).

3.2. Quercetin suppressed the proliferation of YD15 cells

To examine the effects of quercetin on YD15 cell proliferation, YD15 cells were seeded in 60 mm plates and treated with different concentrations of quercetin (0, 7.5, 10, 30 μg/ml) for 24, 60, and 72 hours. As shown in Fig. 3, the cell proliferation rate of YD15 cells were strongly suppressed by quercetin even at low dose (7.5 μg/ml). At 10 μg/ml, cell number was slightly increased after 24 hours of treatment as compare with starting amount (1.2×10^6 vs 1×10^6 cells, increase about 200.000 cells), but after prolonged time of treatment, the number did not increase, maintaining the same number even after 72 hours of treatment. The decrease in cell number was accompanied by the increase of cell debris in the medium suggesting that cell growth at low dose range (< 20 μg/ml) might be involved in cell death. At high dose (30 μg/ml) of quercetin, the number of cells maintained the initial number of cells. Noticeably, under high dose condition (≥ 20 μg/ml), floating cells or cell debris or cells with membrane blebbing (characteristic of cell death) were not observed under a microscope observation compared to lower dose treatment of quercetin, suggesting that the cell growth suppression of quercetin at high dose on YD15 cells was not due to cell death.

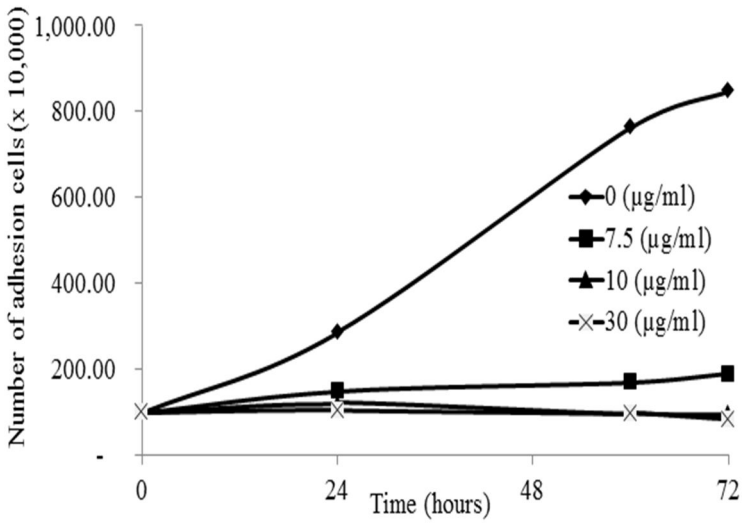

Figure 3. Quercetin suppressed the proliferation of YD15 cells.

YD15 cells were treated with various concentrations of quercetin for indicated hours. Cells were counted by using cell counter nucleocassette (Chemometec, Denmark). Quercetin suppressed the proliferation of YD15 cells.

3.3. The effects of quercetin on YD15 cell morphological changes

Morphological changes of YD15 cells were observed after treating cells with 10, 20 and 30 μg/ml of quercetin for 24 or 48 hours. Images of cell morphology were captured on an optical contrast phase microscopy at magnification of x 100. As shown in Fig. 4A, after treated with quercetin for 24h cell density was decreased, but cells with round shape (characteristic of cells at G2/M phase) was increased at 10 μg/ml, and dramatically reduced at higher dose (20 μg/ml or 30 μg/ml) with apoptotic cells were not much observed. After 48 hours of treatment, cells in G2/M phase were decreased and the cells in apoptosis were increased at 10 μg/ml of quercetin treatment. Interestingly, however, the cells treated with 20 or 30 μg/ml of quercetin were well spread out and strongly attached to the culture dish without apoptotic cell deaths (Fig. 4B).

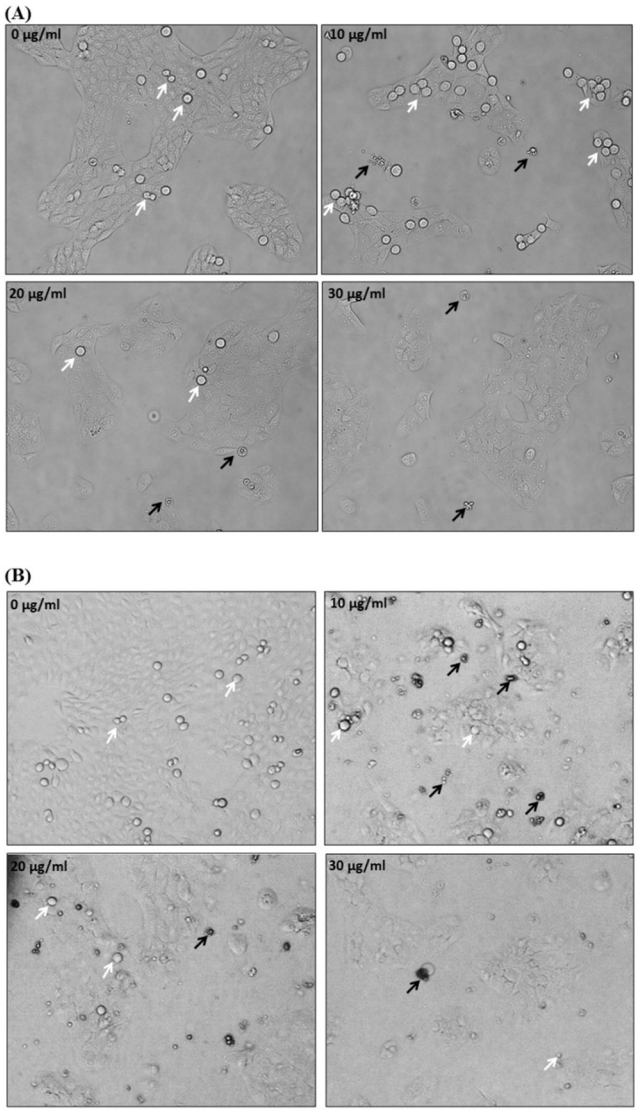

Figure 4. The change of YD15 cell morphology by quercetin.

YD15 cells were treated with indicated concentration of quercetin for 24 hours (A) or 48 hours (B). Cell morphology was observed under a microscopy. The images were captured at magnification of x100. The white arrow indicates the cells at G2/M phase, and the black arrow indicates the cells in apoptosis.

3.4. Effects of quercetin on cell cycle distribution

To further understand quercetin inhibition on cell viability and proliferation, we evaluated cell cycle distribution by using fluorescence activated cell sorting (FACS) analysis after treating with quercetin. YD15 cells were treated with different concentrations of quercetin (0, 10, 20, 30 μg/ml) for 24 hours or 48 hours, and then stained with propidium iodide (PI) for FACS analysis. As shown in Fig. 5, both doses and treatment time of quercetin affected on cell cycle distribution and apoptosis. A 10 μg/ml quercetin induced cell cycle arrest at G2/M phase after 24 hours (50.08 % vs 22.33 % of control sample) while the cell population at Go/G1 phase (14.12 % vs 44.97 % of control sample) and S phase (6.94 % vs 28.05 % of control sample) were decreased. However, after 48 hours of treatment, percent cell population of G2/M phase decreased from 50.08 % to 23.55 % with the corresponding increase of cell population at subG1 phase from 19.19 % to 54.24 %, suggesting that the cell population at G2/M phase arrest at 24 hours shifted to apoptotic cell death after 48 hours treatment. Interestingly, at 20 μg/ml treatment for 24 hours, quercetin arrested YD15 cells at both S phase (42.18 % vs 28.05 % of control) and G2/M phase (31.27 % vs 22.33 % of control) instead of only at G2/M phase as shown at 10 μg/ml, and after 48 hours, cells shifted from S phase arrest (decreased from 42.18 % to 11.3 %) to G2/M phase arrest (increased from 31.27 % to 41.17 %) and apoptotic cells at subG1 phase (11.37 % to 34.66 %). A concentration of 30 μg/ml, the pattern of cell cycle distribution was not much different compared with control cells after 24 hours of treatment. However, after 48 hours of treatment, cell population at G0/G1 phase was decreased with the increase at S phase and G2/M (Fig. 5). Taken together, cell cycle analysis indicates that quercetin has delayed the cell cycle progression of YD15 cells and has different effects depending on both dose and time of quercetin.

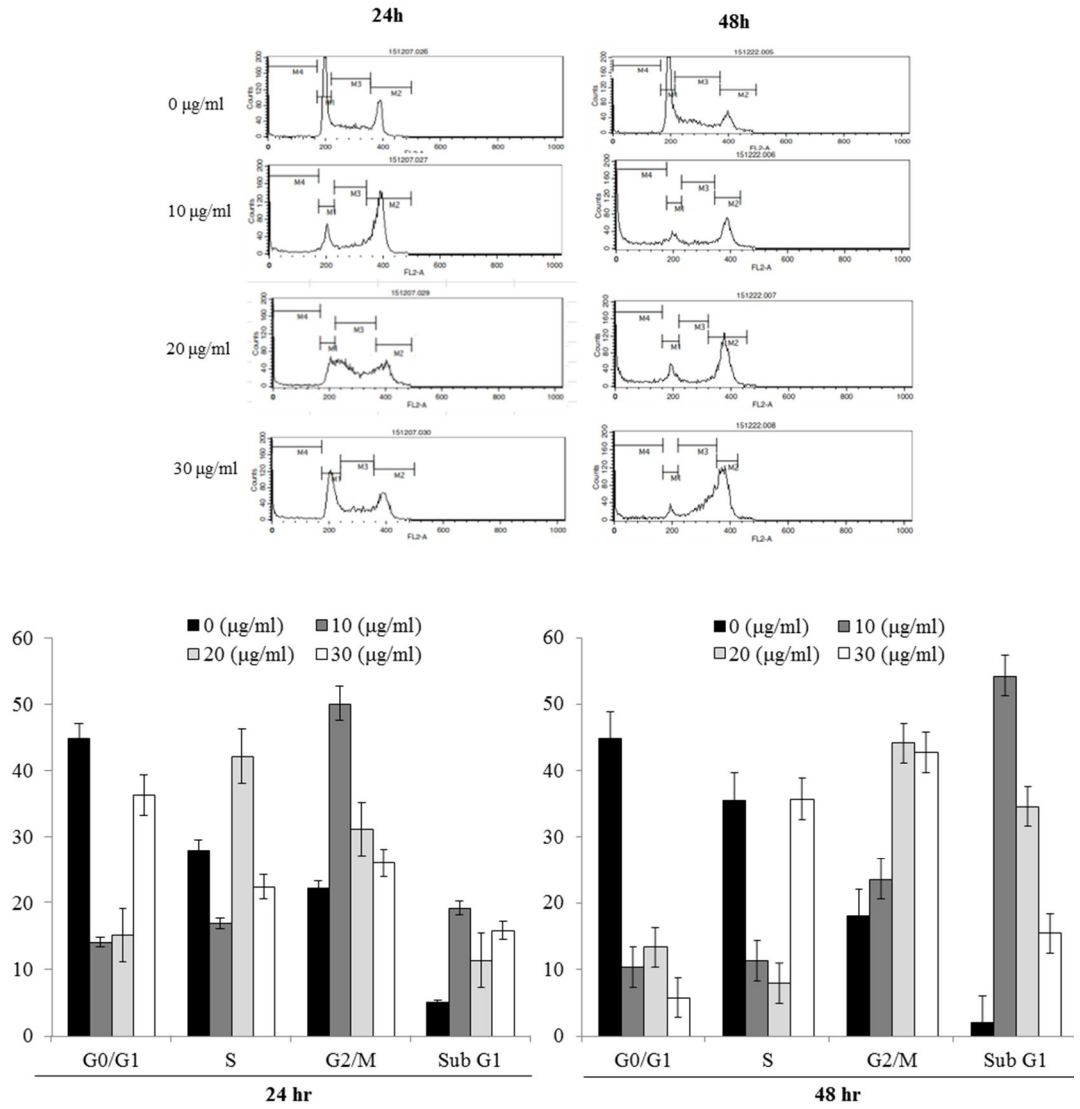

Figure 5. Effects of quercetin on cell cycle distribution.

YD15 Cells were treated with indicated doses of quercetin for 24 hours or 48 hours. The cells were stained with propidium iodide (PI) and evaluated on cell cycle progression by fluorescence activated cell sorting (FACS) analysis. Quercetin induced cell cycle arrest at G2/M or S phase depending on time and dose of quercetin treatment. Black bar, control; dark grey, 10 μg/ml; light grey, 20 μg/ml; white bar, 30 μg/ml.

3.5. Effects of quercetin on cell cycle proteins and cyclin-dependent kinase (cdk) inhibitor proteins

Cell cycle is tightly driven by a family of proteins called CDKs (cyclin-dependent kinases) [161], and these kinases are positively regulated by cyclins (A, B, D, E) and negatively regulated by CDKIs (cyclin-dependent kinase inhibitors) [162]. Since quercetin induces cell cycle arrest was dose- and time-dependent in FACS analysis, we further investigated the expression of these proteins by western blotting using antibodies against cdk4, cdk2, cdk1 (cdc2), cyclin D1, cyclin E1, cyclin A, cyclin B1 (Fig. 6A) and p53, p21, p27 (Fig. 6B). The main function of cdk2-cyclin E complex, together with cyclin D1-Cdk4/6 complex, is to triger G1-S phase transition, promote cells entry into the cell cycle. Cdk2-cyclin A complex drives cells from S phase to G2/M phase. Our data showed that at 10 μg/ml, quercetin did not effect on cyclin D1 expression level, and slightly decrease the expression level of cdk4, cyclin E, cyclin B1 and cdk1. Both cdk2 and phosphorylated cdk2 expression was decreased although the ratio of p-cdk2 / cdk2 is not much different compare with control sample, suggesting that either number of cells that already passed through S phase and G2 phase to entry M phase was increased or cells still stuck at G0/G1 because of quercetin effects. Combined with FACS data, at 10 μg/ml, cells are accumulated at G2/M phase, we suggested that cells already passed through S phase, therefore, the level of cdk2 was decreased at 10 ug/ml of treatment. At higher dose, 20 or 30 μg/ml, quercetin dramatically inhibited the expression of cyclin D1, cdk4 and cyclin B1 while slightly inhibited the expression of cyclin E1, cdk1. The level of phosphorylated cdk2 protein was increased while the cdk2 protein was reduced. This might because cells are still in S or G2 phase due to cdk2 activity is highest when cells at S phase and G2 phase. Combined with FACS data, treatment with quercetin at 20 or 30 μg/ml induced cell cycle accumulation at S and/or G2 phase depending on exposure time. Interestingly, expression of cyclin

A was not affected by quercetin. Thus, quercetin inhibits the expression of cell cycle proteins but not all, therefore cells still can entry into cell cycle progression but slowly than normal, consistent with our FACS data that quercetin delay the cell cycle progression.

Normally, in response to DNA damage or growth factor withdrawal, the expression of p53 protein increases [163] and cell growth is suppressed by cell-cycle arrest and apoptosis [164, 165], [166]. The cell cycle regulation and the DNA repair function of p53 are largely executed by the transactivation of p53-response genes such as p21/Waf1/Cip1 [167],[168]. Since FACS data showed that quercetin induced cell cycle arrest in a dose- and time- dependent manner in YD15 cells, we further investigated whether cells cycle arrest is associated with p53 or not, by examining the expression of p53, p21 and p27. Interestingly enough, as shown in Fig. 6B, the protein expression of p53 was not detected despite increase of p21 expression, suggesting that the p21 expression by quercetin is p53-independent. Quercetin-induced p21 expression was maximized at 10 μg/ml, but reduced at high dose (> 10 μg/ml). Accumulation of p27 has been implicated in G1 arrest. However, in our data, the protein level of p27 was not much affected by quercetin. Thus, the increase of p21 might contribute to cell cycle arrest induced by quercetin.

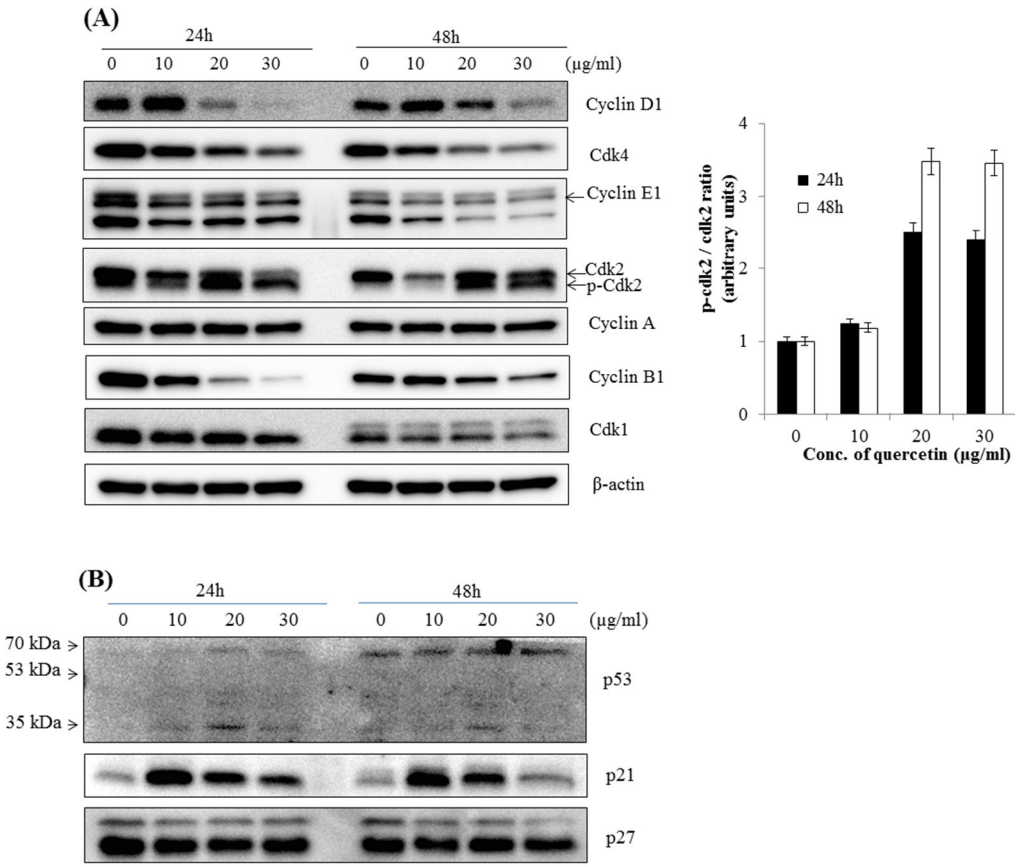

Figure 6. Effects of quercetin on cell cycle proteins and cdk inhibitor proteins.

YD15 cells were treated with indicated concentrations of quercetin for 24 or 48 hours. Total cell lysates (20 μg) were separated on 15 % SDS-PAGE and detected by western blotting. (A), the expression of cell cycle proteins. (B), the expression of p53 and cyclin-dependent inhibitor proteins (p21, p27).

3.6. Effects of quercetin on apoptosis-related proteins

FACS analysis data on cell cycle distribution, quercetin slightly induced the increase in sub-G1 cell population without significant dose-dependency after 24 hours of treatment, but after 48 hours, the sub-G1 cell population at 10 μg/ml was significantly increased. The accumulation of cells at sub-G1 phase indicates for apoptosis. Caspase 3 is considered to be the most important executioner caspases and activated by any of the initiator caspases such as caspase-8, caspase-9, or caspase-10 [24]. Pro-caspase-3 is cleaved into active caspase-3, and then caspase 3 cleaves pro-caspase-6 and pro-caspase-7. Poly (ADP - ribose) polymerase (PARP) is a 116 kDa protein involved in DNA repair as well as chromatin structure formation, and cleaved by caspase 3 (and possibly by other caspases) into an 85 kDa in size fragment during apoptosis [169]. To further understand the quercetin-induced apoptosis mechanism, the expressions of apoptosis-related proteins and the activation of caspases were examined by western blotting. As shown in Fig. 7, pro-caspase 3 was only slightly decreased after 24 hours of treatment with quercetin but decreased significantly after 48 hours. The decrease of procaspase 3 was concomitant with the increase of proteolytically cleaved caspase 7 and PARP. Similarly to p21 expression (described earlier in 3.5), the level of cleaved caspase 7 and PARP was maximized at 10 μg/ml of quercetin treatment is consistent with FACS data (the increased cell population at sub-G). This suggests that quercetin inhibited the proliferation of YD15 cells by cell cycle arrest and apoptosis at low dose (10 μg/ml).

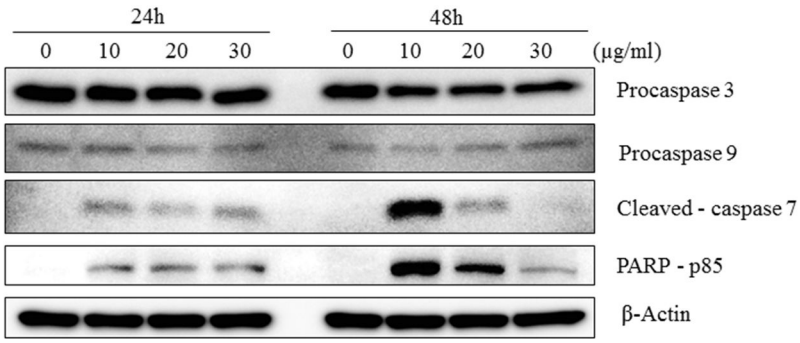

Figure 7. Effects of quercetin on apoptotic-related proteins.

YD15 cells were treated with indicated concentrations of quercetin for 24 or 48 hours. Total 20 μg protein was loaded on 15 % SDS-PAGE for western blotting. β-actin was used as an internal control. Quercetin strongly induced the activation of apoptotic marker proteins at 10 μg/ml but not at higher dose.

3.7. Effects of quercetin on nuclear morphology

Nuclear fragmentation and condensation are one of the characteristic features of apoptosis. To understand quercetin induced apoptotic cell death, YD15 cells were treated with 10 μg/ml or 30 μg/ml of quercetin for 24 hours or 48 hours and then the nuclei were stained with DAPI for the observation by fluorescent microscopy (Fig. 8). Treatment with different doses of quercetin induced the different effects on cell nuclear morphological changes. DAPI staining of YD15 cells at 10 μg/ml for 24 hours showed with visible chromatin condensation. Cells were at anaphase stage of M phase with abnormal mitotic chromatin morphology and sister chromatids could not be pulled toward opposite spindle poles. In addition, cells were multinucleated with abnormal nuclear morphology. After 48 hours of treatment, the number of cells arrested at G2/M phase was reduced with the increase of nuclear fragments and the macro-sized nuclei. This suggested that initially low dose of quercetin (10 μg/ml) induced cell cycle arrest at G2/M phase but as these cells were not able to pass through mitosis, they went to apoptotic cell death. Notably, quercetin treatment at high dose (30 μg/ml) did not induce abnormal mitotic chromatin morphology, multinucleated cells or apoptotic fragments. Instead, nuclei became bigger after 24 hours of treatment and remained even after 48 hours (Fig. 8).

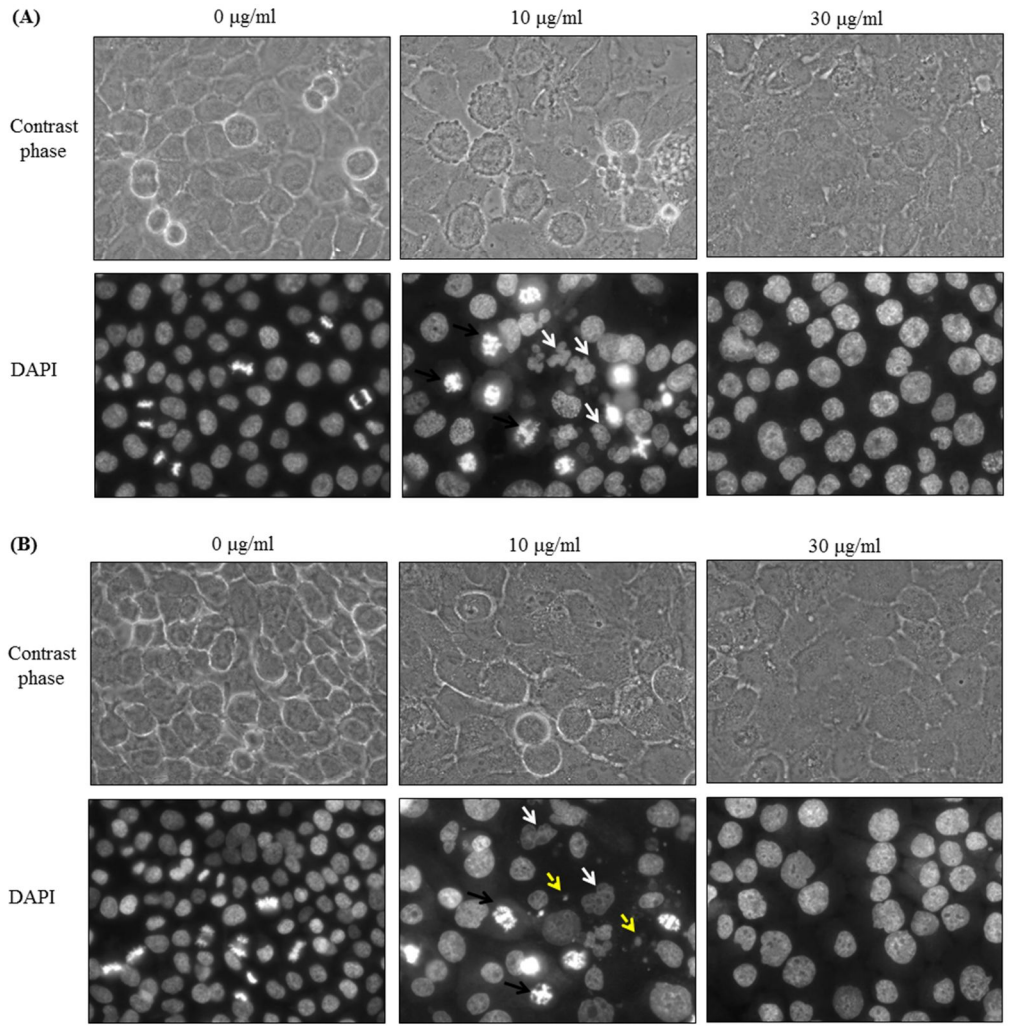

Figure 8. The effects of quercetin on nucleus morphology

YD15 cells were treated with indicated concentration of quercetin for 24 hours (A) or 48 hours (B). Cells were stained with DAPI and cell images were collected by fluorescent microscopy. The images were captured at a magnification of x 400. The black arrows, the nucleus of cells at G2/M phase; the white arrows, multinucleated cells (abnormal nuclei); the yellow arrows, the bodies of apoptotic cells.

3.8. Effects of quercetin on Bcl-2 family protein expression

The intrinsic apoptotic pathway is dominated by the Bcl-2 family of proteins, which governs the release of cytochrome c from the mitochondria [170, 171]. Some of these proteins such as Bcl-2 and Bcl-XL are anti-apoptotic, while others such as Bax, Bad and Bid are pro-apoptotic. The sensitivity of cells to apoptotic stimuli depends on the balance of pro- and anti-apoptotic Bcl-2 family proteins. Thus, we further examined the expression of Bcl-2 family proteins (Bax, Bcl-2 and Bid) by western blotting. The expression of Bcl-2 family proteins was not affected by quercetin (Fig. 9). Further experiment need to be done to examined the expression of Bax, Bcl2 in cytosol and mitochondria to find out whether the apoptosis inducing by quercetin is involve in mitochondria pathway or not.

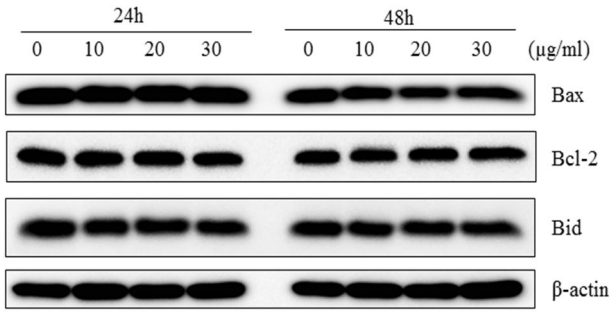

Figure 9. Effects of quercetin on Bcl-2 family protein expression.

YD15 cells were treated with indicated concentration of Quercetin for 24 hours or 48 hours. Total 20 μg protein was loaded on 15 % SDS-PAGE for western blotting. β - actin was used as an internal control. Quercetin did not affect the expression of Bcl2 family proteins.

3.9. Quercetin-induced aberrant mitosis and multinucleation

Previous report indicated that quercetin suppressed the proliferation of endothelial cells chronically through mitotic microtubule polymerization perturbation and led to early M phase arrest [155]. Thus, we hypothesized that quercetin might also induce the aberrant mitotic spindle formation in YD15 cells, causing the cell cycle arrest at G2/M phase and missegregation of chromosomes as observed by cell cycle analysis and DAPI staining of DNA after treating with low dose of quercetin. To test our hypothesis, YD-15 cells were treated with an indicated concentration of quercetin for 24 hours and immunofluorescence staining was performed. Mitotic spindles were visualized by α-tubulin staining (green), centrosomes by anti-pericentrin antibody (red) and nuclei by DAPI staining (blue) (Fig. 10). Aberrant multipolar mitotic spindle cells were observed at 10 μg/ml of quercetin treatment (but not at 30 μg/ml) for 24 hours (Fig. 10A). This was accompanied by the increased number of cells with more than two centrosomes for each cell at M phase of cell cycle (Fig. 10A). Also multinucleated cells with more than one nucleus for each cell were observed (Fig. 10.B). The number of mitotic spindle was equal with the number of centrosome suggesting that the centrosome amplification/de-clustering is the cause of multipolar mitotic spindle formation. Centrosomes in some cells were de-clustered into three, four or even more at concentration 10 μg/ml (Fig. 10A). By contrast, at 30 μg/ml of quercetin, cells with multipolar mitotic spindles or cells at M phase with condensed and visualized chromosomes were not observed. Instead, a large centrosome without mitotic spindle formation was observed (Fig. 10A), suggesting that high dose of quercetin affects differently on YD15 cells compare to low dose of quercetin treatment.

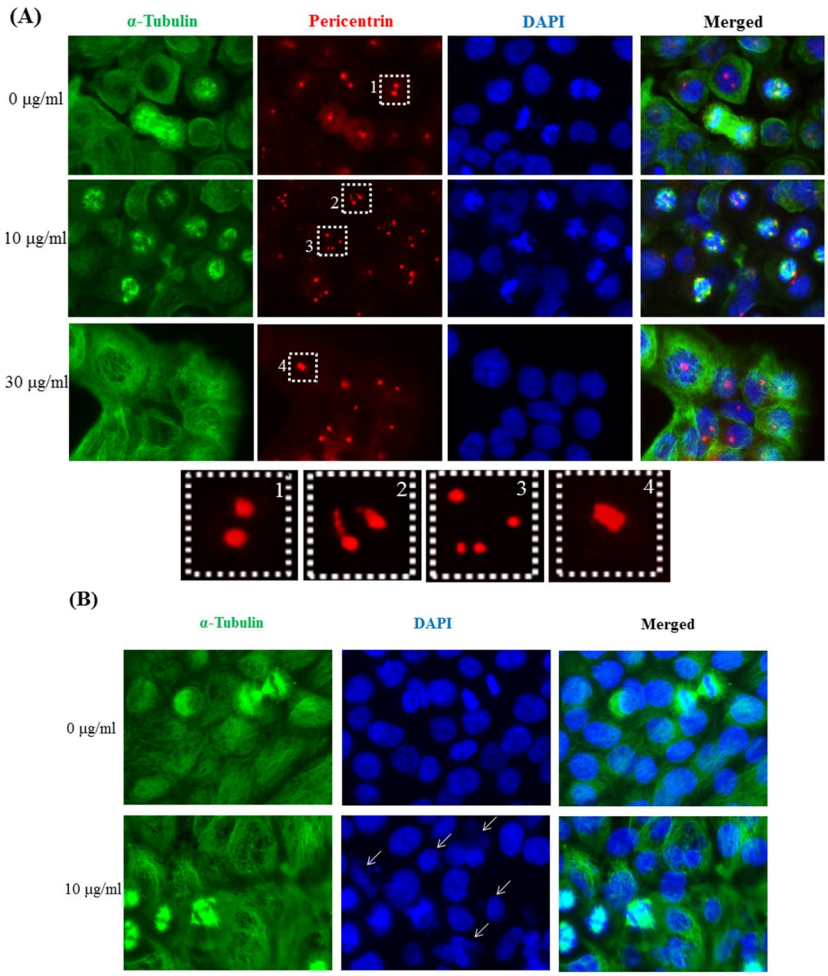

Figure 10. Quercetin induced aberrant mitotic spindle formation and multinucleated cells

YD-15 cells were treated with indicated concentration of quercetin for 24 hours. The cells then were fixed for immunofluorescence staining. Mitotic spindles were visualized by α-tubulin staining (green), centrosomes by staining cells with anti-pericentrin antibody (red) and nuclei by DAPI staining (blue). Origininal images were captured at 600x magnification by Nikon fluorescence microscopy. The white arrows indicate multiple micronuclei cells.

3.10. The aberrant mitotic spindle formation and chromosome missegregation was recovered by removing quercetin.

To clarify that the aberrant mitotic spindle formation and chromosome missegregation are involved in centrosome declustering induced by quercetin, we performed immunofluorescence experiment to see what would happen if quercetin is removed after inducing aberrant mitosis. YD15 cells were treated with indicated concentrations of quercetin for 24 hours, then replaced with fresh medium without quercetin and continue to incubated for 2 or 24 hours. YD15 cells treated with indicated concentrations of quercetin for 26 or 48 hours were used as control. As shown in Fig. 11, after removing quercetin for 2 hours, the multipolar mitotic spindles with amplified centrosomes (Fig. 11a) were disappeared with the newly formed pseudo-bipolar mitotic spindle with two clustered centrosomes (Fig. 11b). This suggestes that quercetin directly affected and regulated the formation of bipolar mitotic spindle, induced the declustering of centrosome to form multipolar mitosis which lead to mitotic catastrophe and eventually cell death. YD15 cells were completely recovered if cells with 10 μg/ml of quercetin were incubated for 24 hours after removing quercetin. This indicated that removing quercetin and incubating for 24 hours, the multinucleated cells which were induced by multipolar mitosis were disappeared and become normal cells (Figure 11 c, d). Interestedly, cells with only one large centrosome without showing mitotic spindle at 30 μg/ml quercetin were also decreased. Instead, cells with two centrosomes which are at separating stage were observed. Cells washed for 24 hours were not much different in nucleus morphology compare with cells washed for 2 hours. Two centrosomes were separated further after 24 hours of washing (Fig. 10 b, d) and the cells at metaphase also occurred indicated that certain amount cells can grow normally after removing quercetin (data not showed). Notably, although two centrosomes of cells were separated, the condensed chromatins were not observed. This suggestes that quercetin at high dose

(30 μg/ml) might damage nucleus more severely than at low dose (10 μg/ml) and certain cells were not able to undergo normal cell cycle progression. Thus, quercetin at high dose (30 μg/ml) might inhibit the separation of centrosome instead of induced centrosome declustering.

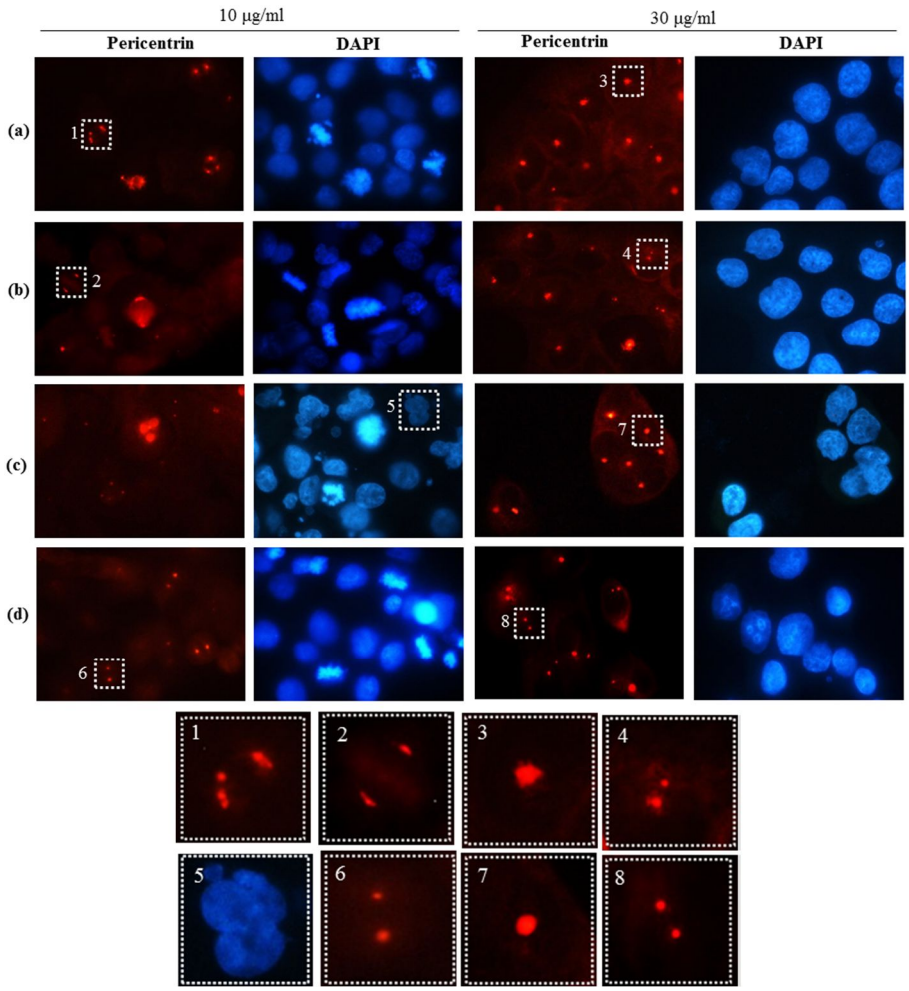

Figure 11. The aberrant mitotic spindle formation and chromosome mis-segregation with centrosome amplification was recovered after removing quercetin.

YD15 cells were treated with indicated concentration of Quercetin for 24 hours, then, keep or changed with fresh medium without quercetin and continue to incubated for 2 (b), or 24 hours (d). Cells treated with indicated concentrations of quercetin for 26 hours (a) or 48 hours (c) were used as control. The cells were fixed for immunofluorescence staining. Centrosomes were visualized by staining cells with anti-pericentrin antibody (red) and nuclei by DAPI staining (blue). Originial images were captured at 600 x magnification by Nikon fluorescence microscopy. 1, centrosome amplification induced by 10 μg/ml quercetin; 2, pseudo-bipolar mitotic spindle with two centrosome formation after removing quercetin and continue to incubate for 2 hours; 3 &7, single large centrosomes induced by 30 μg/ml quercetin; 4 & 8 centrosome segregation after removing quercetin and continue to incubate for 2 or 24 hours; 5, multiple micronuclei induced by 10 μg/ml quercetin, 6, normal bipolar mitotic spindle with two centrosome formation after removing quercetin and continue to incubate for 24 hours.

3.11. Effects of quercetin on senescence and autophagy marker proteins.

Lamin B1, a major structural component of nucleus, plays important roles in regulation of nuclear functions and has been used as a marker protein of cellular senescence in culture and in vivo [63, 172]. Microtubule-associated protein 1A/1B-light chain (LC3) proteins (LC3-A, -B and –C) are structural proteins of autophagosomal membranes, which is widely used as biomarkers of autophagy. Effects of quercetin on senescence and autophagy marker proteins, lamin B1 and LC3B , were investigated by western blotting (Fig. 12A). The loss of lamin B1 was also detected by immunofluorescence staining (Fig. 12B). Our data showed that quercetin decreased the expression of lamin B1 in dose- and time-dependent manner but not on LC3B (Fig. 12A). However, lamin B1 cleavage products (45 kDa in size) was not detected as shown in Fig. 12A, suggesting that reduced expression of lamin B1 was not due to caspase-mediated degradation. The loss of lamin B1 was also confirmed by immunofluorescence staining as shown in Fig. 12B. Cells in senescence cell show increase in nuclear volume (71) and compare with control cells as showed in Fig. 12B. Since quercetin treatment did not affect the expression of autophagy marker, LC3B, it appears that quercetin does not induce autophagy on YD15 cells.

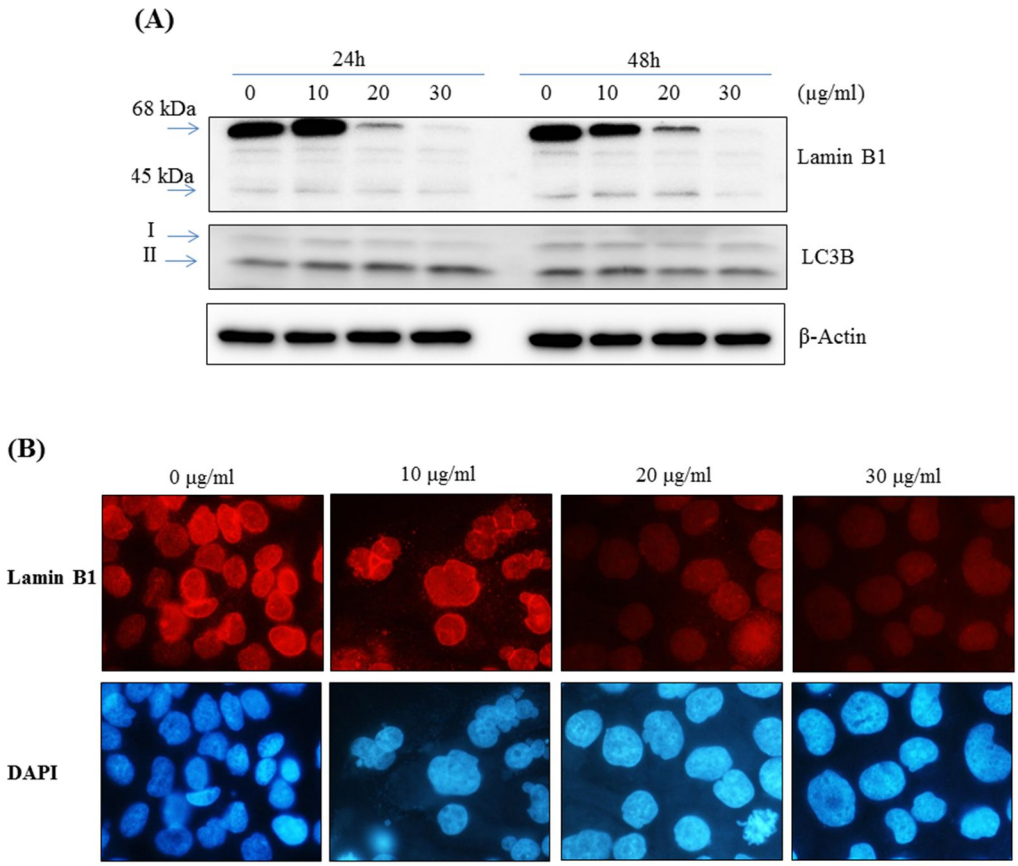

Figure 12. Effects of quercetin on senescence and autophagy marker proteins.

A, YD-15 cells were treated with indicated concentrations of quercetin for 24 hours or 48 hours and total cell lysate (20μg) were loaded on 15% SDS–PAGE and detected by western blotting. Quercetin strongly suppressed the expression of senescence marker protein, lamin B1, in a dose- and time-dependent manner but did not affect autophagy marker protein, LC3B. B, the loss of Lamin B1 was confirmed by immunofluorescence. YD15 cells were treated with indicated concentration of quercetin for 48 hours. Cells were fixed and stained for Lamin B1 by using anti-lamin B1 antibody (red). Nucleus of the same cell was visualized by DAPI staining (blue). Images were captured at 600 x magnification by a Nikon fluorescence microscopy.

3.12. Quercetin-induced senescence on YD15 cells

Senescence cells have β-galactosidase activity, which is histochemically detectable at pH 6.0 and serve as a biomarker to identify senescence cells in vitro and in vivo [58]. In order to confirm whether quercetin induces senescence on YD15 cells or not, we performed senescence-associated β-galactosidase (SA-β-gal) assay. YD15 cells were seeded in 24-well plate and treated with indicated concentration of quercetin for 48 hours. Senescence cells were detected by SA-β-Gal activity assay. As shown in Fig. 13, quercetin significantly induced senescence cells in a dose-dependent manner.

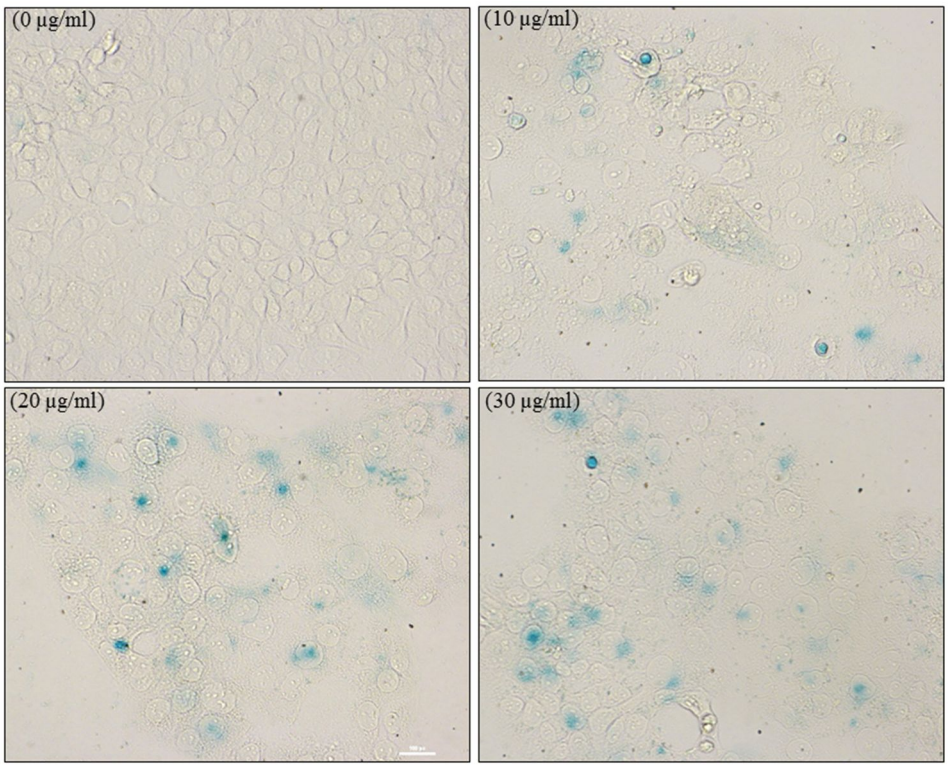

Figure 13. Quercetin-induced senescence.

YD15 cells were seeded in 24-well plate, 1 x 10^5 cell/ml for 24 hours, then treated with indicated concentrations of quercetin for 48 hours. Senescence cells were detected by the senescence-associated β-galactosidase (SA-β-Gal) activity assay. Treatment with quercetin significantly induced the SA-β-Gal activity.

3.13. Quercetin decreased the expression of lamin B1 mRNA

In senescence cells, the loss of Lamin B1 at mRNA level is due to the decrease in mRNA stability not by caspase-mediated degradation observed in apoptosis [63]. To further confirm that quercetin induces senescence on YD15 cells, mRNA of lamin B1 in quercetin-treated cells were detected by RT-PCR. YD15 cells were treated with indicated concentration of quercetin for 24 hours or 48 hours and mRNA was isolated and quantitated. The same amount of total mRNA was used to synthesize cDNA by using reverse transcriptase and GADPH was used as an internal control. Quercetin treatment extremely suppressed the transcription of the lamin B1 gene (LMB1) in YD15 cells in a dose- and time-dependent manner (Fig. 14). The results are consistent with the result of western blot analysis (Fig. 12A). Thus, the suppression of lamin B1 expression at both mRNA and protein support that quercetin induced senescence on YD15 cells.

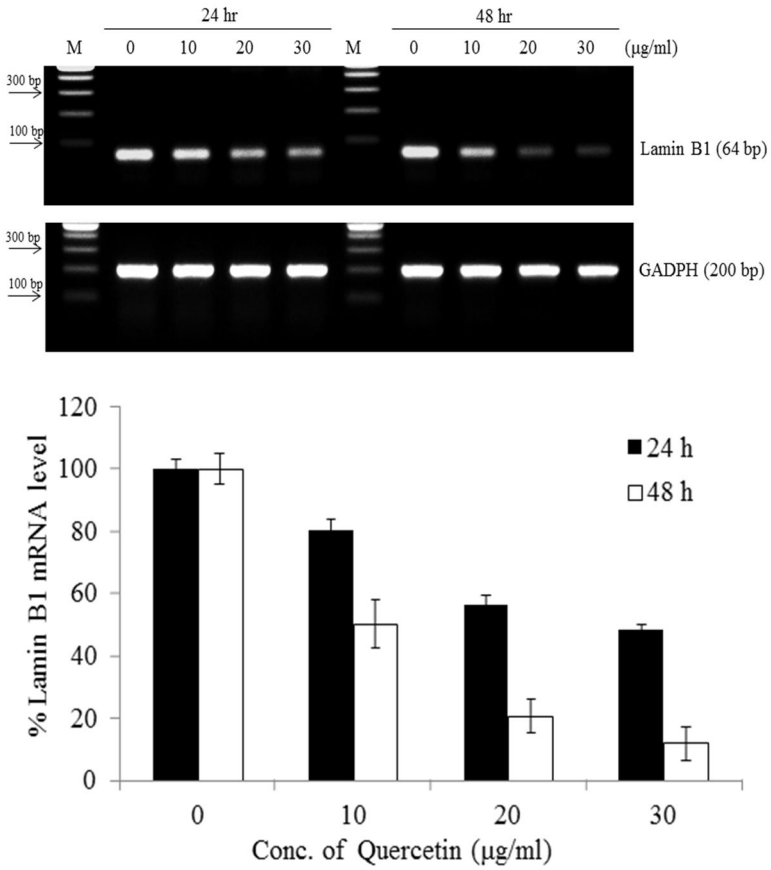

Figure 14. Quercetin induced the decrease of Lamin B1 mRNA.

YD15 cells were treated with indicated concentration of quercetin for 24 or 48 hours. After treatment, mRNA were isolated and quantification. RT-PCR was performed to observe Lamin B1 mRNA expression after quercetin treatment on YD15 cells. GADPH was used as an internal control. mRNA level of lamin B1 was significantly reduced after quercetin-treatment in a dose- and time-dependent manner.

3.14. Effect of quercetin on pAKT and MAPKs

Phosphorylation of Akt is associated with the protection of cells from apoptosis [173]. Mitogen-activated protein kinase (MAPK) families (ERK, JNK, p38) play important roles in various cellular signals such as cell proliferation, signal transduction and apoptosis. The signaling pathway of ERK, JNK and p38 MAPK is activated under certain situations of cellular stress, and implicated in cell death or survival. To find out whether Akt and MAPKs phosphorylations are related to quercetin-induced cell proliferation inhibition, phosphorylation of these proteins were detected by western blotting. As showed in Fig. 15, quercetin decreased AKT phosphorylation but increased ERK phosphorylation. JNK phosphorylation was not much affected, only slightly increased. Quercetin also did not affect p38 MAPK phosphorylation. Thus, reduction of AKT phosphorylation might be involved in the inhibition of cell proliferation by quercetin.

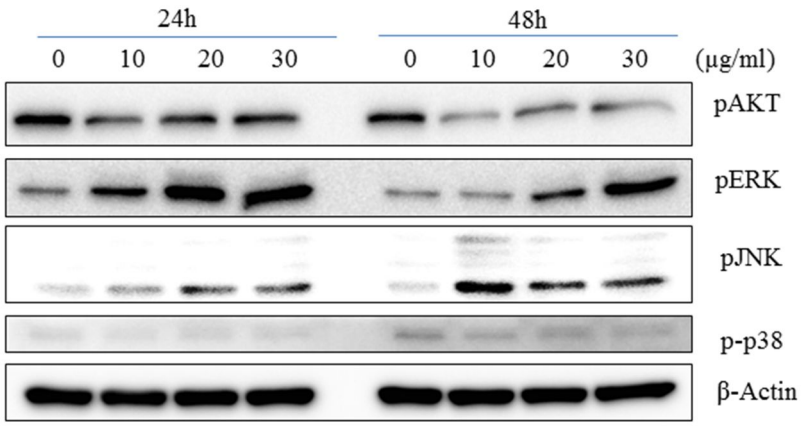

Figure 15. Effect of quercetin on AKT and MAPKs phosphorylation.

YD15 cells were treated with indicated concentrations of quercetin for 24 or 48 hours. Total cell lysate (20μg) were loaded on 15% SDS–PAGE and detected by western blotting.

3.15. Effects of AKT and MAPKs inhibitors on YD15 cell proliferation under the quercetin

To understand the role of AKT and MAPKs phosphorylation in quercetin-induced YD15 cell proliferation inhibition, we further examine the effect of AKT and MAPKs inhibitors on YD15 cell growth (Fig. 16A) and compare the difference between inhibitors co-treated with quercetin and quercetin treated alone (Fig. 16B) by using MTT assay. Lapatinib (a tyrosine kinase activity inhibitor) which inhibits the phosphorylation of ERK and AKT, strongly suppressed the growth of YD15 cells. PD184161 (selective inhibitor of MEK1/2 –ERK1/2 growth signal pathway) also strongly decreased the growth of YD15 cells. LY294002 (an inhibitor of PI3K which is the upstream signal of both AKT and ERK pathway) and SP600125 (an inhibitor of JNK) inhibited the growth of YD15 cells while MK2206 (highly selective inhibitor of AKT activity) and SB203580 (an inhibitor of p38 MAPK) did not affect as shown in Fig. 16A. When cotreated inhibitors with quercetin (10, 30 μg/ml) for different time (24 or 48 hours), the cell viability of YD15 cells was sharp decreased compare with quercetin-treated alone indicated that AKT and MAPKs phosphorylation was involved in cell survival (Fig. 16B). Quercetin inhibited YD15 cell proliferation was a part involved in the decrease of AKT phosphorylation.

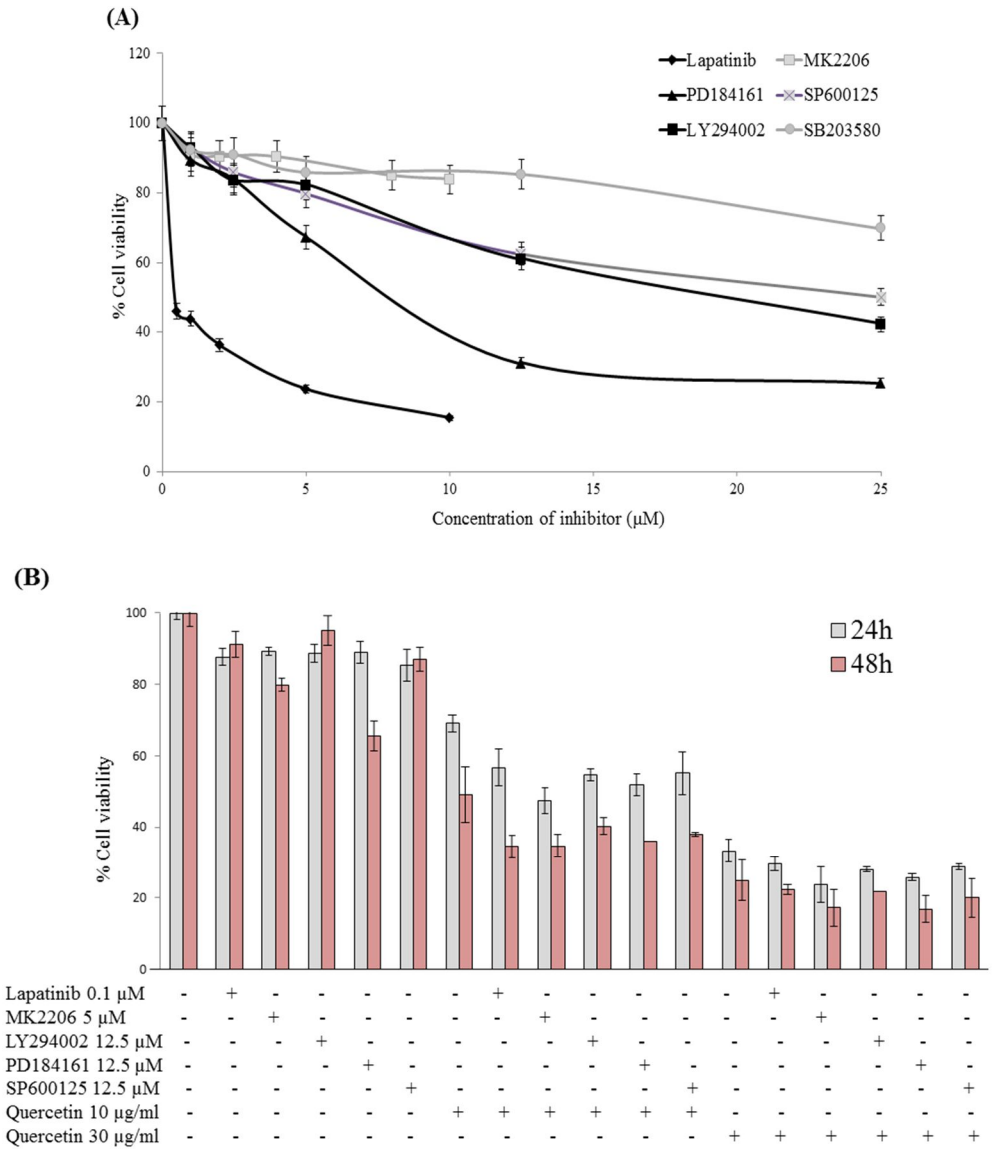

Figure 16. Effects of AKT and MAPKs inhibitors on YD15 cell proliferation under the quercetin

YD15 cells were seeded on 96-well plates (10^5 cells/ml) for 24 hours and then treated with indicated concentration of different inhibitors for 24 hours. The percent cell viability was determined by an MTT assay. Each value is the average of triplicate.

3.16. Effects of quercetin on retinoblastoma and p-Chk2 protein.

Retinoblastoma protein (Rb) serves as a tumor suppressor at a cell cycle checkpoint between the G1 and S phase. The active hypophosphorylated form of Rb (Rb) is primarily found in resting or fully differentiated cells while the inactive, hyperphosphorylated form of Rb (pRb) is primarily found in proliferating cells. The inactivation of Rb through phosphorylation is necessary for cell proliferation. In Fig. 17, quercetin induced an increase in the hypophosphorylation of Rb (Rb) protein level. It has been also reported that activation of Rb pathway was sufficient to induced lamin B1 loss which is a robust hallmark of senescence and serves as an easily detectable biomarker of the senescent state both in culture and in vivo [63]. This data suggested that quercetin-induced the loss of lamin B1 might cause by active retinoblastoma pathway.

Checkpoint kinase 2 (Chk2) is a key component of DNA damage response. Following genotoxic stress, chk2 is activated and triggers the appropriate cellular response such as cell cycle checkpoint activation, induction of apoptosis or senescence. Previous report showed that Chk2 mediates p21 induction through a p53-independent mechanism [174] and quercetin generates mild DNA damage and activates (Chk2 kinase) which plays a crucial role in p21 induction [175]. Our previous data showed that quercetin induced the expression of p21 in p53-independent pathway. So, to examine whether quercetin induced DNA damage and induction of p21 by quercetin was involve in the activation of Chk2, we performed western blotting to detect the expression of pChk2. Our data showed that quercetin induced the phosphorylation of chk2 in dose- and –time dependent manner (Fig. 17). This data suggested that quercetin induced DNA damage on YD15 cells and might contribute to the expression of p21.

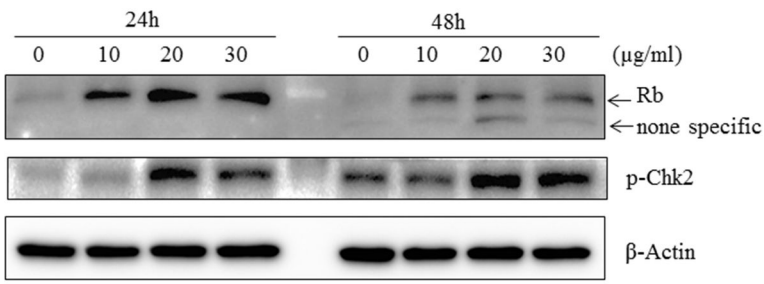

Figure 17. Effects of quercetin on retinoblastoma and pChk2 protein.

YD15 cells were treated with indicated concentrations of quercetin for 24 or 48 hours. Total cell lysate (20μg) were separated on 15% SDS–PAGE and detected by western blotting. Quercetin significantly increased hypophosphorylation of retinoblastoma protein and phosphorylation of Chk2.

IV. DISCUSSION AND CONCLUSION

Quercetin, a naturally occurring flavonol compound, is easily accessable from dietary sources of fruits and vegetables such as apples, blueberries, onions, black tea and red wine [176]. Quercetin is well-known for its anti-oxidant, anti-inflammatory, cardioprotective properties [128, 129]. Understanding quercetin-induced cytotoxicity on cancer cells may also useful for the application of quercetin in cancer prevention. In previous studies, quercetin showed chemopreventive effects on various cancer cell lines such as breast, colon, kidney, ovarian, liver and lung well as leukemia and other malignant tumors [154, 177-184]. The mechanism was mainly through either cell cycle arrest by upregulating cell cycle inhibitors or apoptosis by inhibiting cell signaling. In this study, we investigated the chemopreventive effects of quercetin on YD15 tongue carcinoma cells. Quercetin inhibited the proliferation of YD15 cells by mitotic catastrophe and senescence induction, leading to cell cycle arrest, apoptosis or multinucleated nuclei formation. Quercetin triggered the cell cycle arrest at G2/M phase and/or S phase (Fig. 4) and induced programed cells death as well as senescence, depending on the dose and time of treatment. The evidences of apoptosis was confirmed by nuclear morphological change (Fig. 8), the increased cell population at sub-G1 (FACS analysis) (Fig. 5) and the detection of activated caspase 7 and cleaved PARP (Fig. 7).

In FACS analysis, quercetin induced cell cycle arrest either S or G2/M phase on YD15 cells, shifting dose-dependently from cell cycle arrest to apoptosis and/or senescence. Namely, YD15 cells treated with 10 μg/ml of quercetin for 24 hours induced cell cycle arrest at G2/M phase. However, under 24 hours treatment with 20 μg/ml of quercetin, cell cycle arrest was shifted into S phase and partially M phase. Furthermore, under 24 hour treatment with 30 μg/ml quercetin, cells underwent morphological change, not showing apoptotic body or floating cells and delaying cell cycle progression.

The proliferation of cell is regulated by cell cycle checkpoints by preventing the initiation of downstream cell cycle event until the upstream event is completed. Any mistakes during this process must be corrected [185, 186]. These checkpoints ensure the accuracy of the chromosome replication and segregation processes. The three most important checkpoints are the G1 checkpoint that regulates G1/S transition, the G2 checkpoint that regulates the G2/M transition, and the spindle checkpoint that regulates the transition from metaphase to anaphase of M phase. In response to DNA damage or replication errors, cell cycle checkpoint is activated to repair the damaged DNA and cell cycle arrest may occur. If the damage is irreparable, cells undergo programmed cell death or apoptosis. In mammalian cells, proliferation is tightly regulated at each phase of cell cycle by activation and deactivation of a series of cell cycle proteins such as cyclins, cyclin-dependent kinases (Cdks), and Cdk inhihitors (CdkIs). Cdks are relatively small proteins and binds to a cyclin. Cdk without cyclin has little kinase activity but when it forms cyclin-Cdk complex it becomes active kinase. In mammalian cells, Cdc2 (Cdk1) with its partner cyclin A or B1 can drive the cell cycle [187]. Previous studies showed that quercetin directly interacts with DNA to stabilize its secondary structure, but prolonged exposure to DNA causes DNA damage [188]. The tumor suppressor gene p53 is a key factor in balancing between cell survival and cell death via the regulation of G1 and G2/M portions of the cell cycle [189]. However, in YD15 cells, p53 is mutated at codon 258 with GAA -> GCA (Glu -> Ala) [190]. Quercetin treatment on YD15 cells significantly decreased the expression of cyclin B1 and cdk1, essential components of G2/M cell cycle progression, in a dose-dependent manner (Fig. 6A). Interestingly, quercetin increased the expression of p21 without inducing the expression of p53 (Fig. 5B). This suggests that quercetin induced cell cycle arrest and p21 expression p53-independently. Chk2 is one of key signal transducers in the DNA damage checkpoint signaling. Previous report indicated that quercetin generates mild DNA damage and activates Chk2 kinase which plays a crucial role in p21 induction

[175]. In our result quercetin induced the phosphorylation of chk2 at high dose (Fig. 17) suggested that quercetin induced DNA damage on YD15 cells might not contribute to the expression of p21. The main function of cyclin E-cdk2 complex, together with cyclin D1-cdk4/6, is to trigger G1/S phase transition and pass through G1 checkpoint while cyclin E or cyclin A binding to cdk2 trigger S phase for DNA synthesis. To progress from G2 to M phase, the Cdk1/cyclin B complex must be activated to induce mitosis by phosphorylating and activating enzymes regulating chromatin condensation, breaking down nuclear membrane, reorganizing mitosis-specific microtubule [120]. Quercetin dramatically inhibited the expression of cyclin D1, cdk4, cyclin B1 at high dose of treatment (≥ 20 μg/ml) and less affected at low dose ((10 μg/ml) (Fig. 6A). The ratio of phosphorylated cdk2 / cdk2 was increased at high dose (≥ 20 μg/ml) of quercetin compared to the low dose (10 μg/ml). Decreased cdk2, together with FACS results of accumulated cell population at G2/M phase, may indicate that cells at low dose condition already passed through S phase and G2 phase. Treatment with high dose of quercetin (20 or 30 μg/ml) delayed cell cycle progression because cyclin D1, cdk4 and cyclin B1 expression were inhibited. However cells still gradually passed through the cell cycle phase to S and/or G2 phase because the activities of cdk2 and cyclin E / A was still maintained. Retinoblastoma protein (Rb) plays a crucial role in the negative control of the cell cycle progression which is responsible for G1 checkpoint by blocking S phase entry. The function of Rb protein is regulated by phosphorylation. Rb inhibits actively cell cycle progression when it is dephosphorylated and becomes inactive when it is phosphorylated (hyper-phosphorylated (pRb)), allowing cell cycle progression. It has been reported that activation of Rb pathway was sufficient to induced lamin B1 loss. Lamin B1 is important for maintaining chromatin condensation [79], transcription [78] and organizing replicating chromatin during late S phase [77]. Reducing lamin B1 expression delayed cell cycle and accumulated cells in early S phase [80]. Although high dose of quercetin eventually induced the accumulation of cells at G2/M phase (Fig.

5), but condensed chromatins in cells were not observed by DAPI staining (Fig. 8) suggesting that cells are still in G2 phase. Thus, it appears that quercetin delayed the cell cycle progression without inducing cell death at high dose condition. Namely, cells were able to pass through G1 checkpoint to enter cell cycle and reached to G2 (high dose) or M phase (low dose) and/or some cells at subG1. Cells at high dose of quercetin were not able to enter into M phase, probably due to the loss of lamin B1 and cyclin B1.

Senescence is considered to be an early line of defense against tumor development by preventing proliferation of cells with damaged DNA [51]. Senescent cells develop unique cellular morphology such as increased size of cell and nuclear, flattened cytoplasm and increased senescence-associated beta-galactosidase (SA-βgal) activity. Molecular mechanisms of senescence are involved in p53 and retinoblastoma (Rb) pathways and telomere shortening [60-62]. Activation of either the p53 or Rb tumor suppressor pathway was sufficient to induce lamin B1 loss. Lamin B1 loss is a robust hallmark of senescence and serves as a biomarker of the senescence in culture and in vivo as well [63]. In our result quercetin induced Rb expression (Fig. 17), suppressed lamin B1 at both mRNA and protein level (Fig. 12, 14), induced SA-βgal activity (Fig. 13) and increased nuclear size (Fig. 8). All these results support that quercetin-induced proliferation inhibition is associated with senescence.

DNA damage kills cells through caspase-dependent apoptosis or mitotic catastrophe [45, 81, 82]. The G2 checkpoint is crucial for preventing mitotic cell death and when it is aborted, mitotic catastrophe is potentiated by multipolar mitotic spindles leading to aberrant mitosis [82, 85, 86, 191, 192]. Prolonged spindle assembly checkpoint (SAC) activation also leads to mitotic arrest and often to mitotic catastrophe [96]. During prolonged mitotic arrest, cells undergo one of two consequences either dying in mitosis or moving to the G1 phase without division. The features of mitotic catastrophe can be identified by morphologic characteristics (enlarged and multinucleated

cells) and the presence of mitotic defects (incomplete nuclear condensation, chromosome alignment defects, unequal DNA separation or mitosis in the presence of DNA damage) [193]. In our data, 10 μg/ml of quercetin induced centrosome supernumerary and multipolar mitotic spindle (Fig. 10), missegregation of chromatins and multinucleated cells (Fig. 8), indicating mitotic catastrophe. Multipolar mitotic spindles induced by quercetin were recovered into pseudo-bipolar spindles by re-clustering centrosomes when quercetin was removed, confirming that quercetin disrupted the centrosome clustering process to induce multipolarity of mitotic spindle, leading to mitotic catastrophe. Multipolarity is accompanied by improper kinetochore attachment or insufficient tension, and thereby activating the spindle assembly checkpoint (SAC) and causing metaphase arrest. Centrosome clustering relies on microtubule-based motors and microtubule-bundling proteins that organize spindle poles in both normal and tumor cells with supernumerary centrosomes [116, 194, 195]. Spindle tension is necessary for clustering of supernumerary centrosomes into a bipolar mitotic spindle array [194, 195]. Disturbance of spindle microtubule components reduce the spindle tension, thereby inhibiting centrosome clustering [194]. Previous report showed that quercetin perturbed microtubule polymerization in bovine aortic endothelial cells and prostate cancer cells [155, 156] by perturbing microtubule functions through tubulin binding [157].

Apoptosis is a critical element in defensing against unrestrained cellular proliferation by inducing tumor cell death [14-16]. Activated caspase-3 is a death protease, catalyzing the specific cleavage of many key cellular proteins such as caspase 7, PARP [23]. Bcl-2 family members such as Bax, Bad and Bcl2 are key regulators of intrinsic apoptotic pathway, controlling mitochondrial outer membrane permeabilization (MOMP) [196, 197]. The Bax and Bcl-2 expression which controls the release of cytochrome c from mitochondria to the cytoplasm is the key factor for the

induction of apoptosis through the activation of caspases. It has been reported that quercetin-induced apoptosis is mitochondrial-mediated cell-death pathway [198]. In our study, although quercetin was cytotoxic on YD15 cells (Fig. 2, 3) by inducing sub-G1 cell population (Fig. 5) as well as by activating caspase 7 and cleaving PARP (Fig. 7), quercetin did not affect caspase 9 (Fig. 7) and Bcl-2 family proteins (Fig. 9). In other words, apoptosis induced by quercetin on YD15 cells was not through the mitochondria pathway. Considering that mitotic catastrophe drives DNA-damaged cells to irreversible fate such as apoptosis, necrosis, or senescence [84], quercetin-induced apoptosis (at 10 μg/ml after 24 hours of treatment) might be occurred by mitotic catastrophe. However, it remains to be answered how mitotic catastrophe was signaled to the molecular machineries of apoptosis, necrosis or senescence, and what factors determine the choice among these three onco-suppressive mechanisms [84]. The induction of mitotic catastrophe could be an attractive target for the development of novel anticancer [199]. First, cancer cells are particularly sensitive to the induction of mitotic catastrophe due to their common tetraploidy / aneuploidy, which leads cancer cells more susceptible to mitotic aberrations [199]. Second, mitotic catastrophe is activated at significantly lower doses, unlike cell death induced by multiple chemotherapeutic agents (at relatively high doses) [200]. Third, inhibition of mitotic catastrophe is an important mechanism of chemo-resistance in various cancers [201, 202].

Taken together, our results clearly support that quercetin suppressed the cell proliferation of YD15 cells. Cell proliferation suppression was involved in the delay of cell cycle progression, mitotic catastrophe and senescence (Fig. 18). The delay of cell cycle progression was due to the decreased lamin B1 and cell cycle proteins such as cyclin D1, cdk4, cyclin B1. Quercetin induced centrosome declustering and multipolar mitotic spindles leading to mitotic catastrophe, and mitotic catastrophe caused multinucleated cells and apoptotic cell death. The induction of senescence by quercetin was

further confirmed by senescence-associated beta galactosidase staining and decreased expression of lamin B1 mRNA and protein.

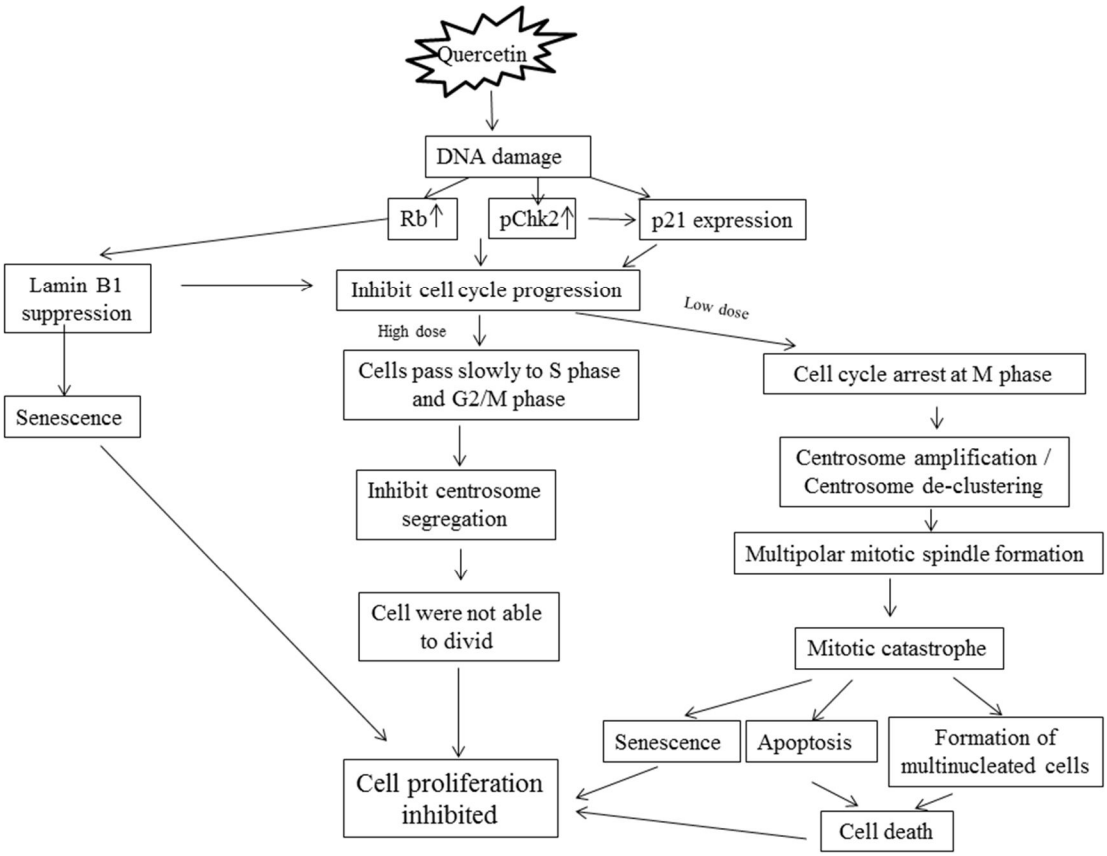

Figure 18. Quercetin-induced inhibition of YD15 cell proliferation

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