บทบาทของหน่วยย่อย PPP2R2B ของโปรตีนฟอสฟาเตสสองเอต่อการเคลื่อนตัวเข้าสู่นิวเคลียสของโปรตีน ATM ในเซลล์มะเร็งศีรษะและคอ

นางสาว โชติกา สุญาณเศรษฐกร

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THE ROLE OF PROTEIN PHOSPHATASE 2A SUBUNIT PPP2R2B IN ATM NUCLEAR LOCALYZATION IN HEAD AND NECK SQUAMOUS CELLS CARCINOMA

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โชติกา สุญาณเศรษฐกร : บทบาทของหน่วยย่อย PPP2R2B ของโปรตีนฟอสฟาเตสสองเอต่อการเคลื่อนตัวเข้าสู่นิวเคลียสของโปรตีน ATM ในเซลล์มะเร็งศีรษะและคอ (THE ROLE OF PROTEIN PHOSPHATASE 2A SUBUNIT PPP2R2B IN ATM NUCLEAR LOCALYZATION IN HEAD AND NECK SQUAMOUS CELLS CARCINOMA.) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : ศ. นพ. อภิวัฒน์ มุทิรางกูร, 90 หน้า.

จากงานวิจัยที่มีมาก่อนหน้านี้ ทำให้ทราบว่าโปรตีนฟอสฟาเตสสองเอมีความสำคัญต่อการดึงหมู่ฟอสเฟทออกจากโปรตีน ATM นอกจากนี้แล้ว การยับยั้งการทำงานของโปรตีนฟอสฟาเตสสองเอด้วยสารยับยั้งจำเพาะได้แก่ okadaic acid ยังทำให้พบโปรตีน ATM ทั้งหมดได้เฉพาะใน cytoplasm ของเซลล์เท่านั้น เป็นที่น่าสนใจว่าพบการแสดงออกของยีน *PPP2R2B* ลดลงในเซลล์มะเร็งศีรษะและคอ ดังนั้นแล้วโปรตีน PPP2R2B อาจจะมีบทบาทสำคัญต่อการเคลื่อนตัวเข้าสู่นิวเคลียสของโปรตีน ATM ในการทดลองครั้งนี้ ผู้ทำการวิจัยได้แสดงให้เห็นว่าการแสดงออกของยีน *PPP2R2B* มีความสอดคล้องกับการเติมหมู่ฟอสเฟทให้แก่โปรตีน ATM และโปรตีน H2AX ในเซลล์มะเร็งศีรษะและคอ เพื่อยืนยันการทดลองดังกล่าว ผู้ทำการวิจัยได้ใส่ siRNA เพื่อทำลาย mRNA ของ PPP2R2B เป็นที่น่าสนใจว่าพบโปรตีน ATM เฉพาะในส่วนที่เป็น cytoplasm ของเซลล์ที่ทำการใส่ siRNA เท่านั้น นอกจากนี้แล้วอัตราการรอดตายของเซลล์ดังกล่าวภายหลังการฉายแสงยังต่ำกว่าในเซลล์ที่มีการแสดงออกของยีน *PPP2R2B* จากการค้นพบทั้งหมดนี้ สามารถชี้ให้เห็นบทบาทของโปรตีน PPP2R2B ต่อการเคลื่อนตัวเข้าสู่นิวเคลียสของโปรตีน ATM

สาขาวิชา ชีวเวชศาสตร์ ลายมือชื่อนิสิต.....

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CHOTIKA SUYARNSESTAKORN : THE ROLE OF PROTEIN PHOSPHATASE 2A SUBUNIT PPP2R2B IN ATM NUCLEAR LOCALYZATION IN HEAD AND NECK SQUAMOUS CELLS CARCINOMA. THESIS ADVISOR: PROF. APIWAT MUTIRANGURA, M.D., Ph.D., 90 pp.

Protein phosphatase 2A (PP2A) has been implicated in radiation-induced activation of cellular responses. Recently, PP2A was found to prevent ataxia telangiectasia mutated (ATM) autophosphorylation and the inhibition of PP2A with okadaic acid (OA), a specific inhibitor to OA, predominantly localyzed ATM within cytoplasm. We found that HNSCC cell lines that had lower PPP2R2B mRNA expression exhibited low basal levels of ATMS1981p and γ-H2AX, an ATM downstream histone phosphorylation target. Interestingly, expression of *PPP2R2B* was found to be down-regulated in head and neck squamous cell carcinoma (HNSCC). Therefore, PPP2R2B may be important in ATM nuclear localyzation. To support our hypothesis, siRNA against PPP2R2B mRNA was employed. Interestingly, ATM of HNSCC cells containing a PPP2R2B siRNA was mainly localyzed within cytoplasm. In addition, $siRNA_{PPP2R2B}$ cells survived less after radiation. Our findings indicate the role of PPP2R2B in ATM nuclear localyzation.

Field of study: Biomedical Sciences Student's Signature:.....
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LIST OF ABBREVIATIONS

A-T	Ataxia telangiectasia syndrome
AD	Alzheimer's disease
ATM	Ataxia telangiectasia mutated protein
ATR	Ataxia telangiectasia related protein
CDKs	Cyclin-dependent kinases
CK2	Casein kinase 2
DNA-PK	DNA-dependent protein kinase
DSBs	DNA double-strand breaks
GTP	Guanosine triphosphate
H2AX	Histone variant H2AX
HAT	Histone acetyl transferase
HEAT	Huntington, Elongation factor 3, PR65/A, TOR
HNSCC	Head and neck squamous cell carcinoma
HR	Homologous recombination
IR	Ionizing radiation
LZ	Leucine zipper
MDC1	Mediator of DNA damage checkpoint 1
MRN	Mre11, Rad50, Nbs1
NES	Nuclear export signal
NHEJ	Non-homologous end joining
NLS	Nuclear localyzation signal
NPCs	Nuclear pore complexs
OA	Okadaic acid
PIKK	Phosphatidylinositol 3-kinase-related protein kinase family

PP2A	Protein phosphatase 2A
RPA	Replication protein A
SCA12	Spinocerebellar ataxia type 12
SCID	Severe combined immunodeficiency
Ser/Thr	Serine/Threonine

CHAPTER I

INTRODUCTION

1. Background and Rationale

PPP2R2B is known as the B subunit of PP2A, a protein phosphatase enzyme that reverse the phosphorylation of serine/threonine residues of proteins. The expansion of CAG triplet repeats within the promoter region of the *PPP2R2B* gene causes the Spinocerebellar Ataxia type 12 (SCA12). SCA12 shares characteristics with Ataxia Telangiectasia (A-T) syndrome, which is characterized by progressive neuronal degeneration (3-5). Lately, the variation in PPP2R2B mRNA levels in head and neck squamous cell carcinoma (HNSCC) cell lines (WSU-HN) were observed (6). We, therefore, evaluated the expression status of *PPP2R2B* in HNSCC by examining microarray data obtained from oncomine (http://www.oncomine.org). From studies by Ginos *et al*. and Pyeon *et al*., we found that *PPP2R2B* was significantly down-regulated in HNSCC, with these studies reporting p values of 8.8×10^{-5} and 2.1×10^{-3}, respectively (1, 2). As a result, decreased PPP2R2B mRNA levels may be important in HNSCC development. Recently, PP2A was shown to reverse a phosphorylation of the ataxia telangiectasia mutated (ATM) protein (7). It was found that, in undamaged lymphoblastoid cells, ATM interacted constitutively with the A and C subunits of PP2A. However, when cells were exposed to ionizing radiation (IR), the ATM and PP2A separated from each other. As a result, phosphorylation of ATM and histone variant H2AX (γ-H2AX) that is responsible for signaling repair proteins to DSB sites was occurred. ATM has been found to localyze predominantly within the nucleus of most proliferating cells (8, 9). However, ATM has also been

reported to localize mainly within the cytoplasm of mouse Purkinje cells, in cells of the human cerebellum and in a subset of cells in the dorsal root ganglia of mouse (10, 11). Lately, the nuclear localization signal (NLS) domain of ATM has been identified to locate within the amino terminal (12). Moreover, interference of PP2A with a specific inhibitor, okadaic acid (OA), was shown to have an effect on the nuclear localization of the endogenous NLS-containing proteins (13). Based on those mentioned information, we thus examined the role of PPP2R2B on ATM nuclear localization.

1.1 PPP2R2B

The product of the *PPP2R2B* gene belongs to the phosphatase 2 (PP2A) regulatory subunit B family. The significant role of the B regulatory subunit is that they modulate substrate selectivity of the PP2A. The *PPP2R2B* gene encodes a beta isoform of the regulatory subunit B55 subfamily. It is located on chromosome 5 (5q31-q32) and consists of 9 exons. *PPP2R2B* gene encodes 6 different mRNA, which finally translate into 4 proteins. Each PPP2R2B protein is different at the first 29 amino acid at the N-terminal (data was analyzed from Pubmed). However, neither tissue specific expression nor substrate specificity of these isoforms-containing PP2A has been examined. The expansion of the CAG non-coding poly-glutamine repeat that lies in the promoter region of the *PPP2R2B* gene causes a rare form of autosomal dominant SCA12, which exhibits similar phenotypes an A-T syndrome, a progression of neuronal degeneration (3-5). This CAG expansion in the promoter region can be found from 7-28 copies and can be expanded to 66-78 copies. The expansion was speculated to obstruct the expression of the gene (14). Interestingly, *PPP2R2B* was found to be significantly down-regulated in HNSCC gene expression microarray (1, 2).

1.2 Protein Phosphatase 2A (PP2A)

In eukaryotic cells, the reversible post-translational modification of proteins known as phosphorylation is a key step in regulating many cellular processes. To trigger these, two protein families known as protein kinases and protein phosphatases are interacted either directly or indirectly. Therefore, a well-balance between kinase and phosphatase activities is crucial for eukaryotic cells to accurately respond their environments, therefore, cells are able to properly regulate their cellular mechanisms.

To date, approximately 500 protein kinases are encoded by the human genome (15). Within these are the Tyr and Ser/Thr protein kinases. In contrast to the Tyr protein kinase activities that functions are opposed by specific phosphatases (16), the Ser/Thr kinase activities are opposed by a small number of protein phosphatase catalytic subunits. Therefore, how these phosphatases present substrate specificity is crucial. The Ser/Thr phosphatases are categorized into three families: PPP, PPM and FCP. The PPP or phosphoprotein phosphatase family itself can be classified into seven subgroups. One of these is the PP2A, which make up 0.1% of the cellular proteins (17). The core PP2A complex ($PP2A_D$) comprises of a 65 kDa scaffold-structural subunit (PR65 or A subunit) and a 36 kDa catalytic subunit ($PP2A_C$ or C subunit). The unique characteristic of the free catalytic C subunits is its high activity but low specificity (18). This substrate-indiscriminate feature of the C subunit, thus, is needed to be guided to prevent a general dephosphorylation. To fix this problem, the B subunits ($PP2A_B$) are required to participate in core PP2A (review in (19, 20)). Whereas both A and C subunits can be classified as α and β isoforms, the B regulatory family can be categorized into four major classes: PR55/B (B55), PR61/B´ (B56), PR72/B´´ and PR93/PR110/B´´´ (19, 21). So far, four PR55/B (22-25), at least eight PR61/B´ (26-32), six PR72/B´´ (33-35), two PR93/PR110/B´´´ (36) and

different spliced variants have been reported (37, 38). As a result, the recruitment of these subunits into $PP2A_D$ can structure more than 75 distinct ABC trimeric complexes. Nevertheless, the actual number of the combinations has not yet been concluded. This is because the B subunit is expressed tissue and developmental stages specifically, while the A and C subunits are mostly expressed in all cells (19).

There are two $PP2A_C$ isoforms, α and β. Both of them share 97% sequence identity but are encoded from different genes (39). The $PP2A_{C\alpha}$ was found to be 10-fold more abundant than $PP2A_{C\beta}$ because of the difference in transcriptional regulation (40). However, Both $PP2A_{C\alpha}$ and $PP2A_{C\beta}$ were shown to have equal affinity with A and B subunits. Additionally, the $PP2A_{C\alpha}$/$PP2A_{C\beta}$ association with the same type of A and B subunits gave indistinguishable phosphatase activities (41).

Similar to the catalytic subunit, the A subunit is composed of α and β isoforms. They are also transcripted from different genes and share only 86% sequence homology (42). The structure of the A subunit is unique, elongated hook-like shape, (20, 43). The mutations of both α and β isoforms have been reported in breast, lung and melanoma primary tumours (44, 45). These mutations were suggested to disrupt the structure of the A subunit, thereby, the heterodimer could not be formed (45).

The third subunit of PP2A that is a key in substrate specificity and localization of PP2A heterotrimeric enzyme is composed of four families; PR55/B (B55), PR61/B´ (B56), PR72/B´´ and PR93/PR110/B´´´. Each family is described to have tissue-specific expression. The PR55/B family has been shown to be enriched in brain (38). The PR61/B´ was revealed to be found in brain and at embryonic development (37) , whereas the PR72/B´´ family was shown to be established in heart and skeleton muscle (46). The fourth family, PR93/PR110/B´´´, was though indicated to be

localized in neuronal dendrites (36). The B subunit, B55 (or PPP2R2B, that we are interested in this study is grouped into the PR55/B family).

1.3 Ataxia Telangiectasia Mutated (ATM)

One of the most cytotoxic forms of DNA lesion is the DNA double-strand breaks (DSBs). However, DSBs are sometimes generated during normal cellular processes, including meiotic recombination and immunoglobulin gene rearrangement (review in (47, 48)). IR can also cause DSBs. If DSBs are not repaired properly, mutations, chromosomal rearrangements and/or cell death may be initiated, eventually leading to the development of cancer in multicellular organisms. Therefore, to maintain genomic stability, cells must detect and repair DNA damage (review in (49-51)). To date, it is well established that ATM, which belongs to the phosphatidylinositol 3-kinase-related protein kinase family (PIKK), is mainly responsible for sensing DSBs generation and transmit the signal of DNA damage to the downstream effectors (review in (51)).

The importance of the ATM protein in response to DSBs was first observed in the genomic instability syndrome Ataxia-Telangiectasia (A-T), which is caused by mutations in the ATM gene (52, 53). After DSBs are induced, ATM is autophosphorylated. Nonetheless, the mechanism of ATM activation is inconclusive. Several groups showed that the total amount of ATM remains unchanged even after exposure to IR (54, 55). Thus, ATM posttranslational modification may be a possible mechanism. From a study of Bakkenist and Kastan, they showed that ATM that formed as an inert dimer was dissociated as an active phosphorylated monomers at Ser-1981 after IR, consequently supporting the posttranslational modification idea (56). Up to now, several mechanisms of ATM autophosphorylation and activation have been proposed, yet needed to be more characterized.

1.4 The role of the PP2A in ATM activation

Recently, a model in which PP2A activates ATM autophosphorylation has been proposed (7) ATM was found to be constitutively interacted with PP2A in undamaged lymphoblastoid cells. However, when cells were exposed to IR, the ATM-PP2A complex that possibly be occupied with any of B subunits was rapidly dissociated. As a result, phosphorylated ATM with kinase activity was detected. Therefore, this model explains that the dimer ATM, which continually kinase to each other at an unexcited stage, is constitutively dephosphorylated by bound PP2A. Once DSBs is induced, PP2A dissociates from ATM through an unknown mechanism, consequently, phosphorylated monomer ATM is detectable. Interestingly, when inhibited PP2A with a specific inhibitor as OA, phosphorylated ATM with no kinase activity is identified, suggesting complex mechanisms involved in the activation of ATM kinase activity. What B subunit occupied in ATM-PP2A complex is undiscovered. However, PPP2R2A that is 70% homology to PPP2R2B was suggested to be involved in. This model is put forward by Guo C.Y. *et al*.. They proposed that IR activated ATM through a signaling pathway implicated the dissociation of PPP2R2A subunit from the heterotrimeric PP2A complex in human T cell lymphoma cell line. Nonetheless, no examination was performed whether the PPP2R2A-engaged heterotrimeric PP2A complex directly interacted with ATM or not (57).

2. Research Questions

2.1 How does PPP2R2B promote ATM autophosphorylation ?

2.2 Are PPP2R2B-deficient cells as radiosensitive as ATM-mutated cells (A-T cells) ?

3. Objectives

3.1 To investigate the role of PPP2R2B in ATM autophosphorylation.

3.2 To investigate whether the PPP2R2B-deficient cells exhibit phenotype as A-T cells or not.

4. Hypotheses

4.1 ATM predominantly localizes within cytoplasm in cells without PPP2R2B.

4.2 PPP2R2B-deficient cells show lower survival rate than PPP2R2B-expressed cells.

5. Keywords

Head and neck squamous cell carcinoma

HNSCC

ATM nuclear localization

Protein phosphatase

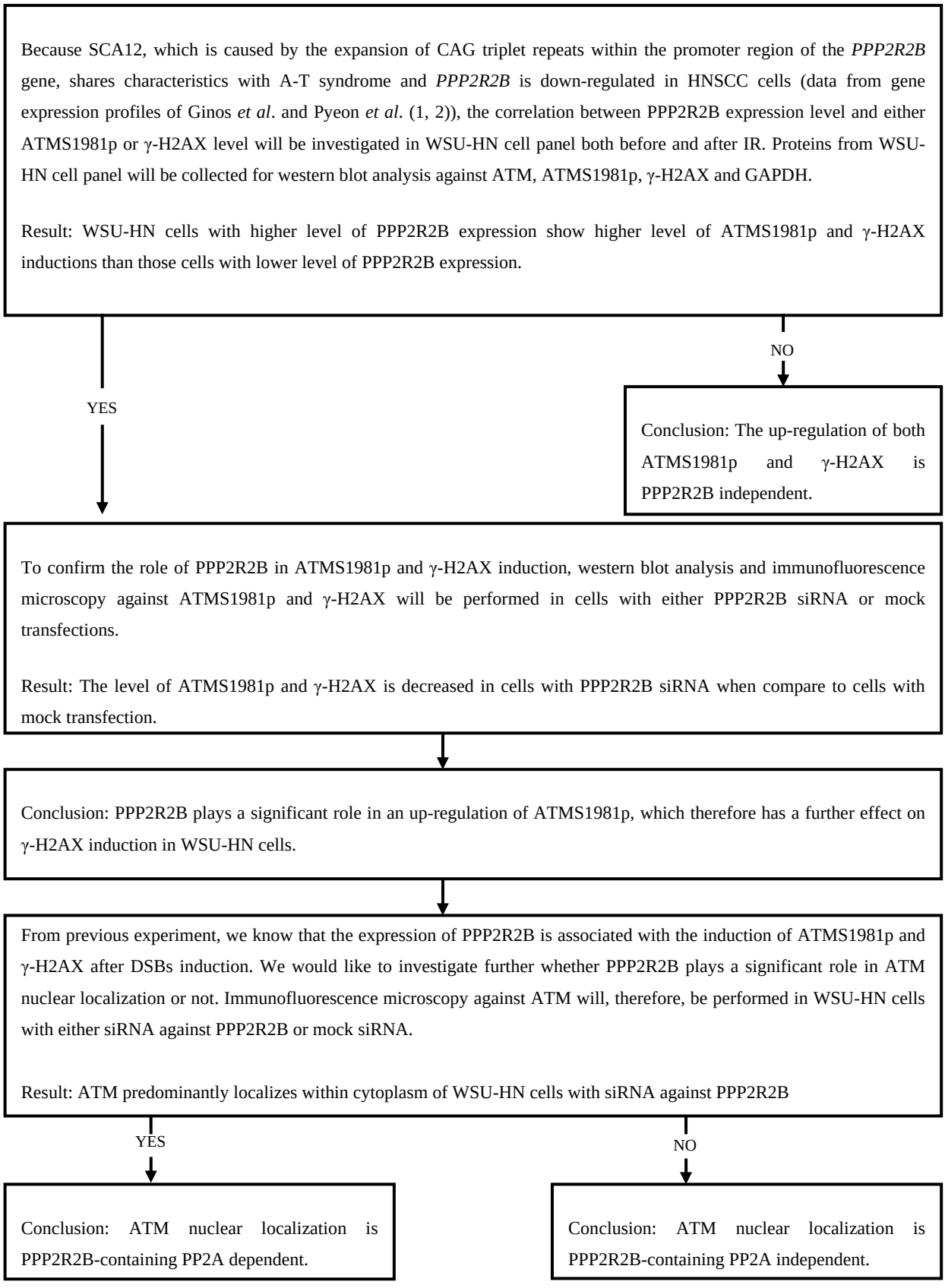
PP2A

B subunit

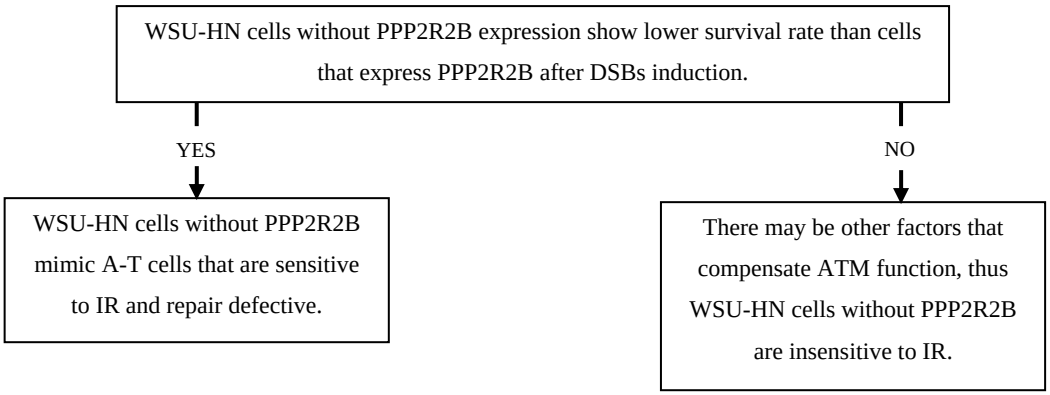
PPP2R2B

6. Conceptual Framework

Research question: How does PPP2R2B promote ATM autophosphorylation ?



Research question: Are PPP2R2B-deficient cells as radiosensitive as ATM-mutated cells (A-T cells) ?



7. Expected Benefits and Applications

The outcome of this study will help us to understand the role of B55β in HNSCC carcinogenesis. This new knowledge may lead to the expansion of novel tumour marker and drug development against HNSCC cancer.

CHAPTER II

LITERATURE REVIEW

1. DNA damage

Maintaining genetic integrity is essential for cells to exhibit normal function. Genomic instability can be caused by errors during DNA synthesis, DNA-damaging agents and several kinds of radiation. Endogenous DNA-damaging agents can be produced during normal cellular metabolisms, including superoxide ions, hydroxyl radicals and hydrogen peroxide (58, 59) DNA can also be damaged by exogenous chemicals, such as anti-tumour agents, including bleomycin (60), cross-linking agents (61, 62) and alkylating agents such as methyl methanesulfonate (MMS) (63). Both ultraviolet and IR are also the cause of DNA damage.

DSBs are a DNA lesion in which both strands in the DNA double helix are broken. It is mainly harmful to the cells because it can lead to gross chromosomal aberrations if it is not rejoined quickly. However, if the repair is too quickly processed, the consequence may be an error-prone and may eventually destroy the host organism. Therefore, mammalian cells have mechanisms for rapidly transmitting the damage signals to the cell cycle arrest, apoptotic machineries and DNA repair mechanisms. DSBs can be induced by either intrinsic sources such as products of cellular metabolisms or extrinsic sources including IR. Two repair mechanisms that are responsible for DSBs are

non-homologous end joining (NHEJ) and homologous recombination (HR).

1.1 Mechanisms of DNA Double-Strand Break Repair: Non-homologous end joining

The non-homologous end joining or NHEJ pathway is required to repair DSBs not only generated by exogenous DNA-damaging agents, such as IR, but also involved in processing the DSB intermediates generated during V(D)J recombination. In contrast to HR, NHEJ pathway does not apply sister chromatid as a complementary template for repair process, as a result, error-prone is easily created. A key component that drives the NHEJ pathway is a trimeric DNA-dependent protein kinase (DNA-PK) enzyme. DNA-PK itself composes of two subunits: a heterodimeric DNA-targeting subunit (KU) and a DNA-dependent protein kinase catalytic subunit (DNA-PKcs). A KU heterodimer consists of KU70 (70 kDa) and KU80 (80 kDa). Both KU70 and KU80 have a high affinity for DNA ends, indicating a significant role in an early NHEJ pathway. Inactivation of either KU70 or KU80, which lead to hypersensitivity to IR and severe combined immune deficiency (SCID) due to impaired V(D)J recombination, has been reported (64-66). DNA-PKcs is a 470 kDa polypeptide with a protein kinase domain near its carboxy terminus. Mutation of DNA-PKcs had been shown to produce a high incidence of T-cell lymphomas in mouse model (67).

The NHEJ pathway can be divided into three steps as shown in figure 1: (1) end binding (2) end processing and (67) ligation. When DSBs occur, KU heterodimer will rapidly binds to DNA end breaks and recruits DNA-PKcs to form a trimeric DNA-PK enzyme. This step is called the end binding. Once bound to broken ends, DNA-PK is

activated and phosphorylates other targets including Artemis and Werner syndrome helicase (WRN). NHEJ pathway that is often described as an error-prone can however precisely repair DSBs. This precisely NHEJ pathway can be happened when breaks are produced by nuclease, leaving complementary overhangs with both 5'phosphates and 3'hydroxyl groups (68). On the other hand, when broken DNA ends cannot be precisely rejoined, several enzymes that have nucleolysis and polymerization functions will be implicated at the broken ends to remove or add in few complementary bases, leading to small deletions or insertions. These enzymes are the MRE11-RAD50-NBS1 (MRN) complex, Artemis and WRN. The role of the MRN complex involves in the unwinding and/or nucleolytic processing of the DNA ends. However, the MRE11 subunit alone functions as a 3'-5' exonuclease. In contrast to the MRE11 subunit, Artemis acts as 5'-3' exonuclease whereas WRN that is stimulated by KU but inhibited by DNA-PKcs possesses 3'-5' exonuclease activity with a preference for recessed 3' DNA ends. In the ligation step, DNA-PK enzyme recruits DNA ligase IV and its partners, XRCC4 and XLF, to close the breaks (69-72).

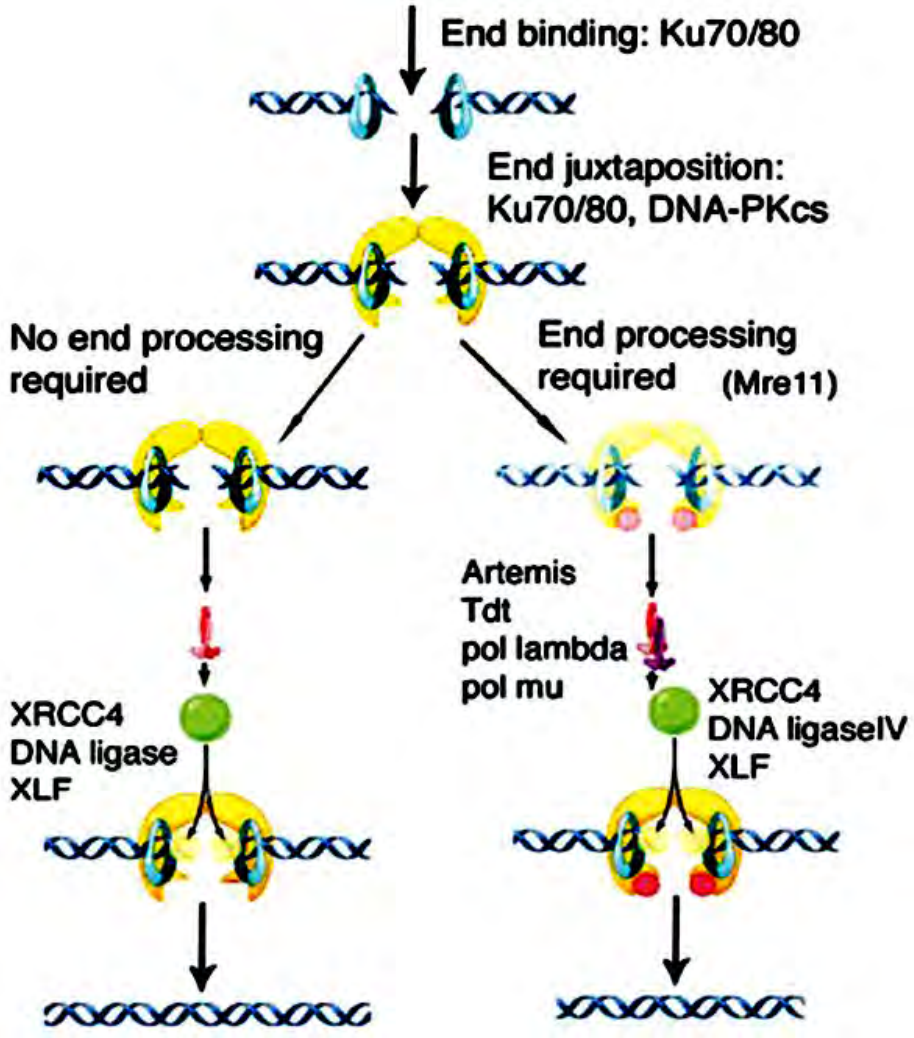


Figure 1 Schematic of non-homologous end joining repair pathway. KU70/KU80 bound to DNA ends recruits DNA-PKcs to the DSB sites. If no further processing of the ends is required, XRCC4, DNA ligase IV and XLF can promote the ligation reaction. However, if the broken DNA requires end processing, the activities of Artemis and/or the DNA polymerase TdT, pol lambda and pol mu are required before the last ligation step (69).

1.2 Mechanisms of DNA Double-Strand Break (DSB) Repair: Homologous Recombination

In contrast to NHEJ, HR pathway is known as an error-free process. This is because the broken ends use homologous sequences, such as sister chromatids, homologous chromosomes or repeated regions on the same or different chromosomes, to prime repair synthesis. Therefore, HR pathway is preferentially occurred at the S and G2 phases of the cell cycle. The process of HR repair pathway, which the basic mechanism is conserved from yeast to human, starts after DSBs detection. The MRN complex that recruits ATM through NBS1 moves to the DSB sites. Here, the MRN complex (RAD50 that has ATPase activity facilitates DNA unwinding, MRE11 mediates 3’ to 5’ exonuclease activity and NBS1 has a high affinity to bind to ATM) resects the broken ends in the 5’ to 3’ direction to yield 3’ single strand overhang. The single strand overhang is coated with replication protein A (RPA), which is then substituted with RAD51 in a reaction mediated by RAD52 and two RAD51 paralogs, RAD55 and RAD57. RAD51 with single strand DNA then search and invade a homologous sequences for base pairing through a facilitation of RAD54, subsequently, strand is extended by DNA polymerase. At this step, if only one of the broken ends anneals with the complementary donor strand (strand invasion), two non-crossover recombinants will be obtained. On the other hand, if both ends invade to each complementary donor strand, double-holiday junction is produced and non-crossover and crossover recombinants will be given (71-74) (as shown in figure 2).

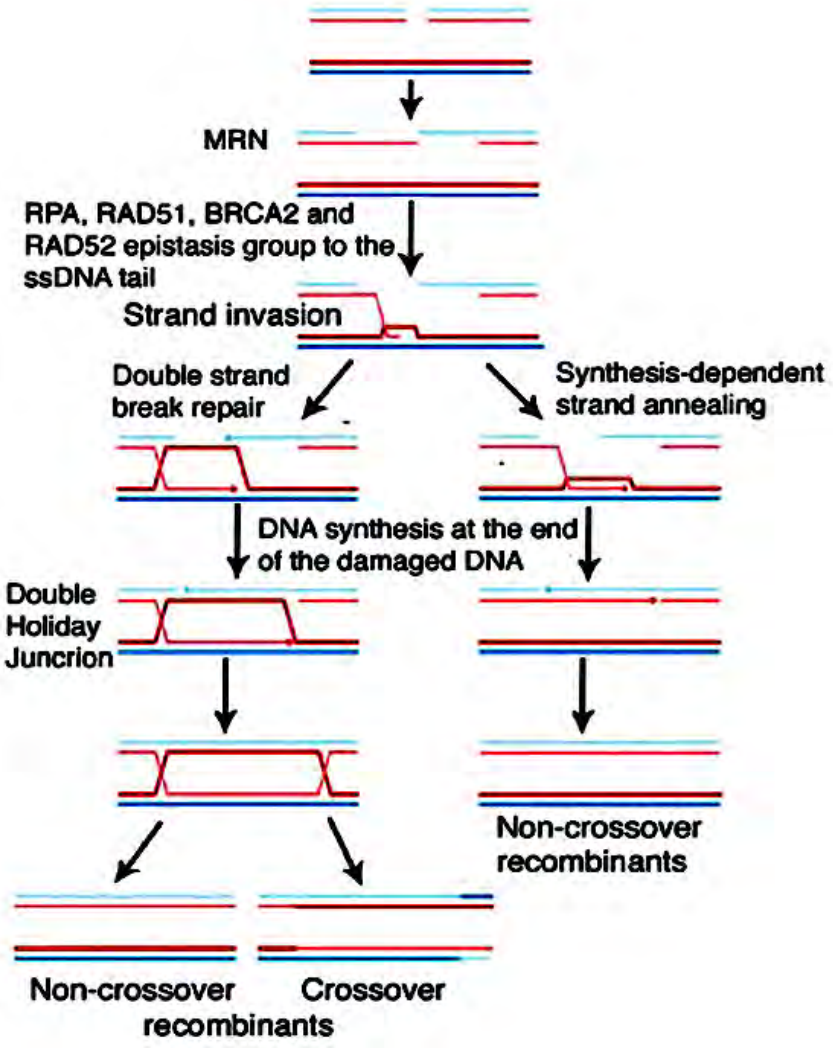


Figure 2 Schematic of homologous recombination repair pathway. DSBs that are repaired by HR pathway require various steps. For the promotion of invasion, RPA binds to 3' single strand DNA overhang, which then is substituted with RAD51 and RAD52 to form nucleoprotein filaments. The nucleoprotein filament then searches for the homologous duplex DNA in the undamaged sister chromatid. This process results in strand inversion, strand exchange and joint molecule formation which stimulate by RAD52 and RAD54. Strand is then synthesized by DNA polymerase and sealed by DNA ligase (69).

1.3 DSB repair pathway choice

It has been known that cell cycle regulates the balanced shift between NHEJ and HR repair pathways (review in (71, 75) HR is preferentially chosen during S and G2 phases of the cell cycle because of template accessibility (summarized in figure 3). However, NHEJ activity remains detectably throughout these phases, suggesting the NHEJ competition for DSBs repair even when homologous templates are in the surrounding area (76). How higher eukaryotes with larger genomes locate a homologous sequences for HR repair is cellular challenging. The control of a nuclear architecture on ordering chromatin organization and chromosome region in nucleus has been suggested to play a significant role (71, 77).

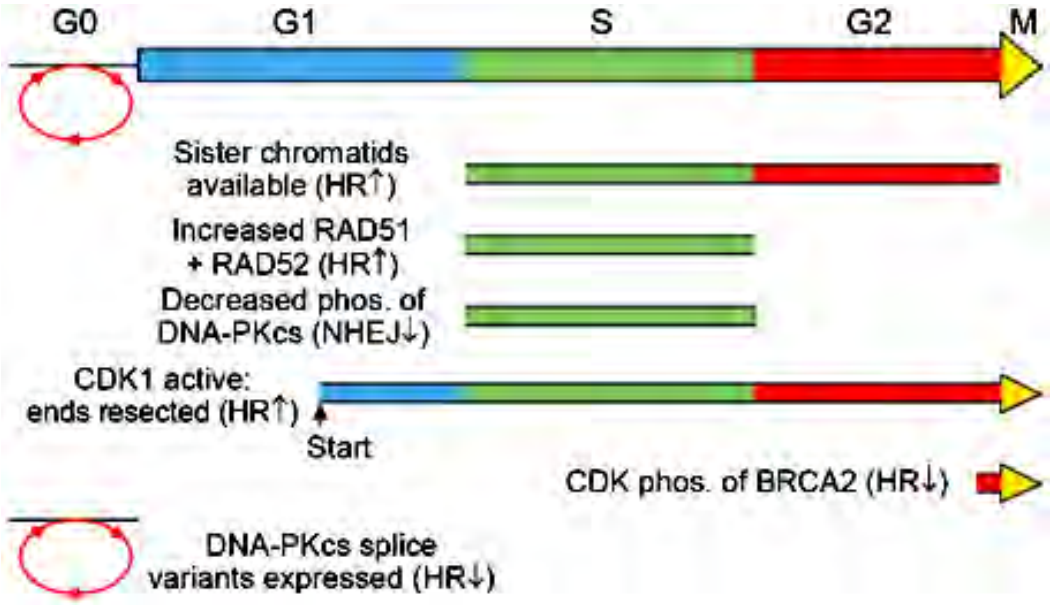


Figure 3 Cell cycle regulation of DSB repair pathway choice. The schematic indicates an influence of each cell cycle phase on the regulation of NHEJ and HR pathways. G0 indicates quiescent (non-cycling) cells (71).

Recently, several studies attempt to elucidate the molecular mechanisms that control the shift between these two repair pathways. Many proteins involved in cell cycle progression and NHEJ/HR repairs have been investigated. Among these proteins are cyclin-dependent kinases (CDKs), the key regulators of cell cycle progression. The phosphorylation activity of CDKs on targeted proteins has been proposed for HR regulation. It was found that CDK-mediated phosphorylation of BRCA2 on Ser-3291 at C-terminal region blocked the interaction with RAD51 (78). This particular phosphorylation was maximized in M phase of the cell cycle, consequently, represents one of the mechanisms by which HR is down regulated in M and early G1 phases (71). Note that BRCA2 interacts with RAD51 to assist the strand invasion during HR repair process (79, 80). On the other hand, the phosphorylation of Ser/Thr clusters on DNA-

PKcs plays a significant role in NHEJ regulation. Several Ser/Thr clusters on DNA-PKcs have been reported to be *trans*-phosphorylated, including T2609 (also called ABCDE) (81, 82), S2056 (also called PQR) (83, 84) and T3950 (85). Phosphorylation of the S2056 and T2609 clusters were reduced in irradiated S phase cells (83), suggesting a regulatory system that play a role in NHEJ down regulation in S phase of the cell cycle. The model in which DNA-PKcs activity was down regulated in S phase has been demonstrated in HeLa cells (86).

Apart from those mentioned proteins, the activity of both ATM and DNA-PKcs has also been suggested to play an important role in the shift between NHEJ and HR pathways. This idea is clearly explained through the mutation in the S2056 cluster of DNA-PKcs. DNA-PKcs autophosphorylates itself at S2056 (87). Mutation in this site increased DSB-induced HR (84). Moreover, the releasing of DNA-PKcs from the broken ends is mediated through the phosphorylation of T2609 of DNA-PKcs by ATM (87). Therefore, HR is stimulated when DNA-PKcs activity is absent and DNA-PKcs-bound broken ends is discontinued by the kinase activity of ATM on DNA-PKcs (71).

1.4 Histone H2AX, a marker for DSB repair

Phosphorylation of histone variant H2AX, also known as γ-H2AX, by a member of the phosphatidylinositol 3-kinase-like-kinase (PI3KK) family is responsible for signaling repair proteins to DSB sites. In mammalian cells, H2AX is phosphorylated by DNA-PKcs, ATM and the ataxia telangiectasia related protein (ATR) on Ser-139 carboxy terminus in response to DSBs (88). Upon DSBs induction, γH2AX is rapidly detected, suggesting a direct role in detection and signaling DNA lesion to cells. When visualize

damaged cells by immunofluorescence after IR, foci formation that represents each local DSB is detected (89, 90). Therefore, the number of γ-H2AX foci scores the approximate number of DSBs induced in cell (91). When DSBs are induced, ATM rapidly phosphorylates histone H2AX. However, DNA-PK fails to initiate this primary histone H2AX phosphorylation when ATM was inhibited with a specific inhibitor (92). This initial induction of γ-H2AX recruits Mediator of DNA damage checkpoint 1 (MDC1). MDC1 that contains two repeated BRCT domains at carboxy terminus structurally interacts with γ-H2AX molecule through phosphor–peptide bound (93, 94). Because MDC1 also directly interacts with NBS1 (95), this leads to the recruitment of MRN complex, then ATM and finally the beginning of DSB repair mechanism. In this way, a positive feed back loop is generated to extend H2AX phosphorylation to almost 2 Mbp regions flanked single DSB site (approximately 2000 γ-H2AX molecules are generated) (88). Therefore, MDC1 is primarily needed to start the loop. The interaction between MDC1 and NBS1 is mediated through phosphorylation of MDC1 by casein kinase 2 (CK2) (96). Loss of MDC1 expression or reduction of its level by siRNA treatment decreases IR-induced histone H2AX phosphorylation. This may be as a result of a defect in ATM recruitment (97-99).

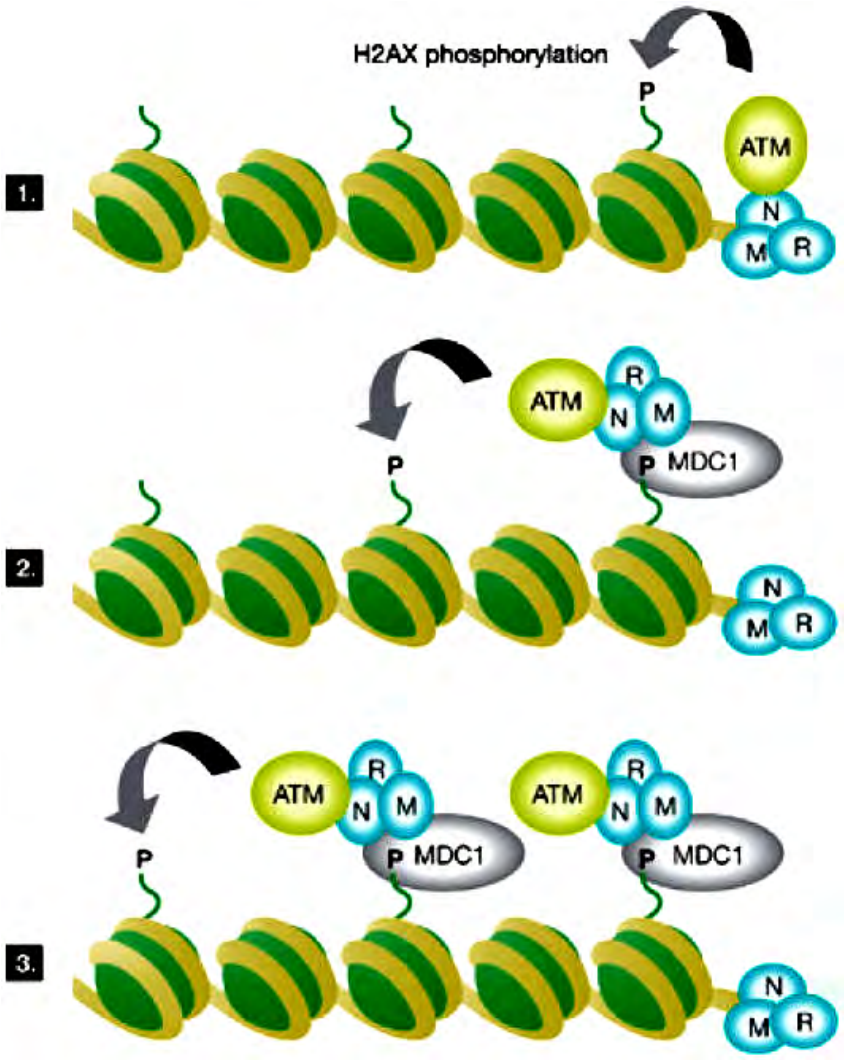


Figure 4 Schematic of MDC1-regulated phosphorylation of histone H2AX. (1) The MRN complex binds DNA ends at DSB sites and recruits ATM, which phosphorylates histone H2AX. (2,3) MDC1 binds phosphorylated proximal histone H2AX and recruits more MRN–ATM. The new pool of ATM phosphorylates more distal histone H2AX (100).

2. Ataxia Telangiectasia Mutated (ATM)

ATM protein is a Ser/Thr kinase. It is activated in response to DSBs and subsequently activates cell cycle checkpoints and regulate DNA repair through phosphorylation of key protein targets. (56) and review in (101). The importance of ATM in response to DSBs was first observed in the genomic instability syndrome called Ataxia Telangiectasia (A-T), which is caused by various mutations in *ATM* gene.

2.1 Ataxia-Telangiectasia

A-T is a rare autosomal recessive syndrome with a complex phenotype. It is characterized by cerebellar degeneration, immunodeficiency, hypersensitive to DSB-inducing agents, thymic and gonadal atrophy, telangiectasia and an increased risk of cancer (102). Therefore, cells from A-T patients represent a severe A-T phenotypes and also emphasize the importance of ATM in response to DSBs. A-T cells showed hypersensitivity with radiomimetric agents and IR treatment but not UV radiation, alkylating and replication block agents (53, 103). They also showed deficiency in cell cycle checkpoint at G1/S and G2/M transitions after exposure to IR (104, 105). To date, more than 300 mutations on *ATM* gene have been reported (106). Among these, approximately 70% are identified to produce premature protein truncations (see also GeneBank entry for *ATM* for list of sequence variations (Q13315)). These truncated forms of ATM are highly unstable and cannot be detected by immunoblotting (107). On the other hand, the other 30% are missense mutations and in-frame deletions/insertions. It is interesting that missense mutations are clustered at the 3’ end of the *ATM* gene, which encodes the regulatory and PIKK-kinase domains (108, 109).

2.2 Structure of ATM

The PIKK proteins can be classified by the existence of several conserved protein domains. The N terminus of each comprises of multiple HEAT domains (110). Each HEAT repeat can form two anti-parallel α-helices, thereby forming superhelical scaffolding matices that can interact with other proteins. Recently, two pairs of HEAT repeat in fission yeast ATM (Tel1) were shown to interact with an FXF/Y motif at the C terminus of Nbs1 (111). Moreover, a conserved C-terminal motif of Nbs1 has lately been proved to recruit ATM to DSB sites in mammalian cells (112). These suggest a possible role of HEAT repeat domain in mediating the interaction between Nbs1 and ATM.

The PIKK proteins also contain the C-terminal conserved FAT/kinase domain/FATC domains. These domains are present as a single unit in all known PIKK proteins (113). The FAT (FRAP, ATM and TRRAP) domain is found to be most weakly conserved among family members, whereas the kinase domain is highly conserved and is responsible for phosphorylating Ser/Thr residues followed by Glu in target substrates (114). The third domain of the PIKK proteins is the 33-amino acid FATC (FRAP, ATM and TRRAP C-terminal) domain. This domain is needed for the kinase activity of the mTor (115) and DNA-PKcs proteins (116, 117), indicating its critical role in regulating kinase activity. Nonetheless, the exact function of the FAT and FATC domains of PIKK proteins is unknown. The schematic structure of ATM protein is shown in figure 5.

2.3 Catalytic activity of ATM

The significance of ATM kinase activity in response to IR was first suggested by Banin *et al*. (54) and Canman *et al*. (118). These two groups showed that p53 was phosphorylated at Ser15 *in vivo* after DSBs induction. Moreover, this activity was markedly enhanced within minutes after IR or a radiomimetic but not UV treatment. The phosphorylation of p53 at Ser15 by ATM was also detected *in vitro* (54, 118). In addition, the kinase activities of both ATM and ATR were shown to depend on manganese *in vitro* (54, 118, 119).Therefore, most of experiments investigating ATM kinase activity were performed under manganese and magnesium additional condition *in vitro*. Like other PIKK proteins, the kinase activity of ATM can be inhibited by wortmannin, a fungal metabolite (54, 120). Apart from wortmannin, caffeine is also known as an effective inhibitor of both ATM and ATR (121-123). So far, it is known that autophosphorylation activates ATM kinase activity (56). Nonetheless, induction of ATM autophosphorylation by OA, a specific inhibitor to PP2A, was recently been shown to prevent ATM kinase activity, suggesting other network factors involved in the initiation of ATM function (7).

A well-characterized ATM autophosphorylation site that initiates its kinase activity is Ser1981 (56). However, several additional phosphorylation sites on ATM in response to DSBs have lately been reported, including autophosphorylation on Ser367 and Ser1893 as shown in figure 5 (124). At Ser1893, phosphorylation was rapid and persistent after IR treatment *in vivo*, paralleling the phosphorylation at Ser1981. Moreover, phosphorylation at this site was dependent on functional ATM and Mre11. All three autophosphorylation sites of ATM were physiological important to DNA damage

response since mutants (S367A, S1893A and S1981A) showed the defect in ATM signaling *in vivo*, DNA repair and cell cycle checkpoint.

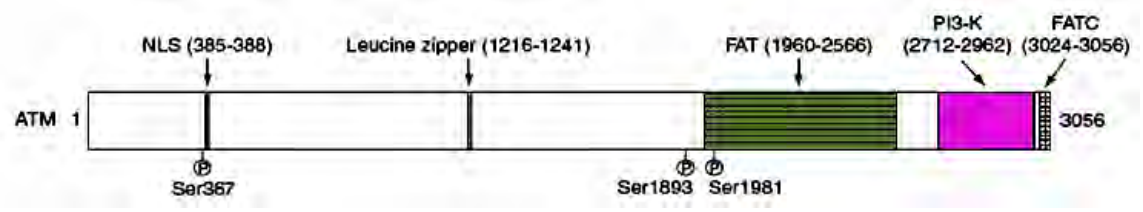


Figure 5 Schematic diagram of ATM protein. ATM is a 350 kDa protein, consisting of 3056 amino acids. "P" indicates the positions of Serine residues that are autophosphorylated (56, 124). As indicated, the identified domains of ATM consist of NLS (12), the leucine zipper (LZ) (125), the FAT domain (113), the kinase domain (125) and the FATC domain (113).

2.4 ATM activation

Apart from PP2A that has been mentioned in chapter I, three more mechanisms, which have been so far proposed to involve in ATM autophosphorylation and kinase activation, are as followed.

2.4.1 The role of Tip60HAT in ATM activation

Tip60HAT is a histone acetyl transferase enzyme. It was found to play a crucial role in autophosphorylation of ATM (126). It was found that ATM was constantly bound

with Tip60HAT. Additionally, only Tip60HAT associated with ATM, not the free Tip60HAT, was activated by DNA damage. Because Tip60HAT's chromodomain interacts with methylated histones that are exposed at DNA damage sites, this explains why Tip60HAT activity is activated by DNA damage and ATM becomes acetylated (126). Tip60HAT was also proposed to be an up-stream regulator of ATM activation, this was according to the up-regulation of Tip60HAT activity did not require a functional ATM's kinase activity. Nevertheless, how acetylation regulates ATM activation is required to be explored. Recently, the regulatory ATM acetylation site was identified at lysine 3016, which was located in the highly conserved FATC domain of ATM, by the systematic mutagenesis of lysine residues (127).

2.4.2 The role of the MRN complex in ATM activation

The MRN complex, which is composed of three proteins, Mre11, Rad50 and Nbs1, is essential for DSBs repair in mammals. The MRN complex is known as a sensor of DSBs because its mutated form did not stimulate ATM activity, consequently, it acts as an upstream regulator of ATM activation (128). Moreover, the association of the MRN complex with DSB sites was found to be ATM independent (129). When DSBs are induced, the MRN complex and ATM, along with other DNA repair transducer proteins, form a highly efficient protein network to signal and mediate the repair pathways in damaged cells. It was found that the conserved carboxy-terminal motif of Nbs1 was required for the interaction with ATM, therefore, ATM was recruited to the sites of DSBs and activated (112).

2.4.3 The role of the chromatin remodeling in ATM activation

It is previously known that there is an association between DSB-induced changes in chromatin structure and the activation of ATM. However, because DSBs generated a rapid, ATP-dependent, chromatin decondensation in an ATM-independent manner, the remodeling of chromatin structure has been put forward as an upstream regulator of ATM activation (130) and review in (131). Nonetheless, a direct molecular mechanism links the relationship of chromatin remodeling and ATM activation is unclear. Recently, HMGN1, the nucleosome-binding protein, has been identified as a modulator between ATM and chromatin interaction both before and after DSBs induction (132). It was found that HMGN1 was required for both IR-induced the acetylation of histone H3 at lysine 14 (H3K14) and ATM autophosphorylation.

3. Protein Phosphatase 2A

PP2A is a Ser/Thr phosphatase with broad substrate specificity and diverse cellular functions. As mentioned in chapter I, PP2A consists of a dimeric core enzyme comprising of the structural A and catalytic C subunits, and a regulatory B subunit. The structural A subunit, a member of the HEAT (Huntington, Elongation Factor 3, PR65/A, TOR) repeat protein family, is the scaffold required for the formation of the heterotrimeric complex. While A and C subunit sequences are remarkably conserved throughout eukaryotes, the regulatory B subunits are more heterogeneous and are believed to play key roles in controlling the localization and specific activity of different PP2A.

3.1 PP2A heterotrimeric structure

The A and C subunits, which represent at least one-third of the total PP2A, can form a stable $PP2A_D$ core dimmer (133). The A subunit contains 15 tandemly repeated HEAT sequences (figure 6). Each repeat consists of ~39 conserved residues that form two anti-parallel α-helices, creating a horseshoe shape when forming as an $PP2A_D$ core dimmer (43). The HEAT repeat 11 to repeat 15 is involved in a strong interaction with the C-subunit through an association of hydrogen bonding, ionic and hydrophobic interactions (134). Mutations located within the $PP2A_D$ interface (either in A or C subunits) that might lead to the defective in B subunits binding have been reported in several cancers (45, 135, 136). From a structural point of view from previous study, the regulatory B subunit controls PP2A substrate specificity through binding to the HEAT repeats at the opposite site of the catalytic subunit, consequently, shielding almost A subunit surface and providing docking site for substrate proteins. With this structural interaction, the active site on the C subunit is facing away from the other two subunits and free for substrate access (19, 134). Unlike the structural A and the catalytic C subunits, the sequence similarity among the regulatory B subunit families is very low. All B subunit members contain a conserved domain with 80% sequence identity. Because of the diversity of these regulatory subunits, which lead to the difference of their structures, the final heterotrimeric PP2A structure is, therefore, fit to each individual substrate protein (137).

How the $PP2A_D$ core enzyme controls the recruitment of the regulatory B subunits is well established. From previous study, it was found that the methylation of the C-terminal carboxyl group of the catalytic C subunits was the one that signal the

recruitment of the B subunits (138). An example of heterotrimeric PP2A formation has been described through A-B56-C complex (134). The methylated amino acid (Leu309) is located among highly negatively charged residues (Glu62, Asp63, Glu64 and Glu101 of the A subunit and Asp306 of the C subunit). Without the C-terminal carboxyl methylation, the charge-charge repulsion between those negatively charged amino acids and the carboxyl group at the C-terminal of the catalytic subunit would not create the docking site for the B56 subunit recruitment. Therefore, methylation allows neutralization of the charge-charge repulsion within this area. Apart from the C-terminal methylation, phosphorylation has also been reported to regulate PP2A (139, 140).

Figure 6 A structural comparison between A subunit alone (grey) and in the trimeric complex (purple) (134).

3.2 Cellular role of PP2A

The use of specific PP2A inhibitors and molecular genetics in yeast, *Drosophila* and mice has revealed roles of PP2A in cell cycle regulation, cell morphology and development. In cell proliferation, PP2A promotes progression through the G1/S phase but inhibits G2/M progression (141, 142). It also controls many enzymes in cell cycle control and is involved in formation of the pre-replication complex in G1 (35, 143), spindle checkpoint control and DNA damage response (25, 143, 144). Additionally, PP2A regulates cell death and is a pro-apoptotic factor. It activated the pro-apoptotic Bad in collaboration with other phosphatases and inactivated the anti-apoptotic Bcl-2 (145). PP2A also activates FOXO that regulates the transcription of many pro-apoptotic and growth inhibiting genes (146).

In addition to its role in the cell cycle progression and apoptotic, PP2A has furthermore been shown to regulate development, transcription, translation, metabolism and cytoskeleton stability, for example review in (19, 61, 142, 147). The reason of why PP2A has multiple roles in various parts of cell is possibly explained through lots of B regulatory subunits that are recruited to $PP2A_D$ core complex, which finally give more than 75 distinct PP2A enzymes as described in chapter I. However, there are some confusions of PP2A activity, for example; growth inhibition and promotion; inhibition and activation of the MAP kinase cascades review in (141, 144). Therefore, research is now focused on establishing the substrate specificity of each individual type of PP2A enzyme.

3.3 PP2A and diseases

At least some of the PP2A enzymes have a tumour suppressor activity. This is because the inactivation of PP2A is necessary for some cancer progression, either through substitutions in PP2A A subunit that is retarded, eliminating interactions with other PP2A subunits or upregulation of oncogenic PP2A inhibitors such as SET and CIP2 (44, 135, 148, 149). Similarly, many DNA tumour viruses, such as the SV40, polyoma virus and papilloma virus, target and inactivate PP2A for viral transformation review in (95, 150). Furthermore, dephosphorylation of oncogenes as c-Myc and Akt and the tumour suppressor p53 is mediated by B56α, B55α and B56γ, respectively. This dephosphorylation marked c-Myc for degradation, inactivated Akt and stabilized p53 (151-153). B56γ has been shown to have some additional couplings to human malignancies. Suppression of B56γ expression could inhibit PP2A-specific phosphatase activity similar to that achieved by the SV40 small t antigen (ST), which commonly interacts with PP2A and confer cells to have the ability to grow in an anchorage-independent fashion to form tumours. This transformation could be reversed by over expressing B56γ (108, 153). Interestingly, a truncated isoform of B56γ has been found up-regulated in malignant melanomas.

Another example of diseases that PP2A involves in is Alzheimer's disease (AD). Tau protein is a microtubule-associated protein that is abundant in neurons in the central nervous system and is less common elsewhere. It is hyperphosphorylated and aggregated in the brain tissue plaques of AD patients. PPP2R2A directs PP2A towards Tau and dephosphorylates it. However, PP2A activity is found significantly lower in AD cells. This may be as a result of loss of $PP2A_C$ methyltransferase, which methylation of $PP2A_C$

C-terminus is required for the formation of PPP2R2A containing PP2A (138, 142, 154).

4. PP2R2B and SCA12

The hereditary spinocerebellar ataxias (SCAs) are a group of autosomal dominant neurodegenerative disorders. The clinical phenotypes among SCAs are variable and overlap with one another. These SCAs can be categorized into 3 groups based on their pathogenesis, according to the position of the trinucleotide repeats; the CAG repeat is translated into an abnormally long poly-glutamine region, the translated poly-glutamine residues disrupt calcium or potassium channel function, and the expanded repeat that located in the non-coding region may have an effect on the gene expression level (4). SCA12 that is classified into the third group is genetically caused by the expansion of the CAG non-coding poly-glutamine repeat lies in the promoter region of the *PPP2R2B* gene. The expansion of this trinucleotide repeat was speculated to obstruct the expression of the gene (14).

SCA12 is unique among all SCAs in which an acting tremor of the upper extremities is the most common symptom. As the disorder develops, patients can be detected with mild cerebellar dysfunction, including gait ataxia and limb and eye movement dysmetria. Moreover, both cortical and cerebellar atrophy can also be identified through MRI and CT scans. The age onset of SCA12 was found to range from 8 to 55 years old. In general with other SCAs, SCA12 patients also show hyperreflexia (155). It is interesting that both SCA12 and A-T syndrome share characteristics, which is a progression of neuronal degeneration (3-5).

5. Nucleocytoplasmic transport of proteins

In eukaryotes, the movement of macromolecules (~more than 40 kDa) between nucleus and cytoplasm through nuclear pore complexs (NPCs) is mediated by a protein family of transport receptors. This transport is triggered by temperature- or signal-dependent mechanisms (156). The transport receptors, which belong to the importin/exportin system, generally recognize those cargo proteins through the NLS sequence or a nuclear export signal (130) sequence review in (157) embedded in the cargo proteins. To enter the nucleus, two basic models are suggested. First, the importin-α recognizes the NLS sequence within the cargo protein, which then forms complex with importin-β1 to enter into the nucleus. Second, the importin-β directly binds to the NLS sequence and transports the cargo protein into nucleus. For nuclear export, the exportin binds to an NES-containing cargo protein and this complex is then exported from nucleus to cytoplasm through the stimulation of the guanosine triphosphate (GTP)-bound form of the guanine nucleotide-binding protein Ran (RanGTP, the GTP-bound form of the small Ras family GTAse, Ran).

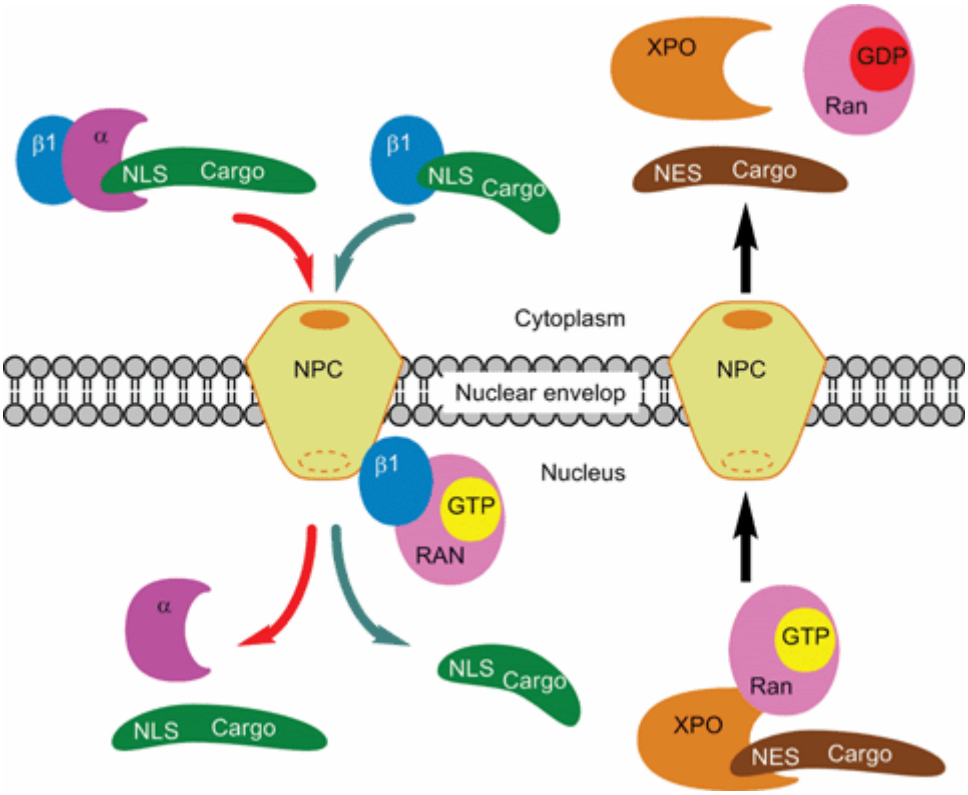


Figure 7 Model of importin/exportin-mediated nucleocytoplasmic transport of macromolecules. The NLS-containing cargo proteins can be imported into nucleus through either importin-α/ β1 complex or importin-β. On the other hand, the RanGTP binds to exportin-bound NES-containing cargo protein to export protein from the nucleus (158).

5.1 ATM nuclear localization

According to ATM role in DSB repair response, it is described to localize predominantly within nucleus of proliferating cells (8, 9). However, ATM has also been reported to localize generally within the cytoplasm of mouse Perkinje cells, a subset of

cells in dorsal root ganglia of mouse and human cerebellum (10, 11). Although the function of the cytoplasmic ATM is unclear, the deficiency of it from cytoplasm causes abnormalities of organelles such as an increase in lysosomal numbers (10). Recently, the NLS of ATM (^{385}KRKK^{388}) has been identified within the amino terminus and was found to be recognized by importin-$\alpha_{1/}$ β1 heterodimer. Both NLS-mutated ATM and truncated ATM containing only the carboxyl terminal half were shown to localize within cytoplasm. Interestingly, this cytoplasmic ATM was unable to phosphorylate itself and its substrates. Therefore, it could not repair DSBs after exposure to IR even after targeted to the nucleus by insertion of the exogenous NLS. These results suggested not only the role of ATM amino terminus in nuclear localization, but also ATM function (12).

5.2 PP2A regulates nuclear import of NLS-containing protein

By taking an advantage of OA, a potent inhibitor of PP2A, it was found that OA blocked the nuclear localization of the NLS-containing proteins, suggesting the role of endogenous PP2A in regulating the nuclear import of the NLS-containing proteins. Interestingly, importin 9 that belongs to importin-β superfamily was identified to interact with the A subunit of PP2A (13). This study supports the earlier unidentified OA-sensitive phosphatase enzyme that is responsible for nucleocytoplasmic transport (159).

CHAPTER III

MATERIALS AND METHODS

1. Cell culture and reagent

Human head and neck squamous carcinoma cell line WSU-HN 4 and 13 were kindly provided by Dr. J Silvio Gutkind (Chief, Oral and Pharyngeal Cancer Branch, DIR; Chief, Cell Growth Regulation Section, and Molecular Carcinogenesis Unit, OPCB, DIR, National Institute of Health, USA). All cells, except the stable transfected cells, were maintained in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% FBS. The stable transfected cells were instead maintained in DMEM supplemented with 250 μg/ml hygromycin B (140) and 10% FBS. Cells were then cultured in 37ºC, 5% CO_2. OA was purchased from LC Laboratories, MA, USA.

2. Ionizing radiation

Cells were seeded for 1×10^6 cells/25 cm^2 flask for 24 hours. Before the radiation treatment, the medium was replaced with ice-cold medium, and cells were then exposed to 2.0 Gy γ–ray, at a rate of 6.22 cGy/min with a ^{60}Co source (Eldorado 78). After IR, medium was replaced with 37°C medium, and cells were incubated further at 37°C, 5% CO_2 before harvesting.

3. RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) assay

Total RNA was extracted from WSU-HN cell lines using TRIzol®LS reagent (Invitrogen, CA, USA). Briefly, media was aspirated and cells were washed once with 1X phosphate buffered saline (PBS), cells were then trypsinized with 1X Trypsin-EDTA. Pellet was wash twice with 1X PBS before RNA extraction process.

For RNA extraction, 1 ml TRIzol®LS reagent was added to 5 to 10×10^6 cells/1.5 ml microcentrifuge tube. Cells were then repetitive pipetting. The homogenized sample was incubated at room temperature for 5 minutes. To separate RNA from DNA and protein, sample was added with 0.2 ml chloroform and shaked vigorously by hand for 15 seconds. Sample was incubated at room temperature for 3 minutes and centrifuged at 12000 g for 15 minutes at 4°C. The aqueous phase (upper layer) was transfered to a fresh RNase-free 1.5 ml microcentrifuge tube. For RNA purification, sample was added with 0.5 ml isopropyl alcohol. Sample was then incubated at room temperature for 10 minutes and centrifuged at 12000 g for 10 minutes at 4°C. Supernatant was discarded and RNA pellet was found at the bottom of the tube. To wash the pellet, 1 ml 75% ethanol was added and then the pellet was vortexed and centrifuged at 7500 g for 5 minutes at 4°C. Excess ethanol was removed and the pellet was air dry. RNA was dissolved in 20 µl DEPC water and incubated at 55°C for 10 minutes before collected at -80°C.

For reverse transcription polymerase chain reaction (RT-PCR), the first strand cDNA templates were prepared with the RevertAid™ first strand cDNA synthesis kit (Fermentas, Vilnius, Lithuania). As described by the manufacturer, 5 µg of total RNA was reversed transcribed in a reaction mixture containing containing 0.025 mg/µl

oligo$(38)_{18}$, 50 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 10 mM DTT, 1 u/ml ribonuclease inhibitor, 1 mM dNTP and 10 u/ml of Moloney murine leukemia virus reverse transcriptase (M-MuLV RT) in a final volume of 20 ml, at 42°C for 60 minutes.

4. Real time PCR

mRNA expression of PPP2R2B was detected by real-time PCR using a QuantiTect SYBR Green PCR Kit (QIAGEN, Basel, Switzerland). To control the cell background effect, the expression of PPP2R2B was normalised to the expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The specific primers for PPP2R2B and GAPDH were designed as followed:

PPP2R2B forward: 5'-GGACATTAAGCCAGCCAACA-3'

PPP2R2B reverse: 5'-TCCCTGGTCATGATATACCTCC-3'

GAPDH forward: 5'-GTGGGCAAGGTCATCCCTG-3'

GAPDH reverse: 5'-GATTCAGTGTGGTGGGGGAC-3'

Each reaction was included with 0.5 μM of each primer and 1X QuantiTect SYBR Green PCR master mix. Amplification was performed up to 40 cycles, with quantification after the extension steps. All reactions were run on a Lightcycler™ instrument (Roche Applied Science).

5. Western blot analysis and antibodies

Media was aspirated and cells were washed once with 1X PBS. Cells were then trypsinized with 1X Trypsin-EDTA. Pellet was wash twice with 1X PBS and total

proteins were extracted into lysis buffer (0.05 M Tris, pH 7.4; 1% SDS), boiled for 5 minutes and immediately placed on ice. Total proteins were electrophoresed on SDS polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to either polyvinylidene difluoride (PVDF) or nitrocellulose membranes.

Here are western blotting conditions of protein detection:

1. To detect PPP2R2B, total proteins were electrophoresed on 8% SDS polyacrylamide gel at 200 Volt for 40 minutes and were transferred onto nitrocellulose membrane at 100 Volt for 1.30 hours.

2. To detect anti-phospho histone H2AX (γ-H2AX), total proteins were electrophoresed on 15% SDS polyacrylamide gel at 200 Volt for 40 minutes and was transferred onto nitrocellulose membrane at 100 Volt for 40 minutes.

3. To detect GAPDH, total proteins were electrophoresed on 8% SDS polyacrylamide gel at 200 Volt for 40 minutes and were transferred onto nitrocellulose membrane at 100 Volt for 1.30 hours.

4. To detect phospho ATM (Ser1981) (ATMS1981p), total proteins were electrophoresed on 5% SDS polyacrylamide gel at 100 Volt for 5 hours and was transferred onto PVDF membrane at 20 mA, overnight.

5. To detect ATM, total proteins were electrophoresed on 5% SDS polyacrylamide gel at 100 Volt for 5 hours and were transferred onto PVDF membrane at 20 mA, overnight.

Blotted membranes were blocked in freshly prepared 1X Tris buffer saline (TBS) with 0.1% Tween 20™ in 5 % nonfat dry milk (TBST-M) for 1 hour at room temperature with constant agitation. To detect interested proteins, the blocked membranes were incubated with specific antibodies overnight at 4°C with constant agitation. Antibodies were purchased as indicated: anti- γ-H2AX monoclonal antibody (Upstate, Charlottesville, VA, USA) (1:1000 in TBST-M), anti-GAPDH antibody (Trevigen, Gaithersburg, MD, USA) (1:3000 in TBST-M), anti-ATM (2C1) antibody (GeneTex, San Antonia, TX, USA) (1:500 in TBST-M), anti-ATMS1981p monoclonal antibody (Upstate, Charlottesville, VA, USA) (1:1000 in TBST-M) and anti-PPP2R2B monoclonal antibody (ABNOVA, Taipei City, Taiwan) (1:500 in TBST-M). The membranes were washed three times, 5 minutes each, with agitation in TBST and then incubated with secondary antibody, either horseradish peroxidase (HRP)-goat anti-rabbit IgG (H+L) conjugated (Zymed® Laboratories, San Francisco, CA, USA) or goat anti-mouse IgG-HRP sc-2005 HRP conjugated (Santa Cruz Biotechnology), for 1 hour at room temperature with constant agitation. The membranes were then washed three times, 5 minutes each, with TBST. Interested proteins were detected using SuperSignal West Pico Chemiluminascent Substrate™ (Pierce). In brief, the two substrate components were mixed at a 1:1 ratio to prepare the substrate working solution. The substrate solution was added to the membranes and incubated for 5 minutes at room temperature. The excess substrate solution was removed and the membranes were covered with plastic wrap. The wrapped membranes were placed in a film cassette with the protein side facing up and exposed to X-ray film (Kodak). Exposure time may be varied to achieve optimal results.

6. Small interfering RNA transfection

For small interfering RNA (siRNA) stable transfection protocol, WSU-HN4 cells were stably transfected with siRNA against PPP2R2B mRNA. The oligonucleotide sequences of the siRNA targeting PPP2R2B are as follows:

Sense strand: 5-GATCCGCGTGATGTGACCCTAGGCTTCAAGAGAGCCTA
GGGTCACATCACGCTTTTTTGGAAA-3

Antisense strand: 5-AGCTTTTCCAAAAAAGCGTGATGTGACCCTAGGCTCTCT
TGAAGCCTAGGGTCACATCACGCG-3

Both sense and antisense strands were dissolved in approximately 100 µl of nuclease-free water. Each strand was diluted in TE in a ratio of 1:100 and absorbance at 260 nm was determined. The concentration of each strand was calculated by multiplying the A260 with the dilution factor and the extinction coefficient (~33 µg/ml). Each template strand was then diluted to approximately 1 µg/µl to be ready for annealing step. At the annealing step, briefly, 2 µl of sense and antisense siRNA template oligonucleotides were mixed with 46 µl of 1X DNA Annealing Solution (Ambion, Austin, Texas, USA) to reach a total volume of 50 µl. The mixture were heated to 90°C for 3 minutes and then placed in a 37°C incubator for 1 hour. To subclone the annealed oligonucleotide templates into the PsilencerTM 3.1 vector (Ambion, Austin, Texas, USA) (see the PsilencerTM 3.1 vector map in appendix A), 5 µl of the annealed siRNA templates were diluted with 45 µl nuclease-free water for a final concentration of 8 ng/ µl. The ligation reaction was prepared as followed: 1 of diluted annealed siRNA templates, 6 µl of nuclease-free water, 1 µl of 10X T4 DNA ligase buffer, 1 µl of

p*Silencer* 3.1 hygro vector (Ambion, Austin, Texas, USA) and 1 µl of T4 DNA ligase (5U/µl). All mixture was mixed and incubated overnight at 16°C. Ligated plasmids were then transformed into *E. coli* DH5α and the transformed cells were plated on LB plates containing 100 µg/ml ampicillin and grown overnight at 37°C. Bacterial clones were selected and cultured for plasmid extraction. Plasmids were performed DNA sequencing to confirm the siRNA sequences. For plasmid transfection process, 2×10^5 of WSU-HN4 cells were seeded in DMEM with 10% FBS to achieve 30% - 60% confluent after approximately 24 hours (in 6-well plate). To prepare complex reagent for plasmid transfection, 3 µl of siPORT *XP-1* were diluted in Opti-MEM I medium (Gibco BRL) in polystyrene tube for a final volume of 100 µl. Mixture was vortexed thoroughly before incubated at room temperature for 5-20 minutes. The mixture was then added with 1 µg of plasmid DNA, mixed by gently flinking the tube and incubated further at room temperature for 5-20 minutes. At the transfection step, WSU-HN4 cells were washed with 1X PBS and rinsed briefly with serum-free DMEM before adjusting the volume of serum-free media to 2 ml. The mixture of siPORT *XP-1*/DNA with Opti-MEM I medium and the plasmid DNA were then dropwised onto the cells. Plates were then gently rocked back and forth to evenly distribute the complexes. Incubation was performed for 2-8 hours in 37°C with 5% CO_2. Media containing siPORT XP-1/DNA complex was then removed and replaced with DMEM with 10% FBS. After transfection for 24 hours, media was changed to DMEM with 10% FBS containing 250 µg/ml of hygromycin (140). Cells were cultured in in media containing hygromycin until all of the cells in non-transfected control culture were killed. The suvived cells were cultured further until cells had reached to the confluency in 6 well plates. Cells were splitted and grown in media

with 150 µg/ml hygromycin to prevent the accumulation of cells that no longer expressed hygromycin resistance. The siRNA transfected-WSU-HN4 cells were harvested to confirm the down-regulation of PPP2R2B protein expression by western blot analysis.

For siRNA transient transfection process, WSU-HN13 cells were seeded in 60-mm tisssue culture dish. Immediately before siRNA transfection process, media was changed to 4 ml DMEM with 10% FBS. The transfection mixture/reaction was prepared by mixing 20 µl of HiPerFect transfection reagent (QIAGEN, Basel, Switzerland) with 5 nM of siRNA (final concentration) against PPP2R2B mRNA. The final volume was made up to 100 µl by serum-free DMEM. The transfection reaction was incubated at room temperature for 10 minutes and dropped wise on media. Cells were then incubated further at 37°C, 5% CO_2 before harvesting at the time that PPP2R2B mRNA was lowest expressed. To monitor the PPP2R2B gene silencing, proteins were collected on day 1 to day 5 after siRNA transfection and western blot analysis against PPP2R2B protein was performed. The siRNA sequences against PPP2R2B (ON-TARGETplus SMARTpool L-003022-00-0005, Human PPP2R2B, NM_181676) was designed by and purchased from Thermo Scientific, Dhamacon. The sequences are as followed:

ON-TARGETplus SMARTpool siRNA J-003022-09, target sequence: UCGAUUACCUGAAGAGUUU

ON-TARGETplus SMARTpool siRNA J-003022-10, target sequence: GGGUCGGGUUGUAAUAUUU

ON-TARGETplus SMARTpool siRNA J-003022-11, target sequence: GAAUGCAGCUUACUUUCUU

ON-TARGETplus SMARTpool siRNA J-003022-12, target sequence: CCACACGGGAGAAUUACUA

A nonsilencing siRNA with no homology to any known mammalian genes (AllStars negative control siRNA, QIAGEN, Basel, Switzerland) was together transiently transfected as a negative control siRNA of each experiment.

7. Clonogical cell survival assay

The $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells, in the exponential growth phase, were seeded at 200 cells per 100-mm tissue culture dish following IR treatment at 0, 1, 2, 3, 4 and 5 Gy γ–ray. After the 11^{th} passage, cells were fixed with 10% trichloroacetic acid (TCA) for 30 minutes and washed three times with tap water. Cells were air dried and stained with 0.057% (w/v) sulphorhodamine B (SRB) for 10 minutes, washed three times with 1% acetic acid. Colonies containing > 50 cells were marked as survivors.

8. Immunofluorescence microscopy

WSU-HN13 cells were seeded for 1.5×10^5 cells/60 mm^2 dish for 24 hours on uncoated 22×22 mm^2 cover glasses. Cells were then exposed to 2.0 Gy γ–ray and incubated further at 37°C, 5% CO_2 for 90 minutes before immunofluorescence staining. Briefly, media was aspirated and cells were washed twice with 1X PBS. Cells were fixed with 4% paraformaldehyde in 1X PBS at room temperature for 10 minutes and wash three times with 1X PBS. Cells were then lysed with 0.05% TritonX-100 in 1X PBS at room temperature for 10 minutes and wash three times with 1X PBS. To block unspecific binding of antibodies, 3% BSA in 1X PBS was added and incubated at room temperature

for 30 minutes. Excess BSA was removed and primary antibody diluted in 3% BSA in 1X PBS was added and incubated for 1.30 hours at room temperature.

Here are list of primary antibodies for immunofluorescence microscopy:

1. anti-ATM (2C1) monoclonal antibody (mouse) was purchased from GeneTex (San Antonia, TX, USA) (Catalog number GTX70103). For immunofluorescence staining, 0.01 μg/μl of antibody was freshly diluted in 3% BSA in 1X PBS before directly applied on cells.

2. anti-phospho ATM (Ser1981) monoclonal antibody (mouse) was purchased from Upstate (Charlottesville, VA, USA) (Catalog number 05-740). For immunofluorescence staining, 0.01 μg/μl of antibody was freshly diluted in 3% BSA in 1X PBS before directly applied on cells.

3. anti-phospho histone H2AX (Ser139) monoclonal antibody (mouse) was purchased from Upstate (Charlottesville, VA, USA) (Catalog number 05-636). For immunofluorescence staining, 0.005 μg/μl of antibody was freshly diluted in 3% BSA in 1X PBS before directly applied on cells.

4. anti-PPP2R2B polyclonal antibody (rabbit) was purchased from Upstate (Charlottesville, VA, USA) (Catalog number 07-334). For immunofluorescence staining, 0.02 μg/μl of antibody was freshly diluted in 3% BSA in 1X PBS before directly applied on cells.

After primary antibody incubation, cells were washed three times with 1X PBS and either Alexa Fluor 488 goat anti-mouse (Catalog number A11001) or Alexa Fluor

546 goat anti-rabbit (Catalog number A 11010) purchased from Invitrogen (CA, USA) was added. Cells were incubated for 1 hour at room temperature without light and washed three times with 1X PBS. Cover glass was mounted with mounting medium with DAPI and edges were sealed with clear nail polish. The fluorescence signal was visualized and imaged using a Zeiss AxioImager Z1 microscope coupled to an ApoTome, using AxioVision 4.6 software.

9. Statistical analysis

A one-way Spearman statistical analysis was used to assess the differences between the amounts of PPP2R2B mRNA and ATMS1981p or γ-H2AX (γ-H2AX and ATMS1981p band densities were normalised to GAPDH and total ATM, respectively), as well as the induction of ATMS1981p and γ-H2AX. Differences were considered significant if $p<0.05$.

CHAPTER IV

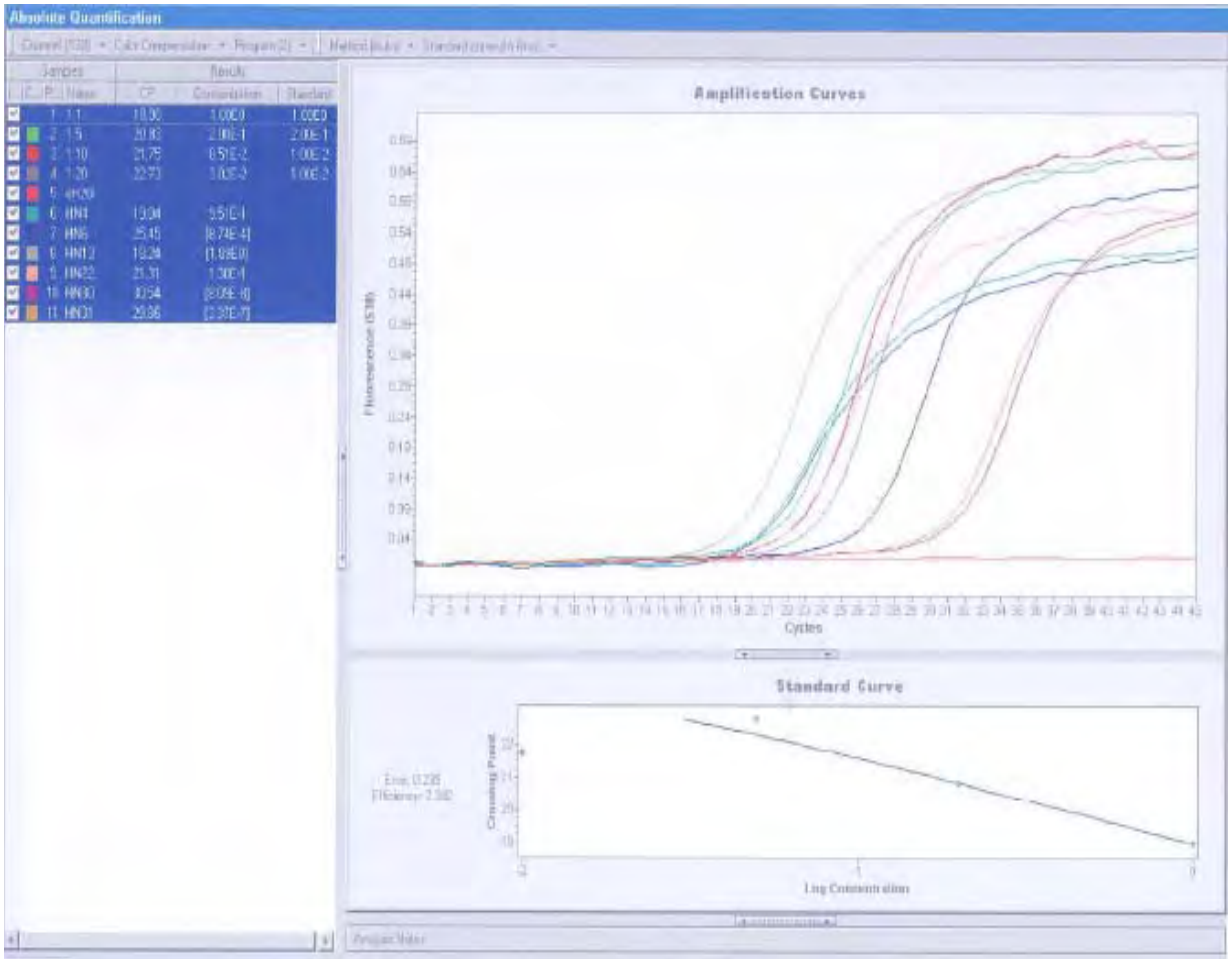
RESULTS

PP2A is a protein phosphatase enzyme that is known to reverse the phosphorylation of serine/threonine (Ser/Thr) residues of proteins, including ATM (7). However, the B subunit of PP2A-associated ATM has not been identified. Recently, inhibition of PP2A by a specific inhibitor known as okadaic acid or OA was shown to predomonantly localize NLS-containing proteins within cytoplasm, suggesting the role of PP2A in NLS-containing proteins nuclear localization (13). We found that HNSCC cell lines that had lower PPP2R2B mRNA expression exhibited low basal levels of ATMS1981p and γ-H2AX, an ATM downstream histone phosphorylation target, and the expression of *PPP2R2B* was investigated to be down-regulated in HNSCC (1, 2). We, therefore, hypothesized that PPP2R2B might play a role in the nuclear localization of ATM. To support our hypothesis, siRNA against PPP2R2B mRNA was employed and the nuclear localization of ATM was then examined. Fascinatingly, ATM was found to mainly localize within cytoplasm of HNSCC cells containing PPP2R2B siRNA. From our data, we are the first group who identified the molecular role of PPP2R2B-containing PP2A and the molecular signal of ATM nuclear localization.

1. PPP2R2B expression is varied in HNSCC cells

Lately, the expression status of *PPP2R2B* in HNSCC was examined by gene expression microarray (1, 2). The data indicated that *PPP2R2B* was significantly down-regulated in HNSCC, with these studies reporting p values of 8.8×10^{-5} and 2.1×10^{-3}. As a result, the decrease of PPP2R2B mRNA expression may be important in HNSCC development. Because, there was a panel of WSU-HN cell lines, we, therefore, investigated the mRNA expression profile of *PPP2R2B* in these cells using realtime PCR. WSU-HN cell lines used in our experiment were WSU-HN4, WSU-HN6, WSU-HN13, WSU-HN22, WSU-HN30 and WSU-HN31. They were originated from HNSCC cells but different part (appendix B). Our results showed that PPP2R2B was highly expressed in WSU-HN4, WSU-HN13 and WSU-HN22 cells. However, the expression of PPP2R2B was hardly detected in WSU-HN6, WSU-HN30 and WSU-HN31 cells (figure 8). Interestingly, our results were found to be related to the gene expression microarray studied by Ms. Leelahavanichkul K. (Oral and Pharyngeal Cancer Branch staff, National Institute of Health, USA) (unpublished data, see appendix C). The role of PPP2R2B in HNSCC development was thus planned to be investigated further.

a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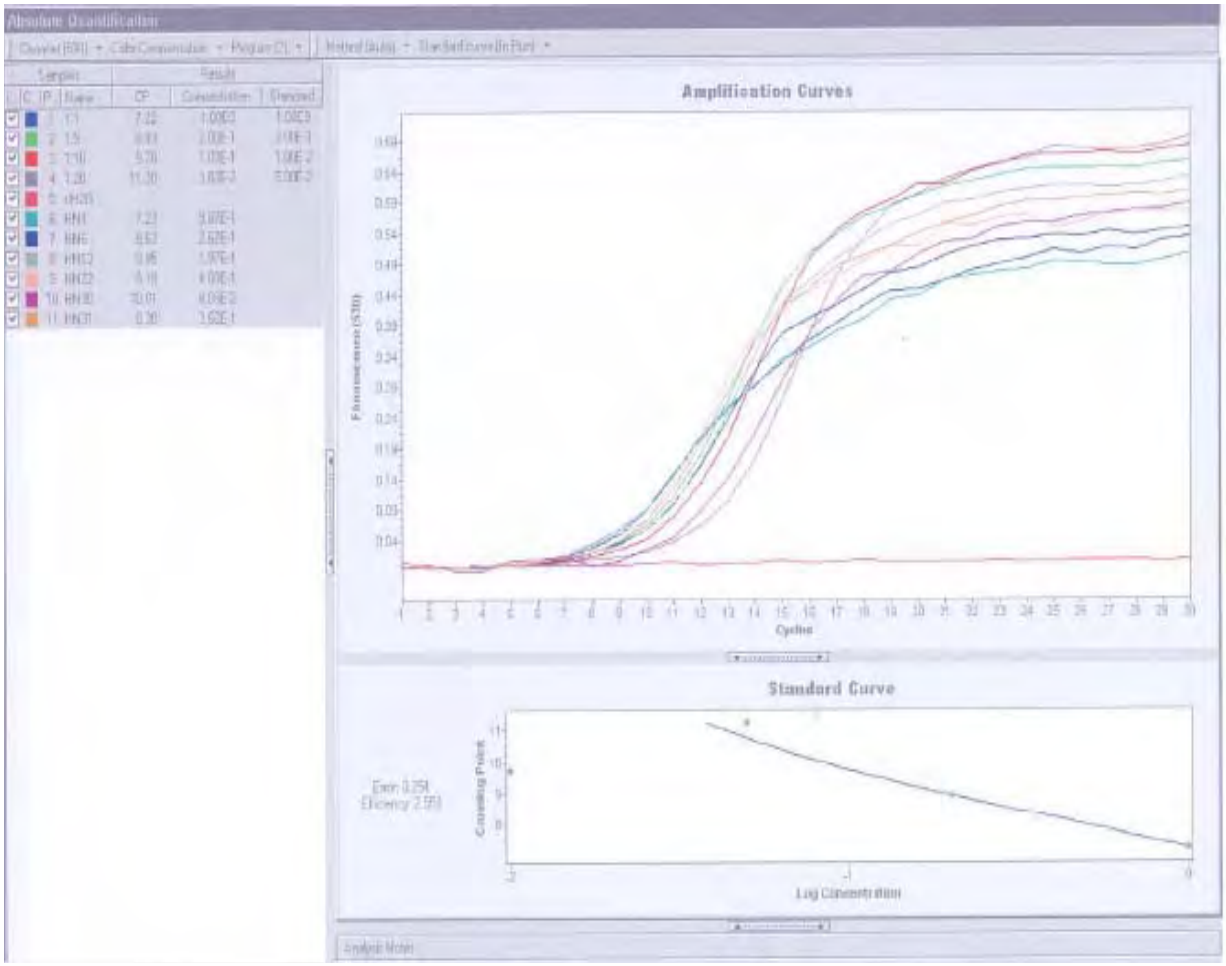
Amplification Curves
Fluorescence (SYBR)
Cycles
Standard Curve
Crossing Point
Log Concentration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Log Concentration

b

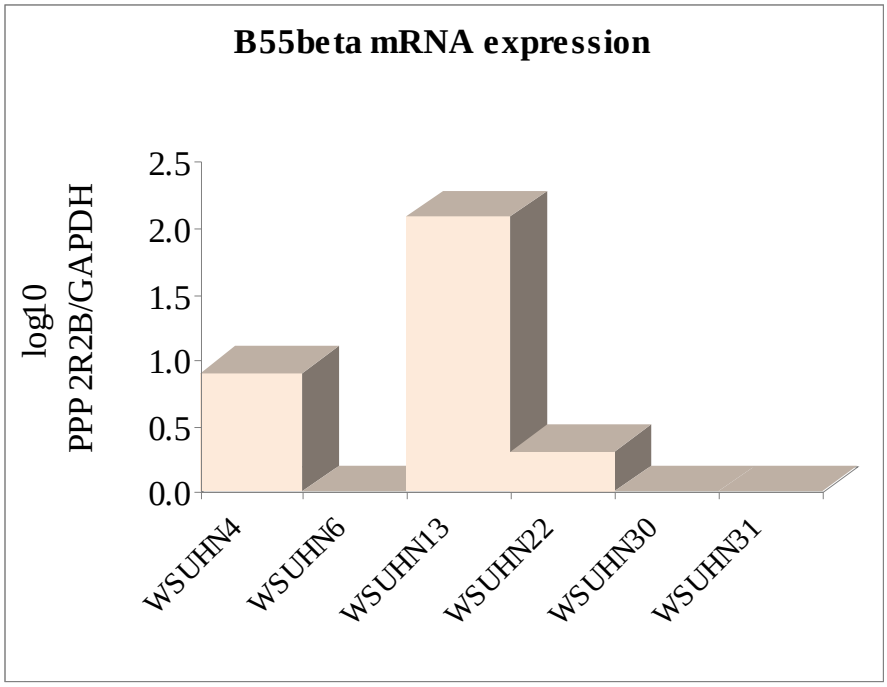


Figure 8 PPP2R2B mRNA expression in WSU-HN cell lines. (a) The mRNA expression of PPP2R2B and GAPDH were identified using realtime PCR. (b) PPP2R2B expression was normalized by GAPDH expression.

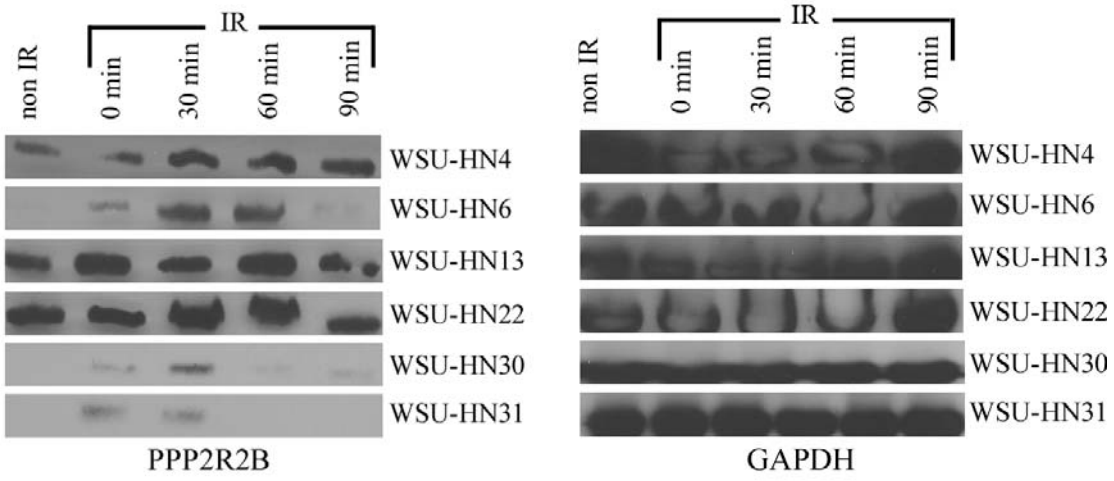
2. The autophosphorylation of ATM is PPP2R2B dependent

PP2A has been found to play a significant role in ATM autophosphorylation and the nuclear localyzation of the NLS-containing proteins (7, 13). From our previous experiment, the expression of PPP2R2B has been found to be varied in WSU-HN cell liness. We, therefore, firstly investigated whether PPP2R2B expression was associated with ATM autophosphorylation or not. Primarily, the induction of γ–H2AX, which is

phosphorylated by ATM and is responsible for signaling repair proteins to DSB sites, was examined. From figure 9a, there was no γ–H2AX detected at basal level (non-IR) of WSU-HN cell lines that expressed low level of PPP2R2B mRNA (WSU-HN6, WSU-HN30 and WSU-HN31). However, γ–H2AX was instead detectable in WSU-HN4, WSU-HN13 and WSU-HN22 cell lines that expressed high level of PPP2R2B mRNA. The profile of γ–H2AX induction was similarly found in WSU-HN cell lines after IR, the phosphorylation of H2AX was induced immediatly after cells were exposed to 2.0 Gy γ–ray (0 min) in WSU-HN4, WSU-HN13 and WSU-HN22 cell lines. This γ–H2AX induction was remained to be seen until 90 minutes after IR that was the last time point observed. On the other hand, γ–H2AX was hardly induced after IR in WSU-HN6, WSU-HN30 and WSU-HN31 cell lines. To normalize γ–H2AX induction, the expression of GAPDH was parallelly investigated. However, the reason why GAPDH bands were saturated because the amount of proteins that was loaded into each well was too high when compare to the amount of substrate enzyme used to detect in immunoblotting process. Because WSU-HN cell lines that expressed high level of PPP2R2B had higher level of γ–H2AX induction than WSU-HN cell lines with low level of PPP2R2B expression, the induction of ATMS1981p was, therefore, examined in all WSU-HN cell lines. As shown in figure 9b, ATMS1981p induction was highly detected in WSU-HN4, WSU-HN13 and WSU-HN22 cell lines at basal level. On the contrary, the induction of ATMS1981p was hardly detectable in WSU-HN6, WSU-HN30 and WSU-HN31 cell lines. To confirm the association between PPP2R2B expression and either γ–H2AX or ATMS1981p inductions and the association between ATMS1981 induction and γ–H2AX induction in WSU-HN cell lines, spearman statistical analysis was performed. Our results

showed that all three associations were proved to be significant, with $p<0.05$ (figure 10). From our previous investigation, all experiments had been done in WSU-HN cells panel that had different cell background effect. To exclude this limitation, WSU-HN4 that expressed high level of PPP2R2B mRNA was stably transfected with siRNA against PPP2R2B. As shown in figure 11a, PPP2R2B protein was down-regurated in WSU-HN4 cells transfected with PPP2R2B siRNA ($siRNA_{PPP2R2B}$) when compare to WSU-HN4 cells trasfected with empty vector ($siRNA_{neg}$). Both $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells were then exposed to 2.0 Gy γ–ray and ATM, ATMS1981p, γ–H2AX and GAPDH were examined. Interestingly, ATMS1981p and γ–H2AX were induced higher in $siRNA_{neg}$ cells than in $siRNA_{PPP2R2B}$ cells after exposure to IR (figure 11b). Our results, therefore, confirm the association of PPP2R2B expression and the induction of ATMS1981p and γ–H2AX.

a



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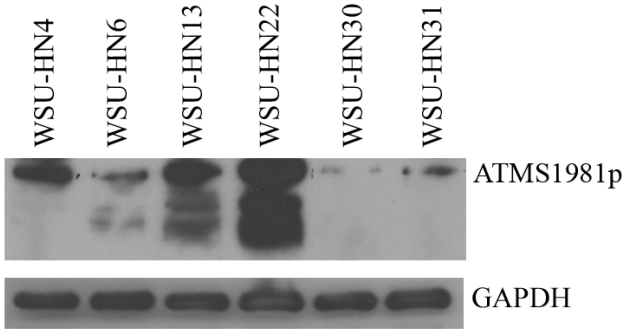


Figure 9 The induction of ATMS1981p and γ–H2AX in WSU-HN cell lines. (a) γ–H2AX induction was examined in WSU-HN cell lines both before and after IR (2.0 Gy γ–ray) at different time points as indicated in figure. (b) ATMS1981p induction was also investigated at basal level in WSU-HN cell lines. Both γ–H2AX and ATMS1981p inductions were normalized by GAPDH expression.

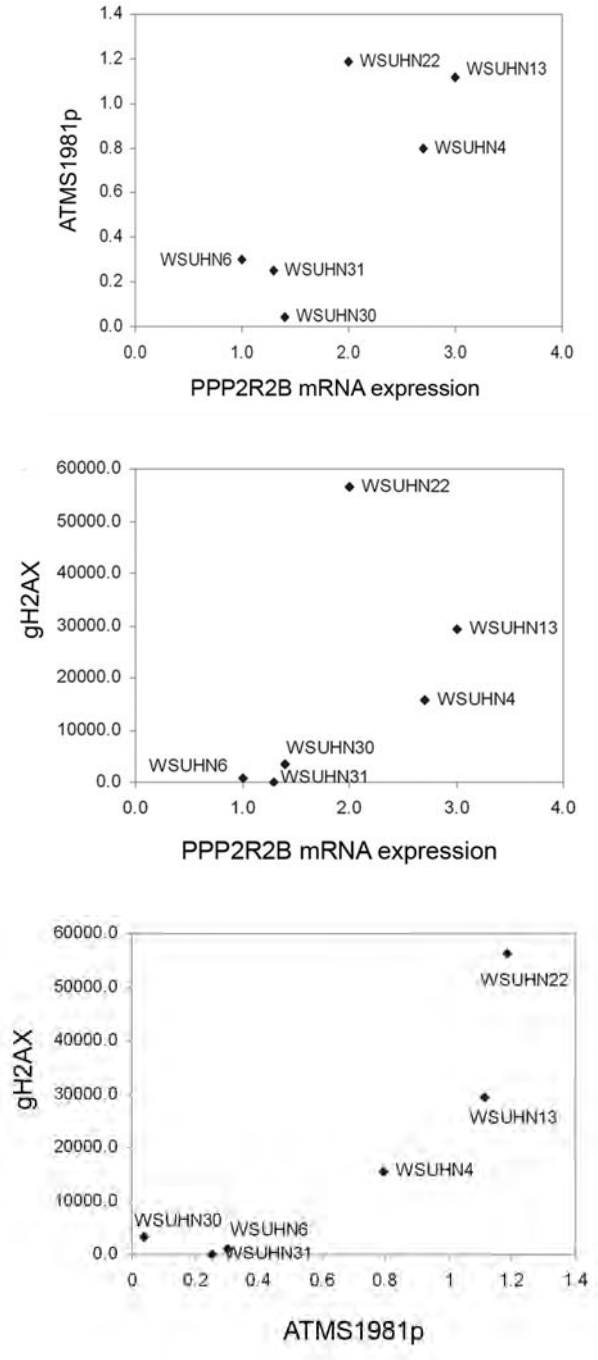
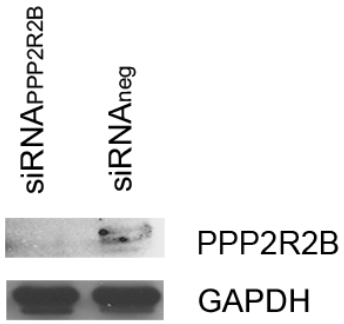


Figure 10 The induction of ATMS1981p and γ–H2AX were associated with the expression of PPP2R2B. Spearman statistical analysis revealed the association between PPP2R2B expression and either ATMS1981p induction or γ–H2AX induction and the association between ATMS1981p induction and γ–H2AX induction in WSU-HN cell lines to be significant, with $p<0.05$.

a



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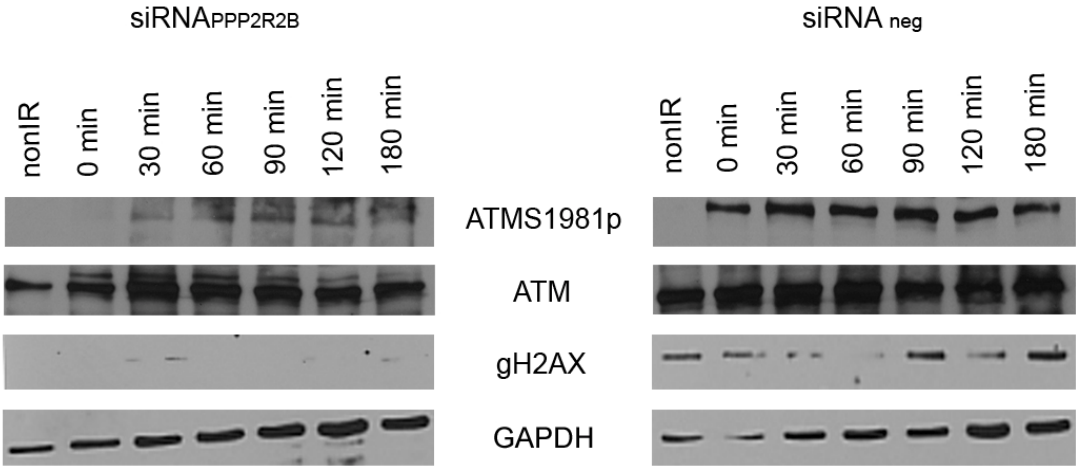

Figure 11 The induction of ATMS1981p and γ-H2AX in $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells. (a) WSU-HN4 cells were stably transfected with siRNA against PPP2R2B and the PPP2R2B protein was proved to be down-regulated in $siRNA_{PPP2R2B}$ cells when compared to PPP2R2B protein in $siRNA_{neg}$ cells. (b) $siRNA_{neg}$ and $siRNA_{PPP2R2B}$ cells were exposed to IR at 2.0 Gy γ–ray and ATMS1981p, ATM, γ-H2AX and GAPDH were examined at different time points as indicated in figure.

3. HNSCC cells with low PPP2R2B expression exhibit low survival rate after exposure to ionizing irradiation

DSB is known to be harmful to the cells because it leads to gross chromosomal aberrations if it is not rejoined quickly and properly. As a result, mammalian cells have evolved systems for rapidly transmitting the damage signals to the cell cycle arrest, apoptotic machineries and DNA repair mechanisms. From our previous results, the expression of PPP2R2B was indicated to be associated with the induction of ATMS1981p, which then triggered the DSBs repair pathway through the phosphorylation of H2AX (γH2AX). We, therefore, investigated the effectiveness of $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells to process DNA repair mechanisms after exposure to IR (from 0.0 to 5.0 Gy γ–ray as indicated in figure 12) through a technique called clonogical cell survival assay. As shown in figure 12, the survival of $siRNA_{neg}$ cells was higher than $siRNA_{PPP2R2B}$ cells after IR at 1.0 and 2.0 Gy γ–ray as indicated on the number of colony formed after IR. This data suggested that $siRNA_{PPP2R2B}$ cells, which had the same cell background effect as $siRNA_{neg}$ cells except PPP2R2B expression, were less effective in DSBs repair than $siRNA_{neg}$ cells. However, the γ–ray at dose 3.0 to 5.0 Gy were too high for both $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells to survive. This experiment showed that $siRNA_{PPP2R2B}$ cells were radiosensitive more than $siRNA_{neg}$ cells and supported our previous result that the expression of PPP2R2B was associated with the induction of ATM autophosphorylation.

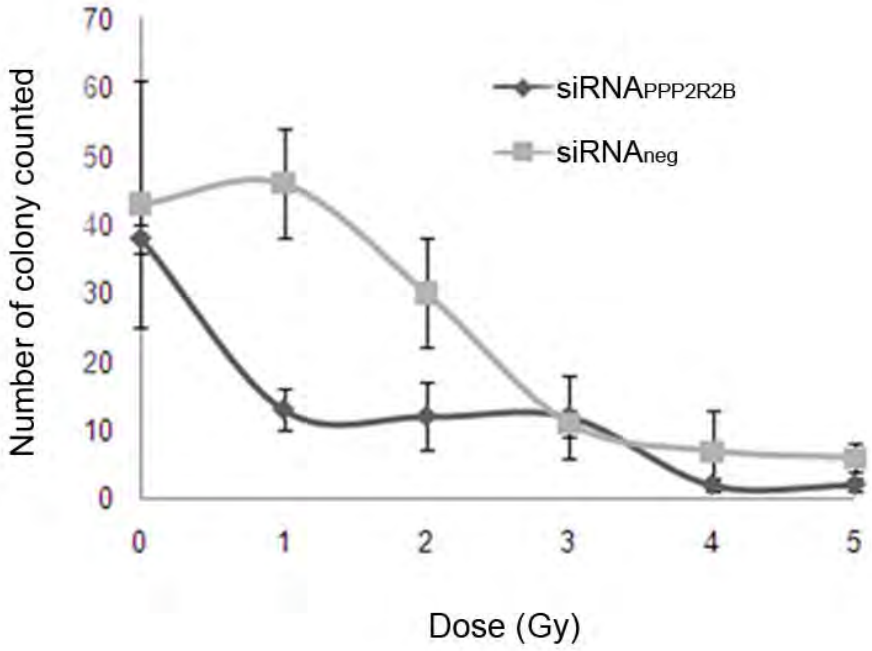

Figure 12 $siRNA_{PPP2R2B}$ cells were radiosensitive more than $siRNA_{neg}$ cells. $siRNA_{PPP2R2B}$ and $siRNA_{neg}$ cells were exposed to IR at 1.0 to 5.0 Gy γ–ray and subjected to clonogenic cell survival assay. Result indicated the role of PPP2R2B in DSBs repair pathway.

4. ATM nuclear localization is PPP2R2B dependent

Because the NLS domain of ATM has recently been identified to locate within its amino terminal (12) and interference of PP2A with its specific inhibitor known as okadaic acid or OA was shown to have an effect on nuclear localization of the endogenous NLS-containing proteins (13), we thus investigated the role of OA on ATM nuclear localization. Primarily, 0.5 μM of OA was added to WSU-HN13 cells, which highly expressed PPP2R2B mRNA, and immunoflorescence staining against ATM was performed. Interestingly, ATM was found to be predominantly localized within cytoplasm after OA addition. On the other hand, ATM was found within both cytoplasm and nucleus of WSU-HN13 cells added with 0.1% DMSO (control) (figure 13). Our result indicated the role of PP2A in ATM nuclear localization. From our previous results, because the expression of PPP2R2B was found to be associated with the induction of ATMS1981p, we thus examined further the role of PPP2R2B in ATM nuclear localization. WSU-HN13 cells were transiently transfected with either PPP2R2B siRNA ($siRNA_{PPP2R2B}$ cells) or a nonsilencing siRNA with no homology to any known mammalian genes ($siRNA_{neg}$ cells) as a control of the experiment. The transfected cells were either unexposed or exposed to IR at 2.0 Gy γ–ray and immunofluorescence staining against PPP2R2B, ATM, ATMS1981p and γ-H2AX were performed. As shown in figure 14, PPP2R2B was mainly localized within cytoplasm of $siRNA_{PPP2R2B}$ cells while it was found within both cytoplasm and nucleus of $siRNA_{neg}$ cells. In $siRNA_{PPP2R2B}$ cells, both ATM and ATMS1981p were detected only within cytoplasm both before and after IR. Moreover, no foci formation of γ-H2AX was found within $siRNA_{PPP2R2B}$ cells even when there was DSBs induction (after IR). On the other hand, ATM and

ATMS1981p were detected within cytoplasm and nucleus of $siRNA_{neg}$ cells and foci formation of γ-H2AX was found after IR within $siRNA_{neg}$ cells toas well. Taken together, our results suggested the role of PPP2R2B in ATM nuclear localization.

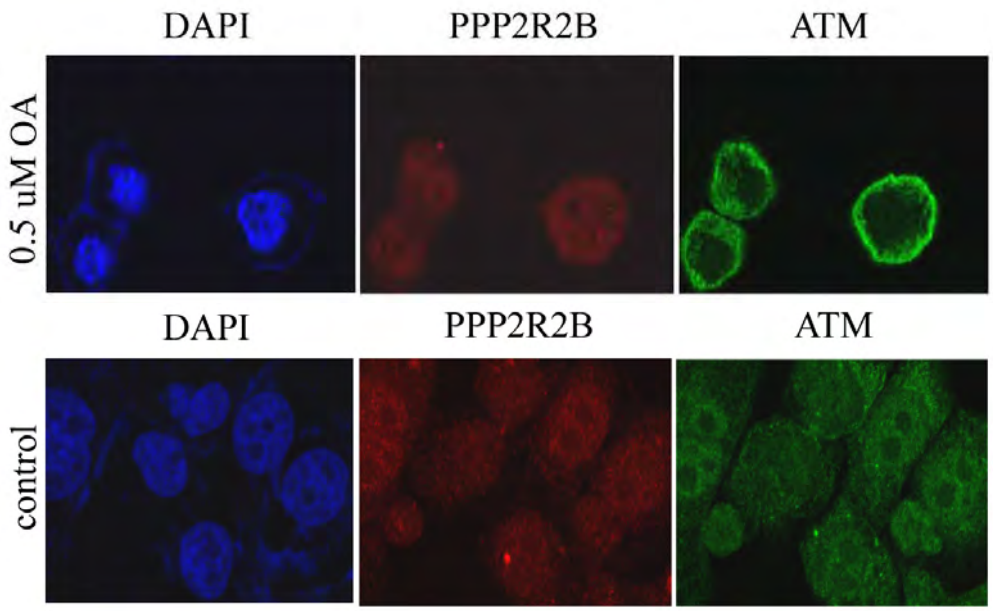

Figure 13 PP2A played an important role in ATM nuclear localization. WSU-HN13 cells were treated with either 0.5 μM OA or 0.1% DMSO as a control of the experiment. Immunofluorescence staining against PPP2R2B and ATM were performed to visualize the nuclear localization of ATM.

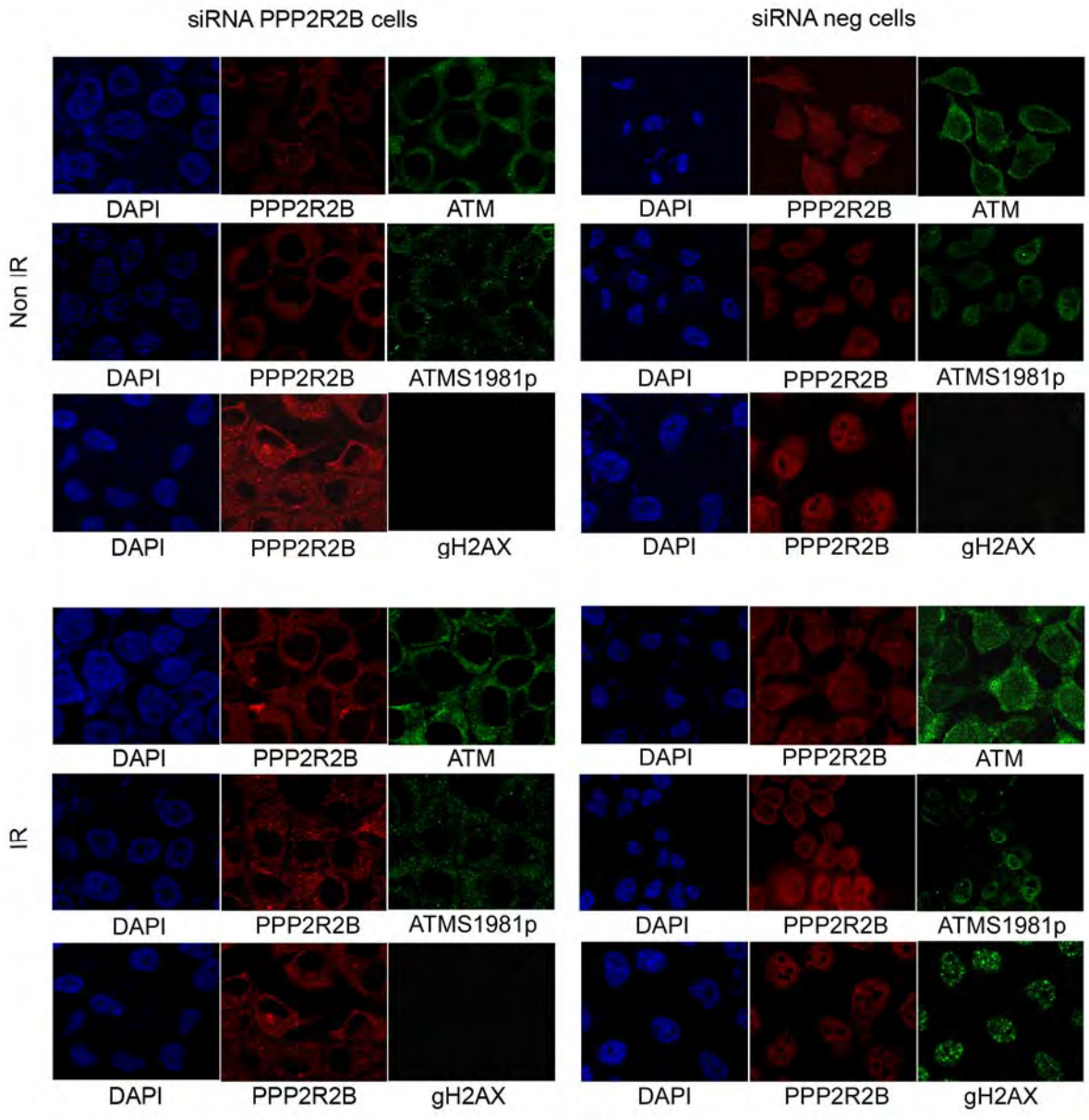

Figure 14 PPP2R2B played an important role in ATM nuclear localization. WSU-HN13 cells were transiently transfected with either PPP2R2B siRNA ($siRNA_{PPP2R2B}$ cells) or a nonsilencing siRNA with no homology to any known mammalian genes ($siRNA_{neg}$ cells). Both $siRNA_{PPP2R2B}$ cells and $siRNA_{neg}$ cells were either unexposed or exposed to IR at 2.0 Gy γ–ray. Immunofluorescence staining against PPP2R2B, ATM, ATMS1981p and γ-H2AX were performed.

CHAPTER V

CONCLUSION AND DISCUSSION

The expansion of CAG repeat at the promoter region of the *PPP2R2B* gene has been identified to cause SCA12, which characterized by a mild cerebellar dysfunction, including gait ataxia and limb and eye movement dysmetria. From our results, we found that HNSCC cell lines without PPP2R2B expression exhibited less efficiency to correct radiosensitivity in response to DSBs. These data were similar to those in cells without ATM function. ATM is a nuclear protein kinase that was discovered as a mutated protein in patients with A-T, a severe genetic disorder characterized by cerebellar degeneration, immunodeficiency, hypersensitive to DSB-inducing agents, thymic and gonadal atrophy, telangiectasia and an increased risk of cancer (102). Following DSB formation, ATM rapidly autophosphorylates on residue Serine 1981, and the inactive ATM dimers are converted into active ATM monomers (56). Active phosphorylated ATM molecules can now interact and phosphorylate downstream proteins that affect cell cycle checkpoints, DNA repair pathway, for example. Recently, the NLS domain of ATM has been identified to locate within its amino terminal. Interestingly, ATM protein that contains only the carboxyl-terminal half not only localized within cytoplasm, but also could not autophosphorylate itself. As a result, neither ATM substrates phosphorylation nor DSBs repair mechanism have occurred (12). Apart from the less efficiency to correct radiosensitivity of low PPP2R2B-expressed HNSCC cell lines, we found that PPP2R2B-containing PP2A also played a significant role in ATM nuclear localization. ATM of

WSU-HN13 cells, which express high level of PPP2R2B, transfected with PPP2R2B siRNA ($siRNA_{PPP2R2B}$ cells) was predomonantly localized within cytoplasm. As a result, ATM could not autophosphorylate itself and, therefore, no γ-H2AX foci formation after DSBs induction. Our results are supported by previous study, inhibition of PP2A that is found to be constitutively interacted with ATM to reverse ATM autophosphorylation, with a specific inhibitor known as okadaic acid or OA has been shown to predominantly localize ATM within cytoplasm. This phenomenon may be explained through OA activity itself. Because OA binds to the carboxyl terminus of the C subunit of PP2A, the methyltransferase is thereby prevented to access to its target site, the C subunit carboxyl terminal. As a result, no trimeric PP2A is formed (25). A significant function of PP2A in signaling nuclear protein transport has been explained. After the addition of staurosporine, the broad specificity protein kinase inhibitor, the nuclear protein transport inhibition was reversed, suggesting that the inhibition of nuclear protein transport is due to the elevation of the phosphorylation of a component of the nuclear transport machinery (159). The other example is that PP2A was found to interact with bone morphogenetic protein (BMP)-Smad1 complex. PP2A-mediated dephosphorylation of the BMP-Smad1 led to the induction of nuclear translocation of Smad1 and thus the amplification of the BMP signal (160). Taken together, PPP2R2B-containing PP2A and may be some other PP2A trimerics play a significant role in ATM nuclear localization.

From our immunofluorescence staining against PPP2R2B (figure 13 and 14), PPP2R2B was found to localize within both cytoplasm and nucleus. However, ATM was mainly localized within cytoplasm of cells without nuclear PPP2R2B (figure 14). Therefore, nuclear PPP2R2B-containing PP2A is the key PP2A that import ATM from

cytoplasm to nucleus. The transport receptors that belong to the importin protein family recognize the NLS-containing proteins through the NLS sequence and the importin 9 that belongs to the importin-β family was identified to interact with the A subunit of PP2A (13). Moreover, the importin β1/α1 heterodimer was found to directly interact with the NLS domain of ATM (12). As a result, PPP2R2B-containing PP2A may interact with ATM/importin β1/α1 complex to import ATM into nucleus. However, importin β1 was also identified to interact with and import the splicing factor PRPF31. Importin is known to exclusively function as a nuclear transport receptor for highly charged nuclear proteins review in (161). Importin β1 may thus target a wide range of nuclear proteins.

The function of PP2A itself is shown to reverse a phosphorylation reaction, including ATM autophosphorylation (7). However, we found that PPP2R2B that is the B regulatory subunit of PP2A is crutial for ATM nuclear localization. Therefore, PPP2R2B-containing PP2A possibly function as one of the ATM nuclear receptor complex that needs phosphatase activity to signal the nuclear import. ATM was identified to constitutively interact with PP2A that reverse the autophosphorylation of ATM (7). Furthermore, Guo *et al.* proposed a model in which IR activated ATM through a signalling pathway involving the dissociation of the regulatory subunit B, alpha isoform (PPP2R2A) from heterotrimeric PP2A (57). It is possible that more PP2A trimerics may involve in ATM nuclear import and maintaining of nuclear ATM dimer at non DSBs induction.

To sum up, ATM plays an important role in maintaining genomic integrity (51). Loss of ATM function has been reported to promote both inherited and sporadic cancers. Here, we showed that HNSCC cells with decreased PPP2R2B expression predominantly

localized ATM within cytoplasm. Because the PPP2R2B subunit was significantly down-regulated in HNSCC (1, 2), it seems reasonable to conclude that PPP2R2B expression is crucial for head and neck squamous cell DSB repair and consequently maintaining genomic integrity. Consequently, a decrease in PPP2R2B expression might contribute to HNSCC genomic instability. Here, we are the first group who identify the molecular signal of ATM nuclear localization and the biological role of PPP2R2B.

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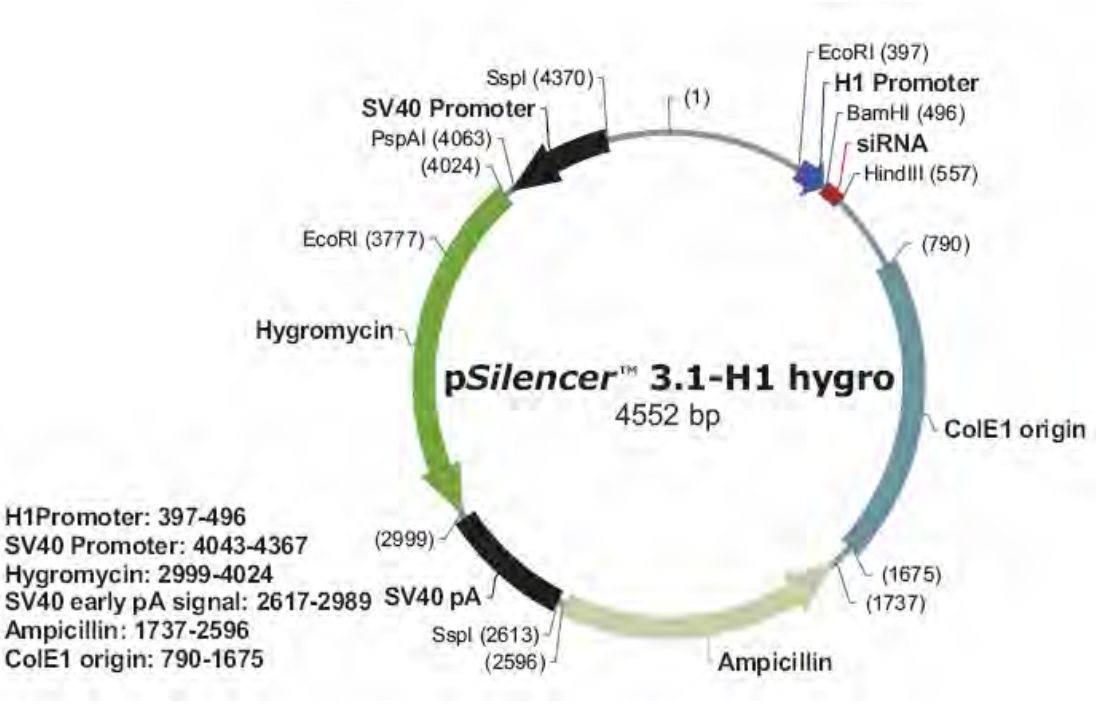
APPENDIX

APPENDIX A

PLASMID

p*Silencer*™ 3.1-H1 hygro



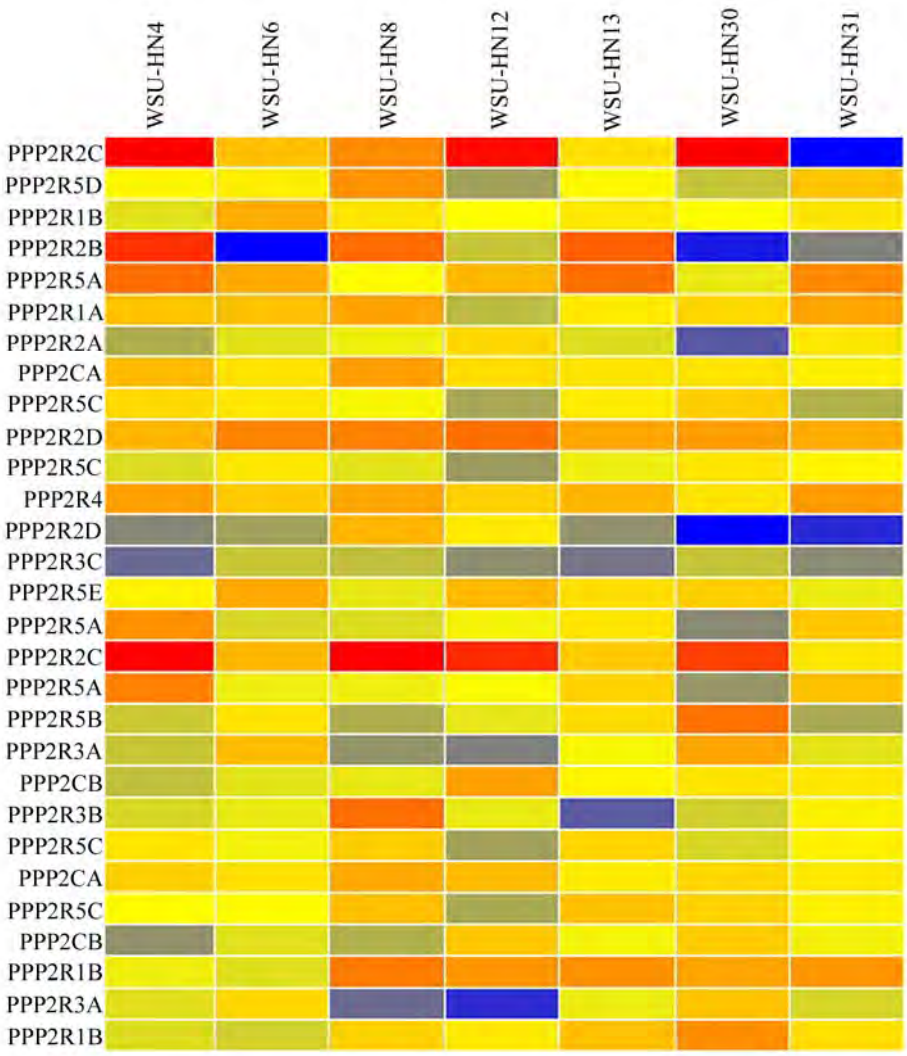
APPENDIX B

LIST OF WSU-HN ORIGIN

HNSCC Cell line	Origin
WSU-HN4	Base of tongue
WSU-HN6	Base of tongue
WSU-HN13	Tongue
WSU-HN22	Epiglottis
WSU-HN30	Pharynx
WSU-HN31	Lymp node

APPENDIX C

PPP2R2B GENE EXPRESSION PROFILE



BIOGRAPHY

Miss Chotika Suyarnsestakorn was born on March 19th, 1976 in Bangkok, Thailand. She received her bachelor degree in Genetic from Department of Botany, Faculty of Science, Chulalongkorn University in 1997. She then continued her master degree in Human Molecular Genetics at Imperial College of Science, Technology and Medicine, University of London, United Kingdom in 1999. After graduated, she has worked as a research assistant at National Center for Genetic Engineering and Biotechnology (BIOTEC), Thailand. Until 2004, she got a Royal Golden Jubilee (RGJ) Ph.D. Scholarship from the Thailand Research Fund (TRF) and participated in Biomedical Sciences, Inter-Department Programme of Biomedical Sciences, Faculty of Graduate School, Chulalongkorn University. During her study, she had an opportunity to do a research at National Institute of Health (NIH), Bethesda, USA, for 5 months.