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利用新一代 EGFR 酪氨酸激酶抑制劑為放射增敏劑，用於膀胱癌的治療

Utilizing New-Generation EGFR Tyrosine Kinase Inhibitor as Radiosensitizer in the Treatment of Urinary Bladder Cancer

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本論文係蔡育傑君（學號 D95421013）在國立臺灣大學臨床醫學研究所完成之博士學位論文，於民國 104 年 6 月 23 日承下列考試委員審查通過及口試及格，特此證明

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誌謝

這趟旅程，比我原本想像的要漫長。幸好一路上都有貴人相助相伴，才得以踏實地走下去。最要感謝的是指導老師鄭安理教授。這些年來我很榮幸能在鄭教授領導的台大醫院腫瘤團隊工作，同時博士班的學業又接受鄭教授指導。鄭教授對於腫瘤學的基礎和臨床研究有非常深入的見解，總是在重要的時刻提供好的意見，也願意不厭其煩地提醒我的缺點。同時我也要感謝另一位指導老師郭明良教授，以基礎醫學研究者的角度指導我如何開始這個計畫，如何把想法和假說實際執行。我的實驗有很多是基於RTK array的分析結果進行，而這部分就是郭老師的建議。

在博士班的前兩年我對於實驗室的工作一竅不通，也很徬徨要如何找到好的研究題目。直到在美國羅徹斯特大學的進修期間，接受張傳祥教授的指導才對膀胱癌的研究有比較好的基礎。本論文的完成要謝謝成佳憲教授的大力協助，剛開始放射生物學的相關研究對我來說非常陌生，成教授帶我走進這個領域，讓膀胱癌放射增敏劑的研究可以成為一個完整的計劃，得到重要的結論。我也要謝謝楊志新教授，他是國際知名的afatinib專家，對這個藥的基礎研究和臨床應用提供精闢的建議。

目前我的臨床工作是以泌尿癌的病人照顧為主，很感謝台大泌尿腫瘤團隊的蒲永孝教授與林家齊副教授的指導，讓我對膀胱癌有更多的認識。對於實驗室工作我要感謝前後任助理宗帆、俊宇、媛媛以及何蓓茵博士與劉為麟博士的全力配合，在一次又一次的嘗試中找到問題的答案。

最後我要感謝家人的犧牲與付出，爸爸、媽媽、姊姊、妻子雅雯和寶貝女兒宜瑾，你們是最棒的。完成這本論文，只是站上夢想的起點，誠摯地希望未來大家可以繼續給我鼓勵，繼續結伴前行。

蔡育傑　謹誌於

中華民國104年8月19日

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ARREVIATION

AJCC: American Joint Committee on Cancer

ANOVA: analysis of variance

BCG: Bacillus Calmette–Guérin

CI: combination index

CT: computed tomography

DAPI: 4',6-diamidino-2-phenylindole

DMSO: dimethyl sulfoxide

DNA: deoxyribonucleic acid

DNA-PK: DNA-dependent protein kinase

DSB: double-strand break

EGFR: epidermal growth factor receptor

EMT: epithelial-mesenchymal transition

FBS: fetal bovine serum

FDG: fluorodeoxyglucose

FITC: fluorescein isothiocyanate

GC: gemcitabine - cisplatin

HER2: human epidermal growth factor receptor 2

IC50: half maximal inhibitory concentration

IGFR: insulin-like growth factor receptor

ISUP: International Society of Urological Pathology

MAPK: mitogen-activated protein kinases

MIBC: muscle-invasive bladder cancer

mTOR: mammalian target of rapamycin

M-VAC: methotrexate - vinblastine - adriamycin - cisplatin

NF-kB: nuclear factor kappa-light-chain-enhancer of activated B cells

NHEJ: non-homologous end joining

NHUC: normal human urothelial cell

NMIBC: non-muscle-invasive bladder cancer

NSCLC: non-small-cell lung carcinoma

PARP: poly (ADP-ribose) polymerase

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PET: positron emission tomography

PI3K: phosphoinositide 3-kinase

PVDF: polyvinylidene difluoride

RT: radiotherapy

RTK: receptor tyrosine kinase

SD: standard deviation

SNP: single nucleotide polymorphism

STR: short tandem repeat

SUV: standardized uptake value

TCGA: The Cancer Genome Atlas

TGFβ: transforming growth factor beta

TKI: tyrosine-kinase inhibitor

TNM: tumor - node - metastasis

TUR-BT: transurethral resection of bladder tumor

WHO: World Health Organization

摘要

膀胱癌是全世界與台灣男性第九常見的惡性腫瘤。對許患有局部肌肉侵襲性膀胱癌的病人，標準的膀胱根除手術並不可行。許多研究的焦點放在如何在”膀胱保存”的治療基準下善用放射線治療。然而相較於膀胱根除手術，放射線治療肌肉侵襲性膀胱癌病人的長期存活率低了約 10%。傳統上化學治療藥物被用來當成放射增敏劑，但這樣的治療有許多廣為人知的毒性。因此我們**有很強大的需求尋找能夠增進膀胱癌放射治療效果，又不增加毒性的藥物**。

一個可以增進膀胱癌放射治療效果的合理方法，是同時使用抑制放射治療相關信息傳導路徑的標靶藥物。表皮生長因子受體(EGFR)是其中最重要的。Cetuximab 是一個抑制 EGFR 的單株抗體，它已被證明在頭頸癌病人身上併用放射治療，可以增加療效。至於膀胱癌，gefitinib 是一個 EGFR 的酪胺酸激酶抑制劑，它被顯示在膀胱癌的細胞模式有中等的放射增敏效果，而在膀胱癌的活體模式則只有些微的放射增敏效果。因此這個主題值得後續的研究。

在前驅性研究中我測試了包括 erlotinib （EGFR 抑制劑)、trastuzumab （HER2 抑制劑)和 lapatinib （EGFR/HER2 抑制劑） 在膀胱癌細胞的放射增敏效果，可惜沒有一個有好的發展潛力。反而是 afatinib，一個新一代可以同時抑制 EGFR 和 HER2 的酪胺酸激酶抑制劑，比較有前途。

在老鼠膀胱癌模式中，我第一次展示了在膀胱癌的細胞及活體模式中，afatinib 都是一個有效的放射增敏劑。這個部分的動物實驗是在免疫正常的老鼠體內進行，比較類

似人類的生理狀態。Afatinib 似乎抑制了放射活化的 EGFR 與 HER2 信息，並增加了細胞 DNA 傷害與凋亡。

基於以上的發現，我假設**在膀胱癌細胞，同時抑制 EGFR 和 HER2 酪胺酸激酶活性的 afatinib，相較於只抑制 EGFR 酪胺酸激酶活性的 erlotinib，會有較佳的放射增敏性。**

為了確認這個假說，第一代的 EGFR 酪胺酸激酶抑制劑 erlotinib 和第二代的 afatinib，第一次在人類膀胱癌細胞中被拿來比較它們的放射增敏性。我展示了在人類膀胱癌的細胞及活體模式中 erlotinib 的放射增敏性的不足以及 afatinib 優越的放射增敏效果。在 HER2 被抑制的人類膀胱癌細胞中，可以看到 erlotinib 顯示了放射增敏效果，所以可能是因為 EGFR 和 HER2 對放射敏感性有協同作用，讓 afatinib 雙重抑制的特性才會變得有效。我也展示了對 EGFR-HER2 異源雙體的抑制可能是 afatinib 放射增敏性機轉的證據。

在”展望”中我提到如何繼續這個研究主題，以及怎樣應用到臨床上。我希望這個研究的結果可以協助達成”增進膀胱癌放射治療效果，又不增加毒性”的目標。

Abstract

Bladder cancer is the ninth most common cancer in the world and in Taiwanese male population. For many patients with localized muscle-invasive bladder cancer, radical cystectomy is not a feasible treatment, and considerable interest was focused on the optimal use of radiotherapy in ”bladder preservation” protocol. However, the long-term survival of patients receiving radiation-based therapy in muscle invasive bladder cancer is about 10% inferior to patients receiving standard radical cystectomy. Traditionally chemotherapeutic agents are used as radiosensitizer but they have many well-known toxicities. **Therefore, there is a strong need to find agents enhancing the radiation effect in urinary bladder cancer treatment while not increasing the toxicities**.

A reasonable way to enhance the outcome of radiotherapy is by concomitantly using agents that inhibit radiation-activated signaling pathways. Epidermal growth factor receptor (EGFR) is the most important target. Cetuximab, an anti-EGFR antibody, has shown clinical benefit in head and neck cancer when combined with radiotherapy. In bladder cancer, gefitinib, an EGFR tyrosine kinase inhibitor (TKI), has moderate *in vitro* and marginal *in vivo* radiosensitizing activities. Therefore the topic deserves further investigation.

In pilot study, I tested the radiosensitizing activities of erlotinib (EGFR inhibitor), trastuzumab (HER2 inhibitor) and lapatinib (EGFR/HER2 inhibitor) in bladder cancer cells.

None of them showed good potential. Instead, afatinib, a new-generation EGFR inhibitor with activity against both EGFR and HER2, is more promising.

In murine bladder cancer model, I demonstrated for the first time the *in vitro* and *in vivo* radiosensitizing activity of afatinib, an EGFR/HER2 dual inhibitor. The animal study was performed in immunocompetent mice and mimic human physiologic status. Afatinib likely mediates its effect on bladder cancer cells by suppressing radiation-activated EGFR and HER2 signals and thereby causing enhanced DNA damage and cell apoptosis.

Based on the findings I hypothesized that **in bladder cancer cells, the concomitant inhibition of EGFR and HER2 tyrosine kinase activity by afatinib has greater radiosensitizing activity than the inhibition of EGFR tyrosine kinase activity alone by erlotinib**

To confirm the hypothesis, in human bladder cancer model the radiosensitizing effects of different generations of clinically useful EGFR TKIs were compared for the first time. I showed the inadequacy of EGFR inhibition alone and the advantage of concomitant blockade of radiation-activated EGFR and HER2 signaling to inhibit the *in vitro* and *in vivo* growth of bladder cancer cells. The radiosensitizing effect of an EGFR inhibitor was much higher in HER2 knocked-down than wild-type cells, therefore HER2 may play a synergistic role with EGFR in determining radiosensitivity. I also showed evidence to support that receptor heterodimerization plays an important role in the radiosensitizing effect of afatinib.

In Prospect I mentioned how to continue current project and apply the data to clinical use. I hope that the results of this study can help to meet the need of enhancing the radiation effect in urinary bladder cancer treatment while not increasing the toxicities.

CHAPTER ONE: INTRODUCTION

1.1 Clinical Overview of Bladder Cancer

1.1.1 Epidemiology

Bladder cancer is the most common cancer of the urinary tract. According to the estimation by GLOBOCAN database of World Health Organization (WHO), there will be 468,351 new cases and 179,753 deaths of bladder cancer in 2015 in the world. (http://globocan.iarc.fr/Default.aspx). By World Cancer Report 2014, it is the 9th most common cancer on the world. In Taiwan, according to the 2012 Cancer Registry Annual Report, there were 2,003 new cases and 807 deaths of bladder cancer this year. The median age of new case and death were 72 and 79, respectively. (http://www.hpa.gov.tw/Bhpnet/Web/Stat/StatisticsShow.aspx?No=201504290001) Bladder cancer is also the 9th most common cancer in male population of Taiwan.

1.1.2 Risk factors

Risk factors for the development of bladder cancer can be classified into: (a) genetic and molecular abnormalities, (b) chemical or environmental exposures, and (c) chronic irritation (Kaufman, Shipley, & Feldman, 2009). Genetic and molecular factors include oncogenes, such as TP63, the epidermal growth factor receptors (EGFR), and Ras p21 proteins. Tumor suppressor genes, including TP53 and RB1 are also important in the pathogenesis of bladder

cancer. Chemical and environmental exposures include aromatic amines, aniline dyes, nitrites and nitrates, acrolein, coal, and arsenic, but the most important environmental factor is cigarette smoking. Other causal factors include chronic irritation, indwelling catheters, *Schistosoma haematobium* infection, and pelvic irradiation.

1.1.3 Pathologic classifications and grading

According to the WHO/International Society of Urological Pathology (ISUP) the histologic types of bladder cancer are (Miyamoto et al., 2010):

- Urothelial carcinoma (also known as transitional cell carcinoma).
- Squamous cell carcinoma
- Adenocarcinoma
- Undifferentiated carcinoma and other rare types

By far the most common histology type of bladder cancer is urothelial carcinoma, and they are graded as low-grade or high-grade according to their cellular characteristics (Lokeshwar, Ruiz-Cordero, Hupe, Jorda, & Soloway, 2015).

1.1.4 Staging

Like most solid tumors, bladder cancer is staged using the Tumor - Node - Metastasis system (TNM system). In anatomy, urinary bladder consists of three layers: the epithelium and the subepithelial connective tissue, the muscularis, and the perivesical fat (peritoneum covering the superior surface and upper part) (Fig. 1-1). According to the seventh edition of staging system by American Joint Committee on Cancer (AJCC), the TNM stage of bladder cancer is defined as:

Primary Tumor (T)

TX: Primary tumor cannot be assessed

T0: No evidence of primary tumor

Ta: Noninvasive papillary carcinoma

Tis: Carcinoma in situ: “flat tumor”

T1: Tumor invades subepithelial connective tissue

T2: Tumor invades muscle

pT2a - Tumor invades superficial muscle (inner half)

pT2b - Tumor invades deep muscle (outer half)

T3: Tumor invades perivesical tissue

pT3a - Microscopically

pT3b - Macroscopically (extravesical mass)

T4: Tumor invades any of the following: prostate, uterus, vagina, pelvic wall, abdominal wall

T4a - Tumor invades prostate, uterus, vagina

T4b - Tumor invades pelvic wall, abdominal wall

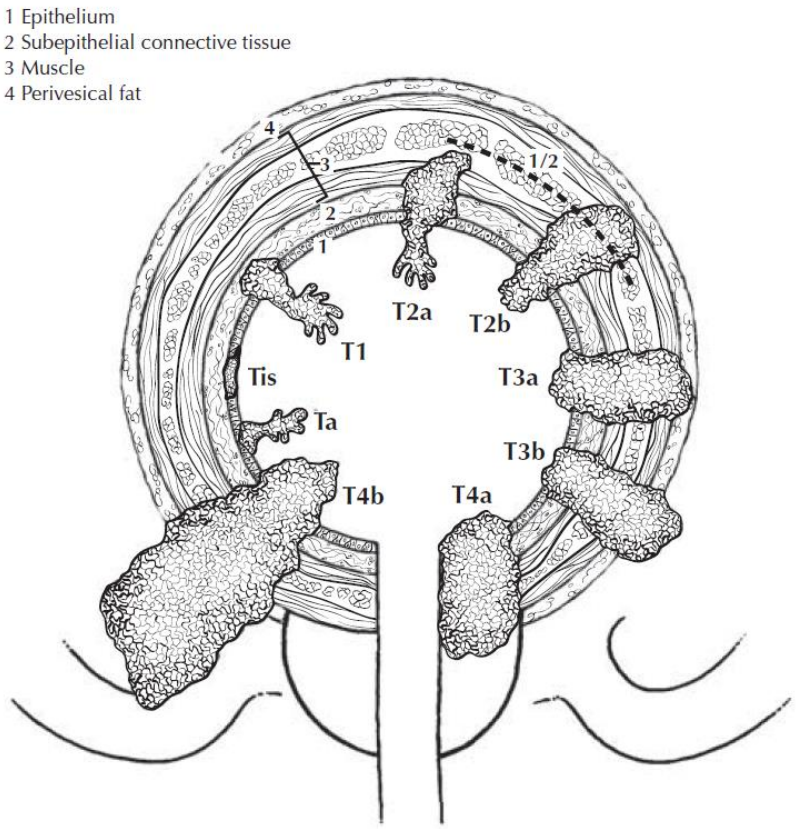

Figure 1-1. The definition of T stage for bladder cancer

(Adopted from AJCC Cancer Staging Manual, Seventh Edition, 2010)

Regional Lymph Nodes (N)

NX: Regional lymph nodes cannot be assessed

N0: No regional lymph node metastasis

Nl: Metastasis in a single lymph node, 2cm or less in greatest dimension

N2: Metastasis in a single lymph node, more than 2cm but not more than 5cm in greatest dimension; or multiple lymph nodes, none more than 5cm in greatest dimension

N3: Metastasis in a lymph node, more than 5 cm in greatest dimension

Distant Metastasis (M)

MX: Distant metastasis cannot be assessed

M0: No distant metastasis

M1: Distant metastasis

The stage grouping is defined as Table 1:

0a	Ta	N0	M0
0is	Tis	N0	M0
I	T1	N0	M0
II	T2a - T2b	N0	M0
III	T3a - T4a	N0	M0
IV	T4b Any T Any T	N0 N1 - N3 Any N	M0 M0 M1

Table 1-1. TNM staging of bladder cancer

(Adopted from AJCC Cancer Staging Manual, Seventh Edition, 2010)

1.1.5 Muscle-invasive versus non-muscle-invasive tumors

At diagnosis the majority of tumors are non-muscle-invasive bladder cancer (NMIBC, including Ta, Tis, T1), and the patients with stage 0 and stage I diseases account for two-thirds of bladder cancer patients in Taiwan (2012 data). Most of them have a favorable prognosis but a high rate of relapse can be found in high-grade disease. In contrary, patients with muscle-invasive bladder cancers (MIBC, including T2, T3 and T4) only comprise one-third of all patients in Taiwan and has a relatively poor prognosis. In particular, 25% of pT2 tumors (defined by invasion of muscle layer of the bladder), 50% of pT3 tumors (defined by invasion of perivesical fat) and 80% of pT4 tumors (defined by invasion into nearby organs and structures) will eventually develop into metastatic disease, while 5-year survival is 67% for pT2 tumors, 35% for pT3 tumors and 27% for pT4 tumors. (Prasad, Decastro, & Steinberg, 2011).

1.1.6a Treatment of non-muscle-invasive bladder cancer

For NMIBC, transurethral resection of the bladder tumor (TUR-BT) is the standard treatment. Due to high rate of recurrence, patients should have cystoscopy and voided urine cytology every 3 months for 2 years, then 6 monthly for 2 years, and then once yearly

indefinitely (Kaufman et al., 2009). Upper tract imaging is suggested because the lifetime risk of upper tract tumor after a diagnosis of bladder cancer is about 5% (Smith, Weaver, Barjenbruch, Weinstein, & Ross, 1989). Intravesical therapy including the immunomodulator Bacillus Calmette–Guérin (BCG) and chemotherapeutic agents such as mitomycin, doxorubicin, thiotepa and gemcitabine was developed to reduce recurrence (Patel, Cohen, Weiner, & Steinberg, 2015).

1.1.6b Challenge in the treatment of localized muscle-invasive bladder cancer

Currently radical cystectomy (radical prostatocystectomy for male patients) is the standard treatment for patients with MIBC and a chance to cure (Herr, Dotan, Donat, & Bajorin, 2007). Patients who undergo radical cystectomy or prostatocystectomy have an inevitable requirement of urinary tract diversion which results in impaired quality of life (Singer et al., 2013). In addition, the median age of bladder cancer at diagnosis is over 70 years old and patients of this age often have substantial comorbidities. Altogether, curative surgery is not usually a viable option for patients with MIBC, especially in old population.

As for the alternative treatment for patients who are not suitable for radical cystectomy, considerable interest focuses on the optimal use of radiotherapy. The “bladder preservation” protocols usually consist of aggressive TUR-BT, concurrent chemoradiation and several cystoscopic examinations (Kaufman et al., 2009; Prasad et al., 2011). However, the long-term

prognosis of this multimodality therapy is still inferior to standard radical cystectomy, with 5-year cancer-specific survival rate 36% versus 40% and 5-year overall survival rate 26% versus 35% (Booth et al., 2014). Moreover, salvage cystectomy may still be warranted for local failure after radiotherapy. The advance of bladder-preservation treatments could provide patients with a chance to improve treatment outcome and quality of life (James et al., 2012).

1.1.6c Systemic chemotherapy of bladder cancer

For metastatic bladder cancer, cisplatin-based combination chemotherapy is the only modality that demonstrated a survival benefit in randomized phase III trials (Loehrer et al., 1992). However the previous standard of methotrexate, vinblastine, doxorubicin and cisplatin (M-VAC) is too toxic for many patients. The doublet of gemcitabine and cisplatin (GC) have similar effects on survival with median overall survival 14.0 months for GC and 15.3 months for M-VAC (von der Maase et al., 2005) Since GC regimen is less toxic, currently it is widely used as first-line treatment of metastatic bladder cancer.

As the rate of eventual metastatic disease is very high in patients with high-risk bladder cancer, neoadjuvant chemotherapy before radical cystectomy had been studied to reduce occult micrometastases. Meta-analyses have demonstrated a small (5%), but sustainable benefit with neoadjuvant platinum-based combination chemotherapy on overall survival at 5 years (Advanced bladder cancer (ABC) meta-analysis collaboration, 2005; Winquist,

Kirchner, Segal, Chin, & Lukka, 2004).

1.2 Molecular Biology of Bladder Cancer

1.2.1 The two-pathway model

In addition to the difference of anatomic location and prognosis between muscle-invasive versus non-muscle-invasive bladder cancers, several lines of evidence support the general concept that the distinct clinical outcomes of NMIBC versus MIBC reflect their distinct molecular causes and potentially their distinct cells of origin (X. R. Wu, 2005). Certain molecular alterations, such as gain of function mutations in fibroblast growth factor receptor 3 (FGFR3), are prevalent in low-grade NMIBC, whereas other alterations, such as p53 loss or mutation, are prevalent in high-grade MIBC (Esrig et al., 1994; Goebell & Knowles, 2010) (Fig. 1-2). Analyses of gene expression profiling (Dyrskjot et al., 2003) and/or genomic alterations (Hurst, Platt, Taylor, & Knowles, 2012; Lindgren et al., 2010) have supported this concept, although it is difficult to establish a mutual-exclusivity model, since some superficial bladder tumors can progress to invasive disease.

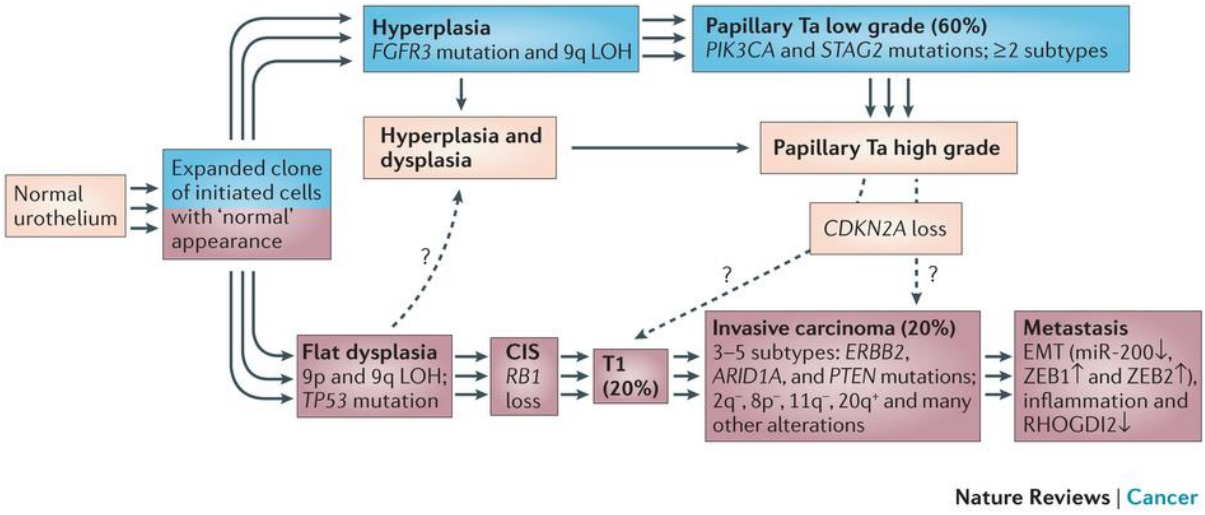

Figure 1-2. The two-pathway model. The blue and purple pathways indicate the two major pathways with distinct histopathological and molecular features that have been recognized (Knowles & Hurst, 2015).

Up to 60% of stage Ta tumors have activating point mutations in FGFR3 (Cappellen et al., 1999; Hernandez et al., 2006), which is associated with favourable outcome. In contrary, FGFR3 mutation is less common in MIBC (10-20% in tumors of stage T2 or above) (Billerey et al., 2001). In cultured normal human urothelial cells (NHUCs), mutant FGFR3 activates the RAS-Mitogen activated protein kinase (MAPK) pathway and phospholipase Cγ, leading to increased survival and proliferation to high cell density (di Martino, L'Hote, Kennedy, Tomlinson, & Knowles, 2009). This *in vitro* phenotype suggests that FGFR3 mutation could contribute to early clonal expansion within the urothelium *in vivo.*

FGFR3 is also implicated in contributing to the risk of bladder cancer development. A single-nucleotide polymorphism (SNP) in an intron of TACC3, which is 70 kb from FGFR3, is associated with bladder cancer risk and with higher risk of recurrence in stage Ta disease, particularly for FGFR3-mutant NMIBC (Kiemeney et al., 2010). One possible mechanism is that altered chromatin structure associated with increased expression of FGFR3 could increase the probability of mutation and/or increase the expression and impact of mutated proteins (Knowles & Hurst, 2015).

In MIBC, the phosphoinositide 3-kinase (PI3K) pathway deserves more attention. Upstream activators include ErbB family receptors. For example, EGFR induces PI3K activation via RAS activation. ErbB3 interacts with p110α, the catalytic subunit of PI3K, and conveys signals from human epidermal growth factor receptor 2 (HER2)-EebB3 heterodimers. Overexpression of EGFR, HER2 and/or ErbB3 in subsets of bladder cancer is associated with higher grade, stage and worse outcome (Jimenez et al., 2001; Kassouf et al., 2008; Kruger et al., 2002). HER2 amplification or overexpression is more common in metastases than in the corresponding primary tumor, implying a role in the metastatic process (Fleischmann, Rotzer, Seiler, Studer, & Thalmann, 2011).

1.2.2 Molecular subtypes of bladder cancer

With the advance of new technology, more sophisticated classifications of bladder cancers are proposed recently. Sjödahl *et al*. studied 308 bladder tumors and defined five urothelial carcinoma subtypes: urobasal A, genomically unstable, urobasal B, squamous cell carcinoma like, and an infiltrated class of tumors (Sjödahl et al., 2012). A further analysis identified that urobasal A subtype shares features with normal urothelium such as keratin 5, P-cadherin, and EGFR expression confined to basal cells, and cell cycle activity restricted to the tumor-stroma interface. In contrast, the squamous cell cancer-like subtype uniformly expresses keratin 5, P-cadherin, EGFR, and cell cycle genes throughout the tumor

parenchyma. The genomically unstable subtype shows proliferation throughout the tumor parenchyma and high HER2 and E-cadherin expression but absence of keratin 5, P-cadherin, and EGFR expression. Urobasal B tumors demonstrate features shared by both urobasal A and squamous cell cancer-like subtypes (Sjödahl et al., 2013). A major transition in tumor progression seems to be loss of dependency of stromal interaction for proliferation.

The Cancer Genome Atlas (TCGA) reported an integrated analysis of 131 urothelial carcinomas to provide a comprehensive landscape of molecular alterations. There were statistically significant recurrent mutations in 32 genes, including multiple genes involved in cell-cycle regulation, chromatin regulation, and kinase signalling pathways. RNA sequencing revealed four expression subtypes, two of which (papillary-like and basal/squamous-like) were also evident in microRNA sequencing and protein data.

TCGA study also identified potential therapeutic targets in 69% of the tumors, including 42% with targets in the PI3K/AKT/mammalian target of rapamycin (mTOR) pathway and 45% with targets (including HER2) in the receptor tyrosine kinases (RTK) /MAPK pathway. Chromatin regulatory genes were more frequently mutated in urothelial carcinoma than in any other common cancer, indicating the future possibility of targeted therapy for chromatin abnormalities (Cancer Genome Atlas Research Network, 2014).

1.3 Radiation Effect and Signal Transduction Pathways

Ionizing radiation induces a variety of deoxyribonucleic acid (DNA) lesions, including oxidized base damage, abasic sites, single-strand breaks and double-strand breaks. These lesions, if unrepaired, will result in cell death through mitotic catastrophe and apoptosis. Radiation also induces complicated biologic responses to cancer cells including repairing DNA damage and counteracting the propagation to apoptosis. Traditionally chemotherapeutic agents are used to enhance radiation effect because these agents can augment DNA damage to cancer cells (Boeckman, Trego, & Turchi, 2005), but the toxicities usually hinder their use in patients with old age or underlying medical diseases.

There are evidences showing that aberrantly activated signal transduction pathways can influence radiosensitivity of cancer cells by modulating apoptotic response and DNA repair mechanism (Begg, Stewart, & Vens, 2011). For example, AKT (Schlessinger, 2000), MAPK (Dent, Yacoub, Fisher, Hagan, & Grant, 2003) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Beg & Baltimore, 1996) signaling can inhibit the apoptotic response after DNA damage by activation of the anti-apoptotic protein MCL1, or inactivation of the pro-apoptotic protein BAD and BIM. On the other hand, links between the AKT, MAPK and transforming growth factor beta (TGFβ) pathways and DNA repair have been found. Activation of the AKT and MAPK pathways leads to the activation of the catalytic subunit of DNA-dependent protein kinase (DNA-PK) (Golding et al., 2007) which is a

central protein in double-strand break repair by non-homologous end-joining (NHEJ) (Kriegs et al., 2010). This protein can also be activated by EGFR after it is translocated to the nucleus (Dittmann, Mayer, Fehrenbacher, et al., 2005). TGFβ is necessary for the full activation of ATM to DNA damage (Kirshner et al., 2006). (Fig.1-3)

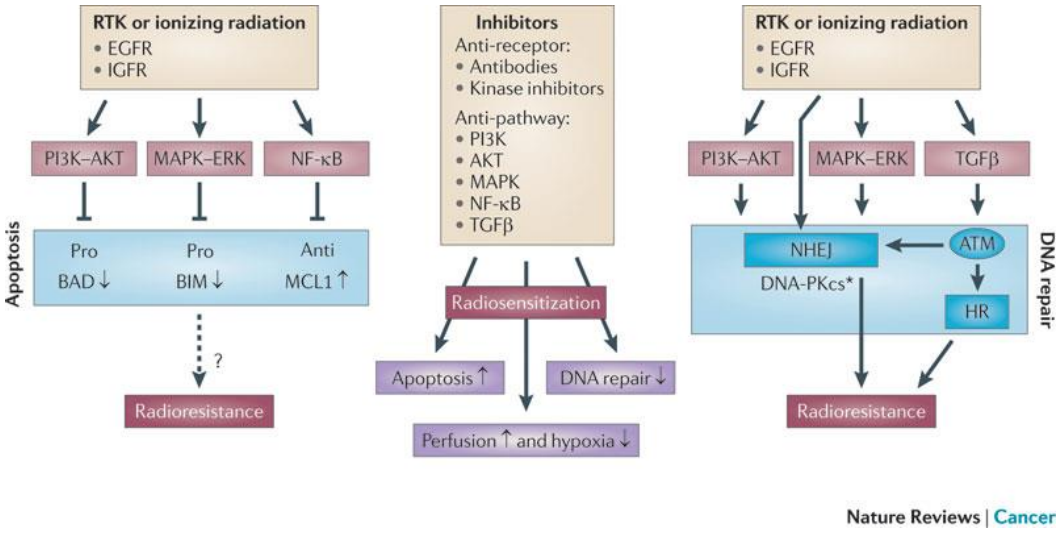

Figure 1-3. Aberrantly activated signal transduction pathways may influence radiosensitivity (Begg et al., 2011)

Receptor tyrosine kinases such as EGFR and insulin-like growth factor receptor (IGFR) share common downstream signaling pathways which may influence radiosensitivity. Multiple lines of evidence indicate that EGFR is an important determinant of radioresponse and has a radioprotective function. Based on current evidence, EGFR-mediated radioprotection can be conceptually divided into three phases (D. J. Chen & Nirodi, 2007): (a) an immediate early phase that involves DNA repair; (b) suppression of DNA damage-induced apoptosis before and after cell cycle arrest, and (c) a tumor repopulation step that

offers a proliferative advantage to tumors emerging from radiation-induced cell cycle arrest (Fig. 1-4).

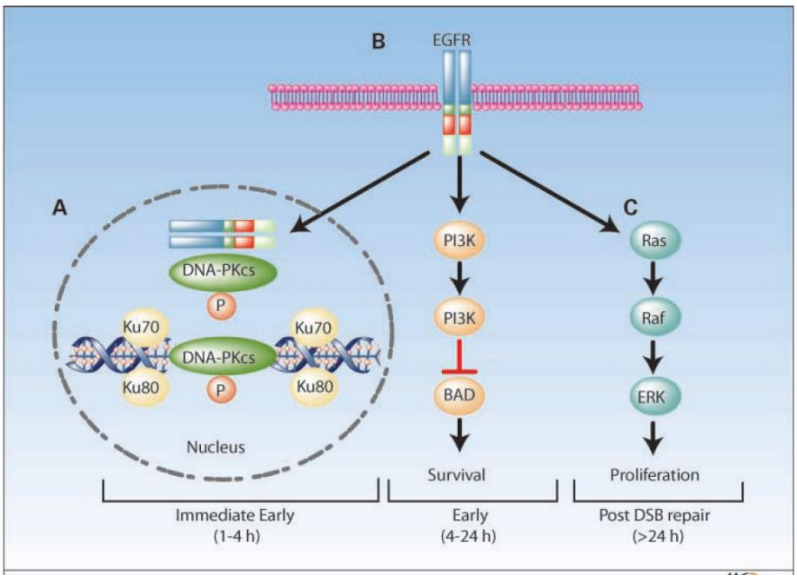

Figure 1-4. A model of EGFR-mediated radioprotection (D. J. Chen & Nirodi, 2007)

The role of EGFR in radioprotection is best demonstrated in a large phase III clinical trial. In this study patients with locoregionally advanced head and neck cancer were randomly assigned to treatment with high-dose radiotherapy alone (213 patients) or high-dose radiotherapy plus weekly cetuximab, an EGFR-specific antibody (211 patients). The median duration of locoregional control was 24.4 months among patients treated with cetuximab plus radiotherapy and 14.9 months among those given radiotherapy alone. Overall survival was 49.0 months among patients treated with combined therapy and 29.3 months among those treated with radiotherapy alone (Bonner et al., 2006). More molecular agents targeting EGFR/ErbB family receptors, angiogenesis and histone deacetylase are under investigation in Phase III studies in combination with radiation therapy (Morris & Harari, 2014).

1.4 New-generation ErbB Family Inhibitors as Radiosensitizers

Preclinical studies showed that cetuximab, an anti-EGFR monoclonal antibody, induces cell cycle arrest in the more radiosensitive G1 and G2-M phases, suppressing growth, radiation-induced DNA damage repair, and tumor angiogenesis (Huang & Harari, 2000). Meanwhile, although EGFR tyrosine kinase inhibitors (TKIs) such as erlotinib enhance the radiation response in preclinical models (Chinnaiyan et al., 2005), their clinical role in radiosensitization is not settled. It has been speculated that the radiosensitizing effect may differ between different classes of EGFR inhibitors (Baumann et al., 2007). EGFR tyrosine kinase inhibition alone may be inadequate to overcome radioresistance.

For bladder cancer, gefitinib, an EGFR specific tyrosine kinase inhibitor, was shown to enhance the growth inhibition of bladder cancer by ionizing radiation. In *in vitro* model, gefitinib has moderate in vitro radiosensitizing activity in bladder cancer cells, and there is a differential response in different bladder cancer cell lines, probably related to EGFR expression (Dominguez-Escrig, Kelly, Neal, King, & Davies, 2004). In *in vivo* model, gefitinib has at best marginal *in vivo* radiosensitizing activity in bladder cancer cells, based on single literature figure (Colquhoun, McHugh, Tulchinsky, Kriajevska, & Mellon, 2007), and the effect of combination therapy lasts only for the duration of gefitinib administration.

One strategy to increase the radiosensitization in urinary bladder cancer treatment is to utilize new-generation ErbB family inhibitors which have more potent activity against EGFR

as well as other ErbB family members such as HER2. Both EGFR and HER2 are key signaling molecules activated by ionizing radiation in literature (Bowers et al., 2001). It is also noteworthy that both EGFR and HER2 are important in bladder cancer progression for their frequent overexpression and association with poor prognosis in bladder cancer (Lipponen, Eskelinen, Syrjanen, Tervahauta, & Syrjanen, 1991; Neal et al., 1990).

Afatinib (BIBW2992) is an anilino-quinazoline TKI designed to irreversibly bind EGFR and HER2, and potently suppresses the kinase activity in wild-type and activated mutant cells (Bean et al., 2008; Li et al., 2008). It is approved as the first-line treatment of patients with metastatic non-small cell lung cancer (NSCLC) whose tumors have EGFR exon 19 deletions or exon 21 (L858R) substitution mutations.

However it remains unclear whether new generation EGFR inhibitors with broader blockade of Erb-B family tyrosine kinase activities like afatinib is superior to first generation inhibitors (which block EGFR kinase activity alone) in enhancing radiosensitivity of bladder cancer cells.

1.5 Hypothesis and Experimental Design

In my PhD work, I first used molecular agents targeting EGFR and/or HER2 to evaluate whether further radiosensitizer experiment is feasible. Meanwhile RTK array was done to detect the change of signaling pathways after irradiation with or without drugs. In murine bladder cancer model, I tested the *in vitro* and *in vivo* radiosensitizing activity of afatinib.

In human bladder cancer cells, I used *in vitro* and *in vivo* models of to test the hypothesis that **in bladder cancer cells, the concomitant inhibition of EGFR and HER2 tyrosine kinase activity by afatinib has greater radiosensitizing activity than the inhibition of EGFR tyrosine kinase activity alone by erlotinib**. To genetically verify the role of HER2, we also tested whether the radiosensitizing activity mediated by EGFR inhibition can be improved by downregulating HER2 expression. Finally I showed evidence to support that receptor heterodimerization plays an important role in the radiosensitizing effect of afatinib.

CHAPTER TWO: PILOT STUDY

2.1 Rationale and Approach

Based on literature and clinically available targeted agents, I chose EGFR and HER2 inhibitors to test the potential of their radiosensitizing activity. First I examined the EGFR mutation status of bladder cancer cells and then performed clonogenic assay to evaluate the radiosensitizing activity of different targeted agents.

2.2 Materials and Methods

Cell Cultures

The NTUB1 cells were cultured with RPMI1640 medium; T24 cells were cultured with DMEM medium with 10% fetal bovine serum (FBS). All of the cells were incubated at 37℃ and 5% CO2. The cells were trypsinized by trypsin-EDTA and collected for further studies.

EGFR mutation sequencing

The cDNA of each cell lines was sequenced their EGFR mutation at exon 18-21, the tyrosine kinase domain of EGFR, by AutoSequencing system. The result sequences were aligned with wild-type EGFR sequence and checked if there have any mutation on this region.

Clonogenic assay

The cells for radiotherapy with or without drug treatment were seeded 1000 cells/well in 6-well plates (in triplicates). Drugs with different dosages as 0, 1nM, 10nM, 100nM, 1μM and 10μM, along with different radiation dosages 0, 2.5Gy, 5Gy, 7.5Gy and 10Gy were tested. Each drug was given three times, namely 24, 48 and 72 hours after the starting time. Radiation was given 48 hours after starting time. After 7 days in culture, colonies were fixed with 10% buffered formalin and stained with 2% crystal violet. The number of colonies were determined and normalized to the number of colonies in controls.

2.3 Results

2.2.1 EGFR mutation analysis

The EGFR mutation status was tested by polymerase chain reaction (PCR) and sequencing. No mutation was found in exon 18, 19, 20 and 21 of NTUB1 or T24 cells.

2.2.2. Clonogenic assay

For erlotinib, the radiosensitizing activity is not significant. (Fig. 2-1 and Fig. 2-2)

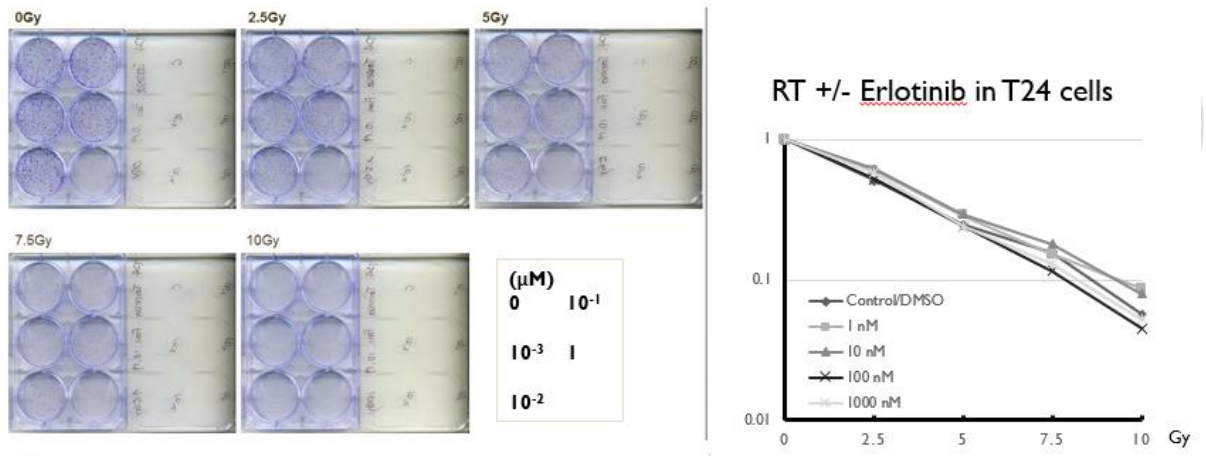

Figure 2-1. Clonogenic assay of erlotinib +/- RT in T24 cells

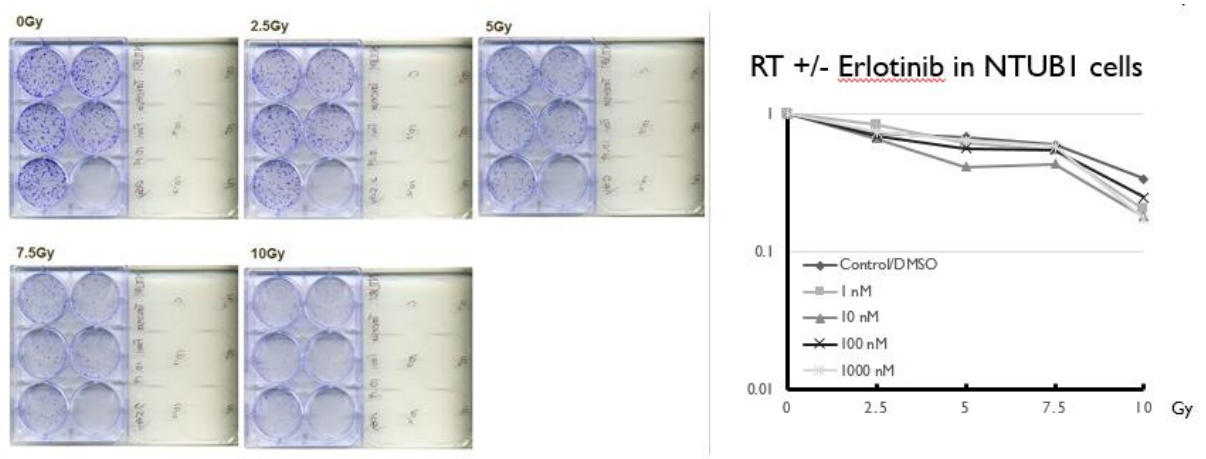

Figure 2-2. Clonogenic assay of erlotinib +/- RT in NTUB1 cells

For trastuzumab, the radiosensitizing activity is not significant, either. (Fig. 2-3 and Fig. 2-4)

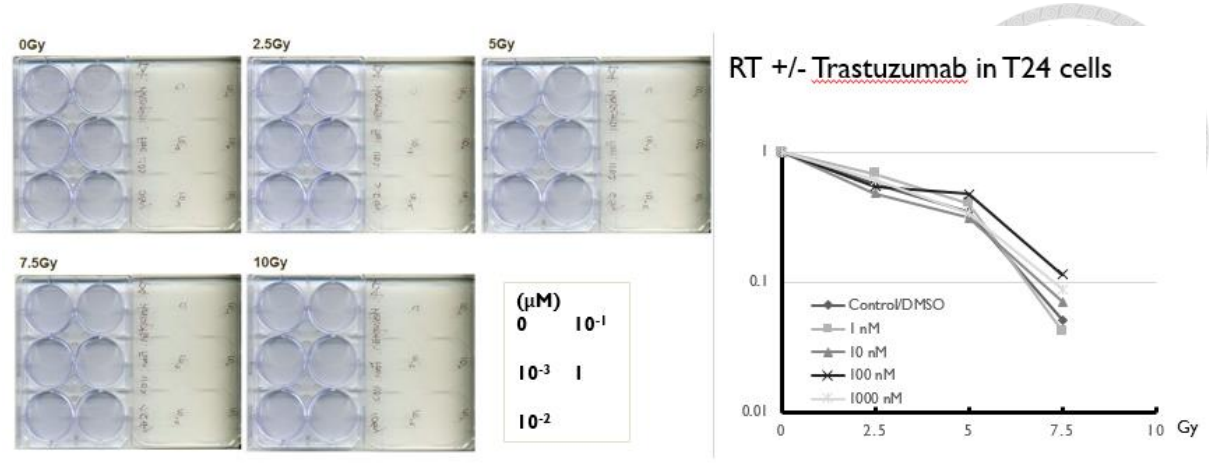

Figure 2-3. Clonogenic assay of trastuzumab +/- RT in T24 cells

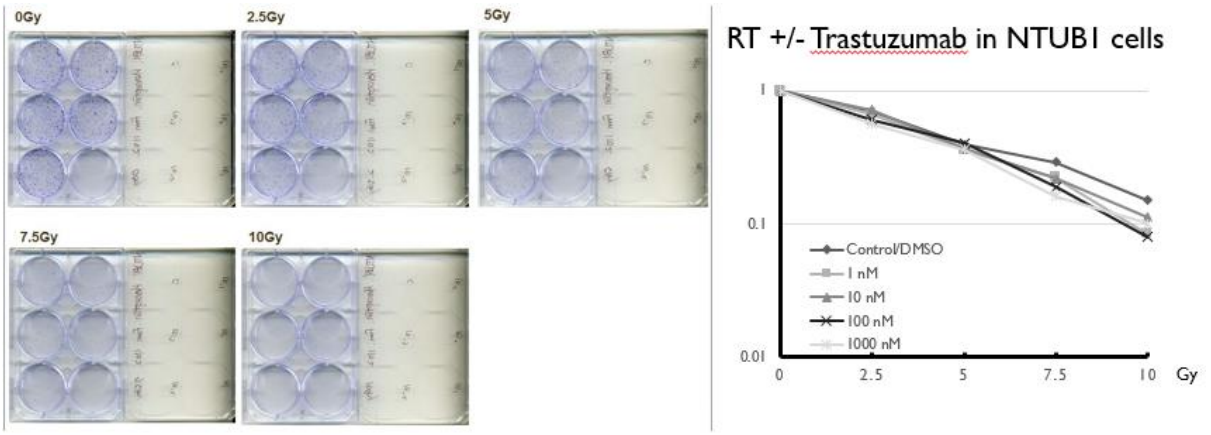

Figure 2-4. Clonogenic assay of trastuzumab +/- RT in NTUB1 cells

For lapatinib, there was no effect of lapatinib in both cell lines. (Fig. 2-5 and Fig. 2-6)

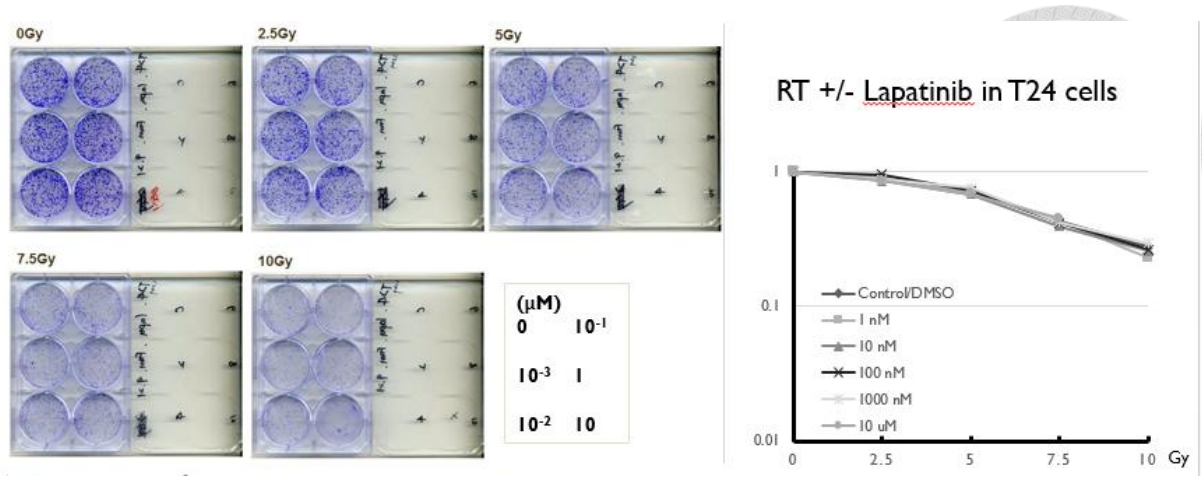

Figure 2-5. Clonogenic assay of lapatinib +/- RT in T24 cells

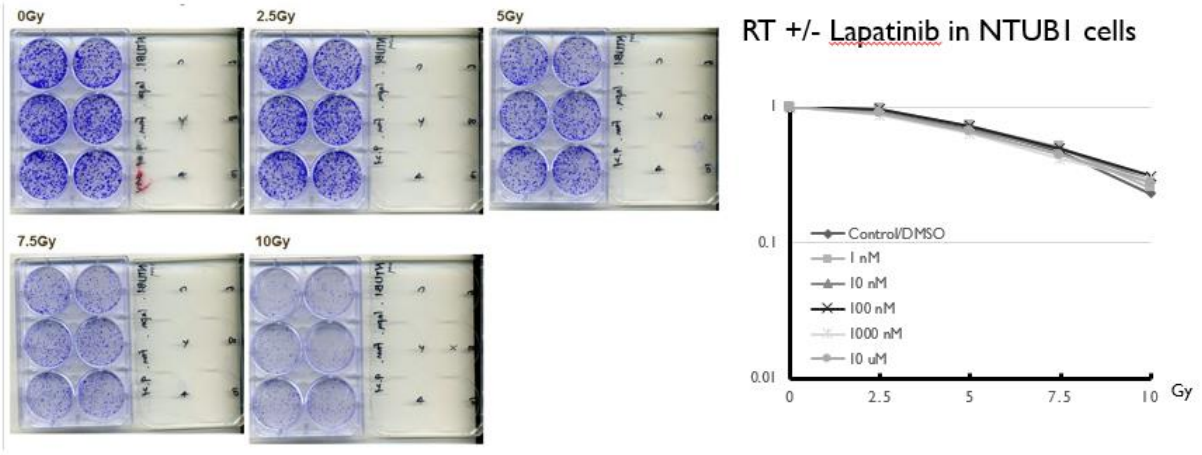

Figure 2-6. Clonogenic assay of lapatinib +/- RT in NTUB1 cells

CHAPTER THREE: RADIOSENSITIZING EFFECT OF AFATINIB IN A MURINE BLADDER CARCINOMA MODEL

3.1 Rationale and Approach

As the mechanism of EGFR-mediated radioprotection may differ in various time sequence, we will test the radiosensitivity after afatinib pretreatment of bladder cancer cells in (a) immediate early phase (1-4 h) by immunofluorescence detection of γH2AX foci, (b) early phase (4-24 h) by flow cytometry analysis, and (c) post double-strand break (DSB) repair phase (>24 h) by clonogenic assay according to a model of EGFR-mediated radioprotection (D. J. Chen & Nirodi, 2007). The efficacy of afatinib will also be validated in mouse xenografts. The advantage of murine bladder cancer model is that the *in vivo* experiments can be performed in immunocompetent animals. The tumor graft in mice will be validated by animal imaging and immunohistochemistry.

3.2 Materials and Methods

Murine bladder tumor cell line

The murine (C3H/HeN) bladder tumor cell line, MBT-2, was obtained from the Japanese Collection of Research Bioresources (Okayama, Japan). Cells were cultured in RPMI-1640 supplemented with 10% fetal bovine serum and 50 U/ml penicillin/streptomycin. Cells were cultured at 37 ℃ in a humidified atmosphere of 5% CO2 and 95% air.

Reagents

Afatinib was purchased from Selleck Chemicals (Houston, TX, USA). For in vitro studies, stock solutions of afatinib were prepared in dimethyl sulfoxide (DMSO) and diluted in culture medium containing 10% fetal bovine serum. For in vivo studies, afatinib was suspended in a vehicle (0.5% methylcellulose [wt/vol] and 0.4% Tween 80 [vol/vol] in sterile water) for oral administration to C3H/HeN mice bearing xenograft tumors.

Irradiation of cells

MBT-2 cells in culture flasks were irradiated with different doses of radiation, using a 6-MV photon linear accelerator. The distance from the radiation source to the bottom of the flask was set at 100 cm.

Colony formation assay

Cells (500/well) were seeded in six-well plates and treated with different doses of radiation following 30-min pretreatment with various doses of afatinib (200–1000 nM) or DMSO vehicle. Cells were then cultured for an additional 7 days, after which the number of colonies (clusters of more than 50 cells) was counted in each well using an inverted phase-contrast microscope at 100X magnification and photographed. The effect on colony number was analyzed using CompuSyn software (ComboSyn, Inc., Paramus, NJ, USA).

Western blot analysis

Aliquots of cell lysates containing 90 μg of protein were separated by SDS–PAGE (6–15% polyacrylamide) and then transferred onto polyvinylidene difluoride membranes and immunoblotted with various antibodies. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by enhanced chemiluminescence (ECL, Boehringer Mannheim, Mannheim, Germany). Antibodies to the phospho-HER2 and phospho-EGFR were obtained from Epitomics, Inc. (Burlingame, CA, USA), EGFR and HER2 from GeneTex, Inc. (Irvine, CA, USA), poly(ADP-ribose) polymerase (PARP) and cleaved PARP from Cell Signaling Technology (Danvers, MA, USA), beta-actin from Santa Cruz Biotechnology (Santa Cruz, CA) and histone variant H2AX, phospho-H2AX (Ser139) and clone JBW301 from Millipore Corporation (Billerica, MA, USA).

Cell cycle phase analysis

The distribution of cells among the phases of the cell cycle was determined by quantifying the cellular content of propidium iodide-stained DNA. Cells (106/ml) were treated as indicated, harvested by centrifugation, stained with propidium iodide (PBS containing 0.5% Tween 20, 15 μg/ml propidium iodide and 5 μg/ml DNase-free RNase), and analysed using a Becton Dickinson FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson Immunocytometry Systems, San Jose, CA, USA).

γH2AX immunofluorescence microscopy

Cells were plated on polylysine-coated coverslips, allowed to attach overnight and exposed to ionizing irradiation of 2.5 Gy either alone or combined with 100 nM afatinib. After treatment, cells were incubated for 30 min, washed three times with ice-cold phosphate-buffered saline (PBS), fixed in 4% formaldehyde/PBS for 30 min, permeabilized in 0.5% Triton X-100 in PBS for 1 h, blocked in 5% bovine serum albumin for 1 h at room temperature, incubated with the antibody (fluorescein isothiocyanate [FITC] conjugated anti-phospho-Histone γH2AX [Ser139; 1:1500; Millipore, Billerica, MA, USA]) for 2 h at room temperature in the dark, washed with PBS and mounted in Vectashield mounting medium containing diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA). γ-H2AX foci were examined

using a Zeiss Axio Imager A1 fluorescence microscope. In each sample, the number of γ-H2AX foci per nucleus was counted by focicounter under high power field and the average of 150 nuclei was calculated. The average number of γ-H2AX foci per nucleus represents the amount of double strand breaks.

In vivo studies

Female C3H/HeN mice (6 weeks of age) were obtained from the National Laboratory Animal Center and used for ectopic (subcutaneous) xenograft implantation. Body weights were measured weekly. Mice from each group were sacrificed on day 8. The tumor was fixed in 10% neutral buffered formalin and processed for histopathological and immunohistochemical evaluations. Tumor volumes were measured with a set of calipers and calculated using a standard formula: $width^2 \times length/2$. All experimental procedures using these mice were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee.

Ectopic tumor model

Ectopic tumors were established by subcutaneous injection of MBT-2 cells (2 × 106) into the right hind leg of mice. As the tumors became established (mean starting tumor volume = 162 mm^3), the mice were randomized into 4 groups to receive the following treatments: (1)

methylcellulose/Tween 80 vehicle; (2) afatinib (10 mg/kg/day of body weight) on day 1–7; (3) methylcellulose/Tween 80 vehicle plus 15 Gy of radiotherapy on day 4; (4) afatinib plus radiotherapy. Small animal positron emission tomography/computed tomography (PET/CT) scans with [18F]-2-fluoro-2-deoxy-D-glucose (FDG) were performed on the 8th day of treatment. The mice were intravenously injected with 14 MBq (378 Ci) of FDG in saline via the tail vein.

Irradiation of mice

Mice were immobilized using a customized harness. With the body shielded, the thigh tumor was irradiated with a half-beam rectangular field. A 6-MV photon beam from a linear accelerator was used to irradiate the thigh tumor with 15 Gy on day 4.

Histological evaluation

After fixation, tumor tissues were embedded in paraffin blocks and sectioned (5 μm). Tumor cells were detected in representative stained sections. The expressions of phospho-EGFR (Cell Signaling Technology, Inc., Danvers, MA, USA) and phospho-HER2 (Abcam PLC, Cambridge, UK) were evaluated after immunohistochemical staining using specific antibodies.

Statistical analysis

The tumor volume data satisfied the assumptions of normality and homogeneity of variance for parametric analysis; thus, group means on day 21 for the ectopic tumor models were compared with a one-way analysis of variance (ANOVA) followed by Fisher's least significant difference method for multiple comparisons. Differences were considered significant at $p < 0.05$.

3.3 Results (Tsai et al., 2013)

3.3.1 Radiosensitization of MBT-2 cells by afatinib

Clonogenic cell survival decreased dose-dependently either with irradiation (2.5–10 Gy) or afatinib treatment (200–1000 nM (Fig. 3-1A). To determine if the interaction between afatinib and radiation was synergistic, combination index (CI) values were calculated from the dose–response data. In MBT-2 cells treated with highest doses of irradiation and afatinib, CI values of < 1 were achieved and indicative of synergism (Fig. 3-1B).

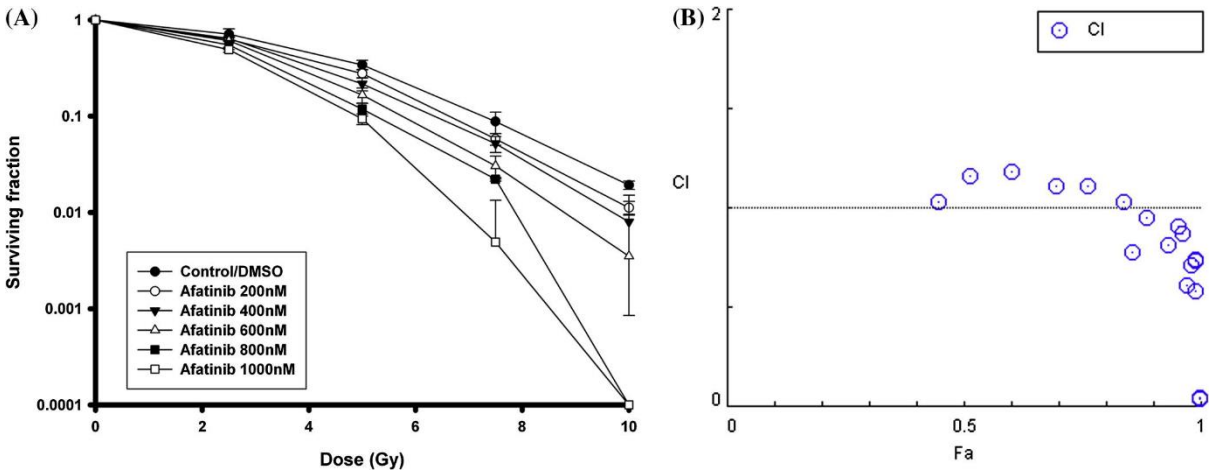

Figure 3-1. Radiosensitization of MBT-2 cells by afatinib. (A) Quantitative results of the clonogenic assays after combination treatment with afatinib and irradiation. Cells were cultured at a density of 500 cells per well in six-well plates and pretreated with different doses of afatinib (200–1000 nM for 30 min and then irradiated with different doses (2.5–10 Gy). After 7 days, the cells were fixed, stained and photographed (100X). The images were used to count the number of colonies containing more than 50 cells in each well. The number of MBT-2 colonies at each dose level is expressed as a percentage of those in the

corresponding control group. Lines, mean (n = 3); Bars, S.D. (B) CI for each dose level of irradiation and afatinib were calculated and plotted as a function of the MBT-2 cell fraction affected (Fa). CI values < 1 indicate synergism.

3.3.2 Radiation activates EGFR/HER2 and Akt protein expressions in a time-dependent manner

It has been reported that receptor tyrosine kinases, such as Erb-B family proteins, are activated by irradiation. Besides, activation of the PI3K/Akt pathway is associated with radioresistance. We found in Western blotting assays that levels of both HER2 and EGFR proteins increased time-dependently, starting at 2 h and 6 h after irradiation in MBT-2 cells with 2.5 Gy and 10 Gy, respectively (Fig. 3-2). Similarly, the increased expression of phospho-Akt was induced in a time-dependent manner.

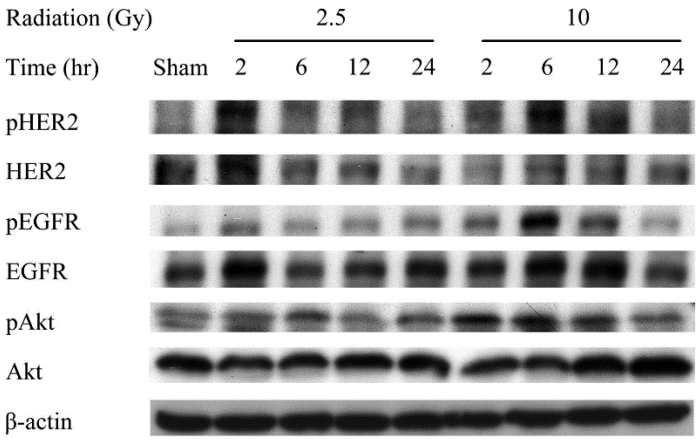

Figure 3-2. Radiation activates EGFR and PI3K/Akt pathways at both low and high doses. MBT-2 cells were treated with irradiation (2.5 Gy and 10 Gy). Cell lysates were prepared for Western blotting of the phosphorylation forms of EGFR, HER2 and Akt, and the effects of

irradiation can be seen to unfold in a time-dependent manner.

3.3.3 Afatinib inhibits radiation-induced EGFR/HER2 and Akt protein expressions in MBT-2 cells

Since radiation induces Erb-B family protein expression, we investigated whether the dual EGFR/HER2 inhibitor, afatinib, can suppress induced expression of these proteins. In MBT-2 cells that received irradiation (either alone or in combination with afatinib), the increased expression of HER2 and EGFR proteins as well as expression of activated phospho-Akt were inhibited by afatinib at 6 h (Fig. 3-3).

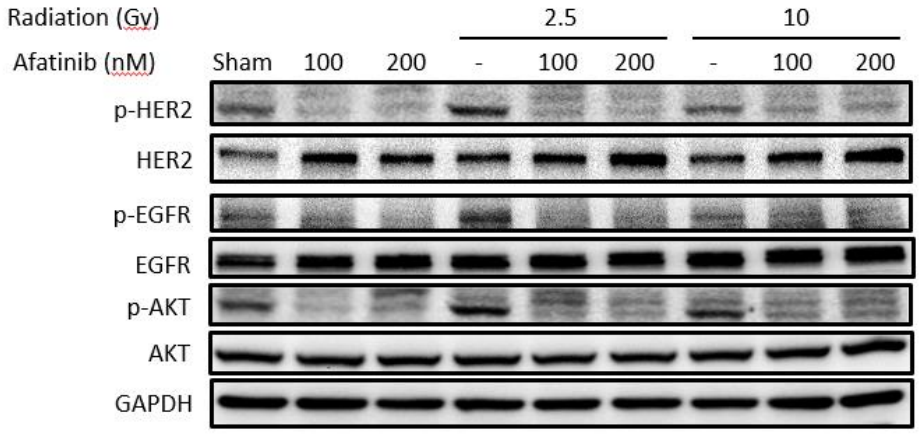

Figure 3-3. Afatinib inhibits the radiation-activated EGFR phosphorylation and PI3K/Akt pathways. MBT-2 cells were pretreated with afatinib (100 nM and 200 nM for 24 h and then irradiated (2.5 Gy and 10 Gy). After 6 h, cell lysates were prepared for Western blotting of phosphorylated forms of EGFR, HER2 and Akt, with β-actin as a loading control.

3.3.4 Afatinib combined with irradiation increases the apoptosis of MBT-2 cells

Our analysis of the cell cycle distribution of MBT-2 cells at 6 h after irradiation (10 Gy) with or without pre-treatment of afatinib (100 nM, 30 min) revealed that the combination significantly increased the sub-G1 population ($p < 0.05$), indicating apoptotic cell death (Fig. 3-4A). Radiation alone failed to cause a statistically significant increase in the sub-G1 population, but it did insignificantly increase the proportion of cells in G2/M phase arrest and insignificantly decrease the proportion of cells in S phase. Afatinib alone, however, did not cause any significant change in the cell cycle phases. Moreover, Western blot analysis of cleaved PARP revealed that pretreatment with afatinib strongly increases the expression of this apoptotic marker in response to irradiation (Fig. 3-4B).

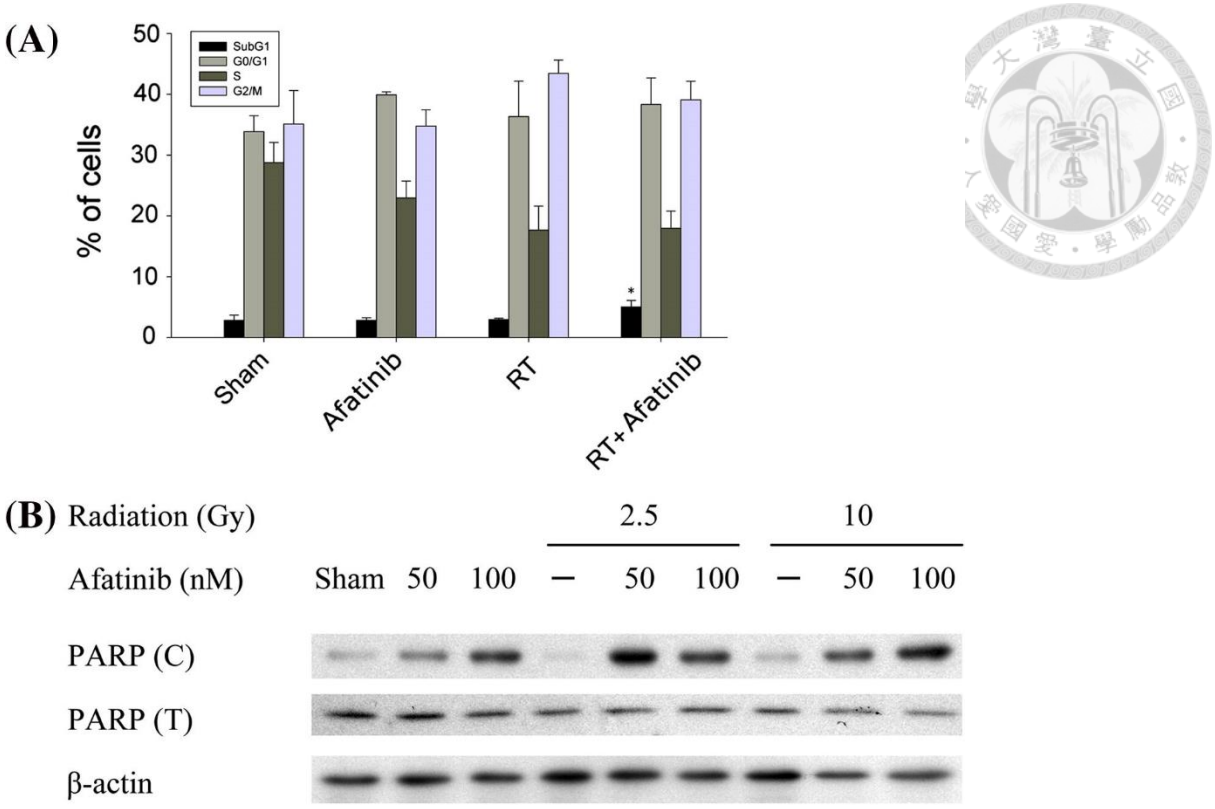

Figure 3-4. Afatinib enhances radiation-induced apoptosis in MBT-2 cells. (A) MBT-2 cells were pretreated with afatinib (200 nM for 30 min and then with radiation (RT; 10 Gy). The cell cycle distributions were assessed 6 h after afatinib alone, RT alone and in combination. Columns, mean (n = 3); Bars, S.D. ∗, $p < 0.05$. (B) MBT-2 cells were pretreated with afatinib (50 nM and 100 nM for 30 min and then with RT (2.5 Gy and 10 Gy). After 6 h, cell lysates were prepared for Western blotting to detect the apoptotic marker PARP (cleavage form).

3.3.5 Afatinib combined with irradiation increased DNA damage of MBT-2 cells

Fig. 3-5A and 3-5B show the result of immunofluorescence staining of γ-H2AX, a marker of DNA double-strand breaks. While sham-irradiated cells exhibited a minimal number of γ-H2AX foci (0 ± 0.05/cell), radiation alone induced immediate increases in γ-H2AX foci (13.0

± 0.28/cell) that were evident at 30 min as a result of cellular DNA damage. In contrast, treatment with afatinib had no effect on γ-H2AX foci (0 ± 0.03/cell). However, in cells pretreated with afatinib prior to irradiation, the number of γ-H2AX foci was significantly increased over that observed after irradiation alone (20.0 ± 0.46/cell versus 13.0 ± 0.28/cell, $p < 0.001$). Western blot assay revealed dose-dependent changes in γ-H2AX levels in MBT-2 cells pre-treated with afatinib (50 or 100 nM for 30 min followed by irradiation (2.5 or 10 Gy; Fig. 3-5C).

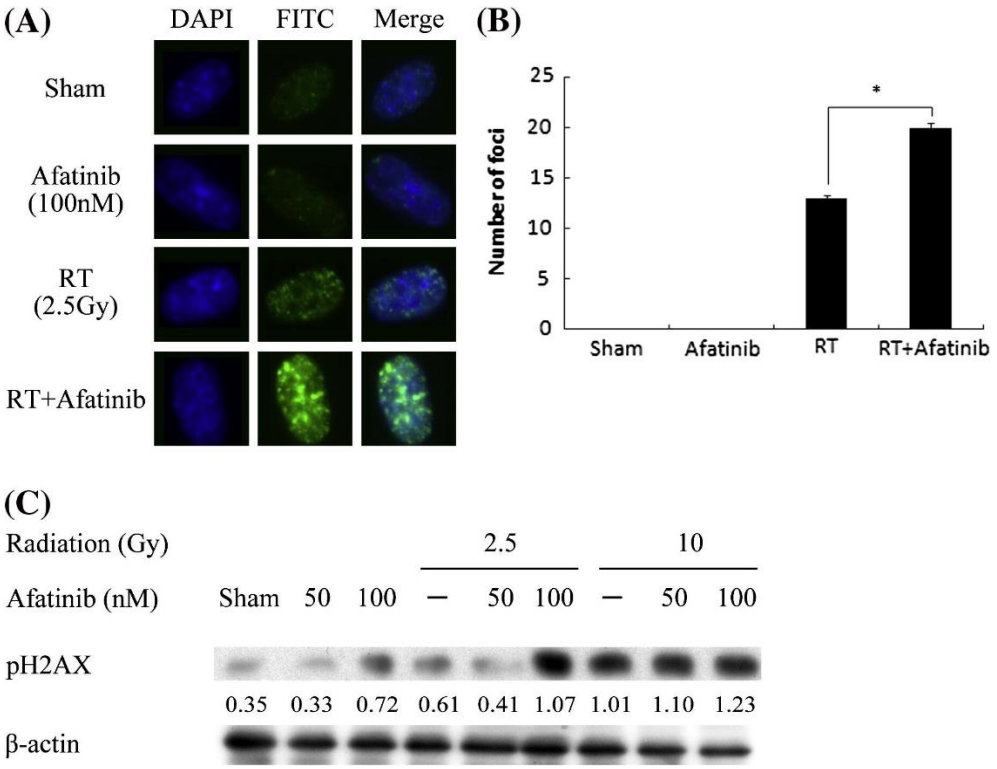

Figure 3-5. Afatinib enhances radiation-induced DNA damage of MBT-2 cells. (A) Micrographs (1000X) of γ-H2AX foci, a marker of DNA double-strand breaks, of MBT-2 cells at 30 min after pretreatment with afatinib (100 nM for 30 min and then with radiation (RT; 2.5 Gy) show the 4',6-diamidino-2-phenylindole (DAPI) staining for cells, FITC for γ-

H2AX (green foci) and the merged images. (B) The number of γ-H2AX foci counted in 150 cells per group. Data presented are the mean number of foci per cell in each group. Columns, mean; Bars, S.D. ∗, $p < 0.05$. (C) MBT-2 cells were pretreated with afatinib (50 and 100 nM for 30 min and then RT (2.5 Gy and 10 Gy). After 30 min, cell lysates were prepared for Western blotting to detect phospho-γ-H2AX (p-H2AX) and H2AX (loading control).

3.3.6 The combination of afatinib and radiotherapy exhibits an enhanced ability to control the growth of ectopic MBT-2 xenograft tumors

Daily oral treatment of mice with afatinib (10 mg/kg for 7 days) in combination with radiotherapy on day 4 suppressed the growth of xenograft tumors to a greater extent than radiotherapy alone (Fig. 3-6A). Afatinib itself did not satisfactorily control growth. Treatment with afatinib enhanced radiation-induced suppression of MBT-2 tumor growth by 64%.

One day after the treatment (day 8), thigh tumors were imaged by micro-PET/CT with 18F-FDG. Tumor viability was decreased after combined afatinib and radiotherapy, when compared to either modality alone or sham treatment (Fig. 3-6B). The treatment with afatinib alone failed to reduce metabolic tumor volume, but radiotherapy to thigh tumors (15 Gy) by itself partially reduced tumor size. Importantly, co-treatment with afatinib at 10 mg/kg significantly improved this radiotherapeutic effect.

The expressions of HER2 and EGFR were assessed immunohistochemically in MBT-2 tumors harvested at 8 days after initiation of treatments. Radiotherapy itself increased the expressions of both HER2 and EGFR (Fig. 3-6C). Moreover, combined treatment with radiotherapy and afatinib suppressed radiation-activated expression of HER2 and EGFR in tumor tissues.

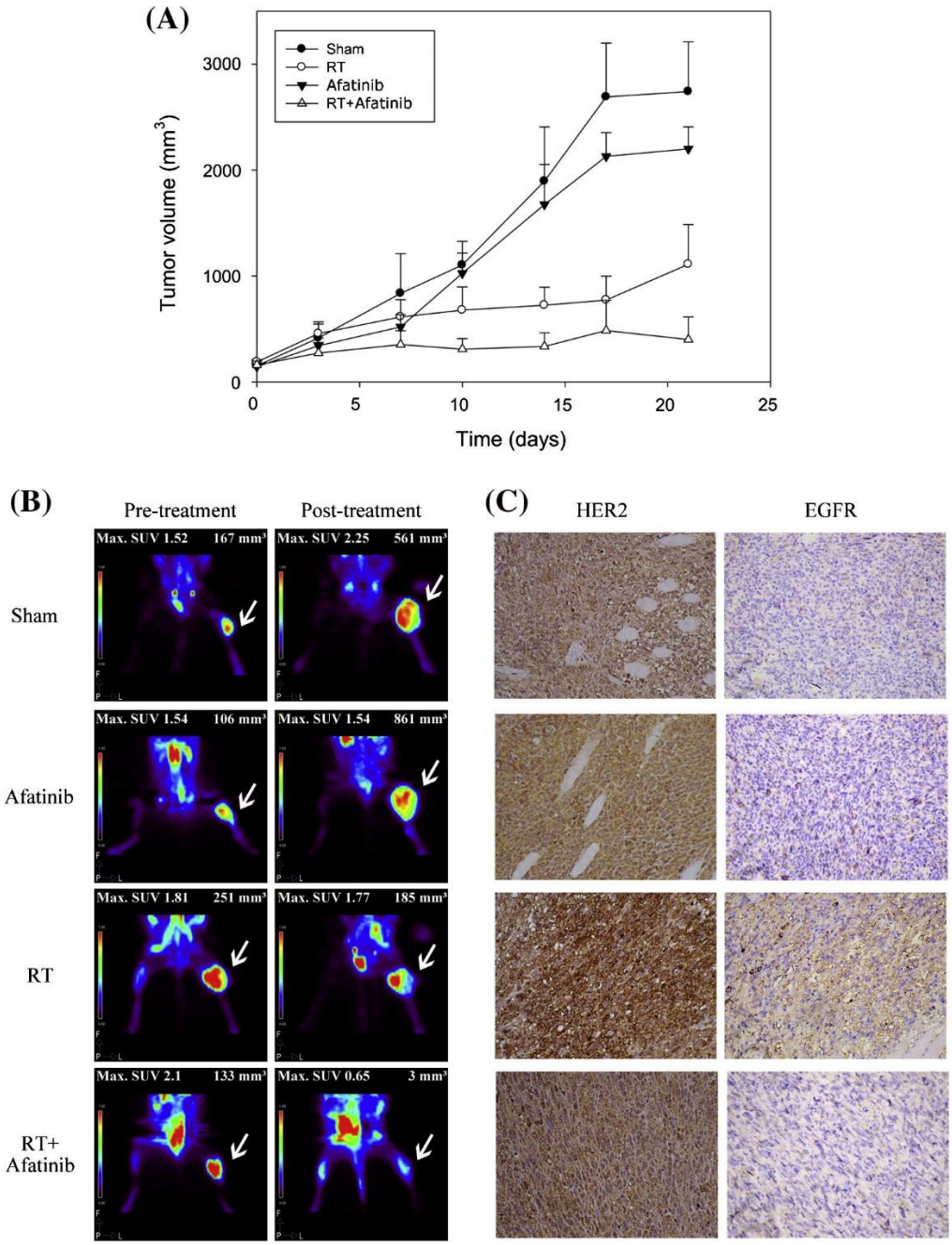

Figure 3-6. Combined afatinib and irradiation (RT) enhances tumor suppressive activity in ectopic murine bladder tumor model. (A) C3H/HeN mice bearing subcutaneous MBT-2 tumors were randomized into 4 groups (n = 5 in each group) to receive RT alone (15 Gy on day 4), oral afatinib (10 mg/kg/day from day 1–7) alone, combined afatinib and RT or control treatment (sham). Data presented are the mean tumor volume for each group measured on the indicated days. Points, mean; bars, S.D. (B) Mice bearing ectopic MBT-2 tumors were randomized as in (A) to receive afatinib (10 mg/kg/day) alone, RT alone, combined RT and afatinib or sham treatment. Mice were scanned by positron emission tomography/computed tomography to determine tumor metabolism on day 8. Images of representative mice from each group are shown. Arrows indicate viable right thigh tumors. The standardized uptake value (SUV) and volume of each tumor were shown on top of the panel. (C) Mice bearing ectopic tumors were sacrificed on day 8. Microscopic images (200X) of tumor tissue sectioned and immunohistochemically stained with antibodies against HER2 (left panel) and EGFR (right panel) from a representative mouse in each group are shown.

CHAPTER FOUR: RADIOSENSITIZING EFFECT OF AFATINIB IN HUMAN BLADDER CANCER MODELS

4.1 Rationale and Approach

Erlotinib (Maemondo et al., 2010) is a first-generation EGFR tyrosine kinase inhibitors and have well-established efficacy in non-small cell lung cancer patients with EGFR activating mutations. Afatinib, on the other hand, is a second-generation EGFR tyrosine kinase inhibitor and found to be of benefit to patients with advanced lung adenocarcinoma who failed previous gefetinib or erlotinib (Miller et al., 2012). First RTK array was done to detect the change of signaling pathways after irradiation with or without drugs. Then the experimental design is similar to murine bladder cancer model and includes: (a) clonogenic assay, (b) flow cytometry analysis, (c) immunofluorescence detection of γH2AX foci and (d) animal study. The advantage of human bladder cancer model is that the result may benefit patients clinically. Finally the mechanism was explored.

4.2 Materials and Methods

Cell lines

The human bladder urothelial carcinoma cell line, T24, was purchased from the American Type Culture Collection / Bioresource Collection and Research Center (Hsinchu, Taiwan) in 2011. The cells were authenticated in BCRC by short-tandem repeat (STR)-PCR profiling. They were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Another human bladder carcinoma cell line, NTUB1, was established from human bladder cancer tissue and kindly provided by Dr. Hong-Jeng Yu (Yu, Tsai, Hsieh, & Chiu, 1992). It was not authenticated. NTUB1 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum. Both cell lines were incubated at 37℃ in a humidified atmosphere of 5% CO_2 and 95% air. We sequenced the cDNA of both cell lines, and none of the common EGFR mutations was found.

Reagents

Afatinib and erlotinib were purchased from Selleck Chemicals (Houston, TX). For *in vitro* experiments, afatinib and erlotinib stock solutions were prepared in DMSO and 50% acetonitrile, respectively. Both compounds were diluted in culture medium before dosing. For *in vivo* experiments, afatinib and erlotinib were suspended in a vehicle (0.5% methylcellulose [wt/vol] and 0.4% Tween 80 [vol/vol] in sterile water) for oral administration to ICR nude

mice (BioLASCO, Ilan, Taiwan) bearing tumor xenografts.

Irradiation of cells and animals

T24 and NTUB1 cells cultured in flasks were irradiated with various doses of ionizing radiation, using a 6-MV photon beam from a Siemens Primus linear accelerator (Siemens Oncology Medical Systems, Inc., Concord, CA). Mice were immobilized using a customized harness. With the body shielded, the thigh tumor was irradiated with a half-beam rectangular field. The distance from the radiation source to the bottom of the flask or the thigh tumor of nude mice was set at 100 cm.

RTK signaling antibody array

The PathScan® RTK signaling antibody array kit from Cell Signaling Technology (Danvers, MA) contained 39 antibodies against phosphorylated forms of receptor tyrosine kinases or key signaling proteins. T24 cells were first treated with 100 nM afatinib or erlotinib for 30 min, and then with 10 Gy of radiation. After 24 h of incubation, the cells were processed for RTK array analysis according to the manufacturer's instructions. The membrane was developed with LumiGLO® and Peroxide reagent (Cell Signaling Technologies), and RTK spots were visualized using a UVP imaging system and densitometrically quantified with ImageProPlus software. Each kinase array dot was manually selected, and an average

intensity for each kinase was calculated. For comparison of different stimulation conditions, sets were normalized to allow equal intensities of positive controls.

Clonogenic assays

T24 or NTUB1 human bladder cancer cells (1×10^3 per well) were cultured in 6-well plates, treated with different doses of radiation following 1-h pretreatment with afatinib or erlotinib on day 1, re-treated with the drugs on day 2 and day 3 using the same concentrations, incubated for 7 days, and stained with 0.5% crystal violet (Sigma-Aldrich; St. Louis, MO) in 10% methanol for 30 min at room temperature. Colonies with more than 50 cells were counted. At each drug concentration, the surviving fraction was determined by dividing the total number of colonies after irradiation by the number of colonies without irradiation. Each point on the survival curve represents the mean surviving fraction from 3 independent experiments.

Cell-cycle analysis

Cell cycle stages were analyzed using a BD FACSCan Flow Cytometer (Becton Dickinson; Franklin Lakes, NJ). In brief, T24 or NTUB1 bladder cancer cells were pretreated for 30 min with vehicle, 200 nM afatinib, or 200 nM erlotinib, irradiated (2.5 Gy), incubated 24 h, fixed in 70% ethanol, and stained with a solution containing 50 μg/mL propidium iodide and 0.1

mg/mL RNAase (both from Sigma-Aldrich) in the dark for 30 min. Ten thousand events were examined for each determination. The relative proportions of cells in different cell cycle phases were determined using WinMDI software.

Determination of apoptosis with fluorescence microscopy

Apoptotic cells were detected using the annexin V/FITC apoptosis detection kit (AVK050, Strong Biotech, Taipei, Taiwan) according to the manufacturer's instructions. The annexin V-positive cells were examined using a Zeiss Axio Imager A1 fluorescence microscope. Representative images from different treatment groups were taken into account and at least 50 cells were calculated in every group. The portion of annexin V-positive cells was calculated as the ratio of positively stained cells divided by the total cell numbers.

Western blotting and immunoprecipitation

Aliquots of T24 and NTUB1 bladder cancer cell lysates containing 50 μg of protein were separated by SDS-PAGE (8–15% polyacrylamide), and the separated proteins were transferred to polyvinylidene difluoride (PVDF) membranes and immunoblotted with various antibodies. For immunoprecipitation experiments, we used the Catch and Release v2.0 Reversible Immunoprecipitation System (Millipore) according to the manufacturer's instructions. The immunoprecipitates (50 μg) of cells were eluted, resolved by 8% SDS-

PAGE, electrotransferred to PVDF membranes and incubated with primary antibodies. For whole-cell preparations, tumor tissue from individual animals was homogenized with a motor driven pestle and then lysed in 0.2 ml of RIPA lysis buffer/20 mg tissue. The homogenate was then centrifuged (13,000 *g*) for 10 min and the supernatant was used as whole-cell extract. Bound antibodies were detected using appropriate peroxidase-coupled secondary antibodies followed by enhanced electrochemiluminescence (Roche Diagnostics; Basel, Switzerland). The antibodies used were EGFR, HER2, phosphor-Akt (Ser473), caspase-3, PARP, cleaved PARP (Cell Signaling Technology), phospho-EGFR (pY1086), phospho-HER2 (pY1139) (Epitomics, Burlingame, CA), and beta-actin (Santa Cruz Biotechnology, Santa Cruz, CA).

Chemical cross-linking

Samples for the cross-linking analysis were obtained at 60 minutes after various treatments. The cells were washed with PBS three times and incubated for 60 min at room temperature with 5 mM Suberic acid bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt (Sigma-Aldrich) in PBS, and the reaction was terminated using 20mM Tris-HCl, pH 8.5 for 15 min at room temperature. Subsequently, cells were washed with PBS and solubilized in lysis buffer. The collected proteins were subjected to western blotting using EGFR or HER2 antibody.

HER2 RNA interference and stable transfection

To knockdown HER2 gene expression, we used a target-specific lentiviral vector plasmid encoding a 19–25 nt hairpin shRNA (Santa Cruz Biotechnology, cat. no. sc-29405-SH). One day before transfection, T24 and NTUB1 bladder cancer cells were seeded into 6-well culture plates and grown until 70–90% confluent for transfection. A mixture of HER2 shRNA plasmid or scramble plasmid (Santa Cruz Biotechnology, cat. no. sc-108083), blank Dulbecco's modified Eagle's medium, and lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA) at a ratio of 1 μg:98 μl:2 μl was added to the T24 and NTUB1 cells, according to the manufacturer's instructions. The cells were incubated 5–7 h at 37°C, harvested, and incubated with 2 μg/ml puromycin (Invitrogen) to select stable clones. The efficiency of HER2 knockdown in T24 and NTUB1 cells was confirmed by Western blot.

Mouse xenograft models

Male athymic ICR nude mice (6- to 8-weeks old; BioLASCO, Ilan, Taiwan) were housed in sterilized cages under 12 h light/dark cycles and given ad libitum access to food and water. NTUB1 human bladder cancer cells (2×10^6 cells per site) were suspended in Matrigel (BD Biosciences, San Jose, CA) and injected into the right hind leg of mice. As the tumors became established (mean starting tumor volume = 101 mm^3), mice were randomized into 6 groups (n=6) to receive the following treatments: (1) methylcellulose/Tween 80 vehicle; (2) oral afatinib (daily 10 mg/kg of body weight) on days 1–7; (3) oral erlotinib (daily 50 mg/kg

of body weight) on days 1–7; (4) vehicle plus 10 Gy of radiotherapy on day 4; (5) oral afatinib (daily 10 mg/kg of body weight) on days 1–7 plus 10 Gy of radiotherapy on day 4; (6) oral erlotinib (daily 50 mg/kg of body weight) on days 1–7 plus 10 Gy of radiotherapy on day 4. Tumors were measured twice a week using calipers, and tumor volume was calculated by the formula: Tumor volume (mm^3) = 0.5 * tumor length (mm) * (tumor width $[mm]^2$). Mice were sacrificed when tumor volume reached 1500 mm^3. All experimental procedures using these mice were performed in accordance with protocols approved by the National Taiwan University Institutional Animal Care and Use Committee.

Animal imaging

Small animal PET/CT scans with 18F-FDG were performed on day 0 and 14. The mice were intravenously injected with 14 MBq (378 Ci) of FDG in saline via the tail vein. After the PET/CT images, mice were sacrificed and tumors were excised for tissue Western blot or fixation in 10% neutral buffered formalin.

Statistical analysis

The paired-comparisons were assessed using the Student *t* test. Differences were considered significant at $p < 0.05$.

4.3 Results (Tsai et al., 2015)

4.3.1 Concomitant EGFR and HER2 tyrosine kinase inhibition significantly suppresses radiation-activated signaling pathways

To understand the impact of irradiation on the phosphorylation of RTK and key signaling proteins, we used an RTK signaling antibody array to determine the change in intensity of phosphorylated proteins 24 hours after irradiation in T24 bladder cancer cells. Figure 4-1A and 4-1B demonstrates that phosphorylation of EGFR, HER2, and downstream signals were enhanced after irradiation of these bladder cancer cells. Treatment with 100 nM afatinib 30 min before irradiation significantly suppressed the level of phosphorylated EGFR, HER2, Akt (Thr308 and Ser473), MAPK, and S6 ribosomal proteins. In contrast, 100 nM erlotinib pretreatment followed by irradiation suppressed only levels of phospho-EGFR and phospho-MAPK. These results showed that afatinib (a tyrosine kinase inhibitor with activity against EGFR and HER2) inhibits radiation-activated signaling pathways and is a more efficient suppressor than erlotinib (a tyrosine kinase inhibitor blocking EGFR activity) in blocking the phosphorylation of HER2, Akt, and S6 ribosomal protein.

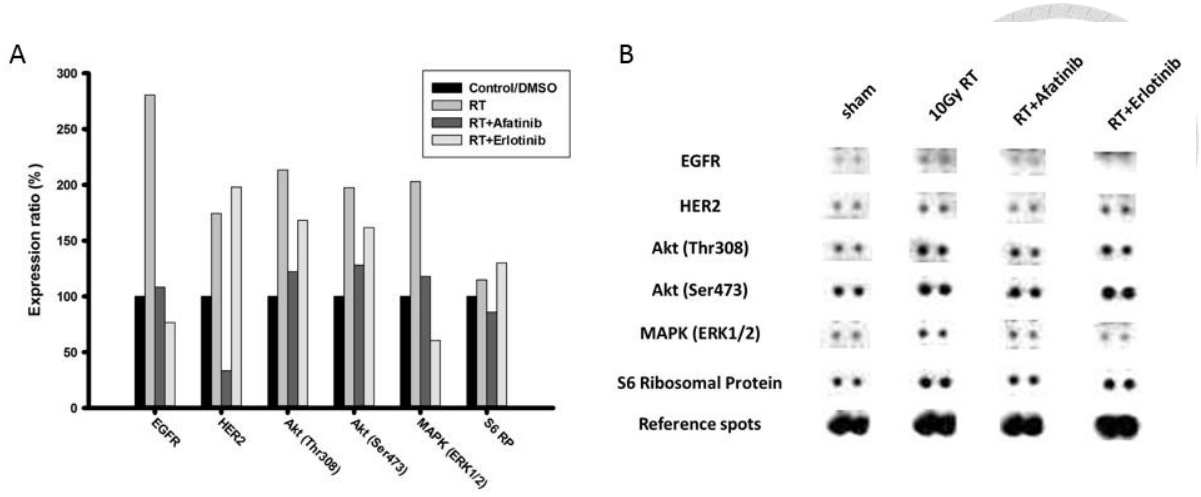

Figure 4-1. The effect of combining radiation with EGFR TKIs on radiation-sensitive signals. (A) Treatment of T24 cells with irradiation stimulates phosphorylation of EGFR, HER2, and downstream signals as detected by the PathScan® RTK Signaling Antibody Array Kit 24 hours after treatment. Pretreatment with 100 nM afatinib suppresses the phosphorylation of EGFR, HER2, Akt, MAPK, and S6 proteins. In contrast, pretreatment with 100 nM erlotinib only inhibits the phosphorylation of EGFR and MAPK. (B) The original results of PathScan® RTK Signaling Antibody Array.

4.3.2 Dual blockade of EGFR and HER2 tyrosine kinases significantly radiosensitizes bladder cancer cells

The clonogenic assays of T24 and NTUB1 bladder cancer cells after treatment with afatinib or erlotinib once a day for three consecutive days and irradiation on the first day demonstrated that afatinib dose-dependently decreased the clonogenic survival of both cells (Fig. 4-2A and 4-2B) whereas erlotinib had no such effect, even at 1500 nM (Fig. 4-2C and 4-

2D). The result indicated that EGFR/HER2 dual inhibitor (compared with EGFR inhibitor) was a better radiosensitizing agent of bladder cancer cells.

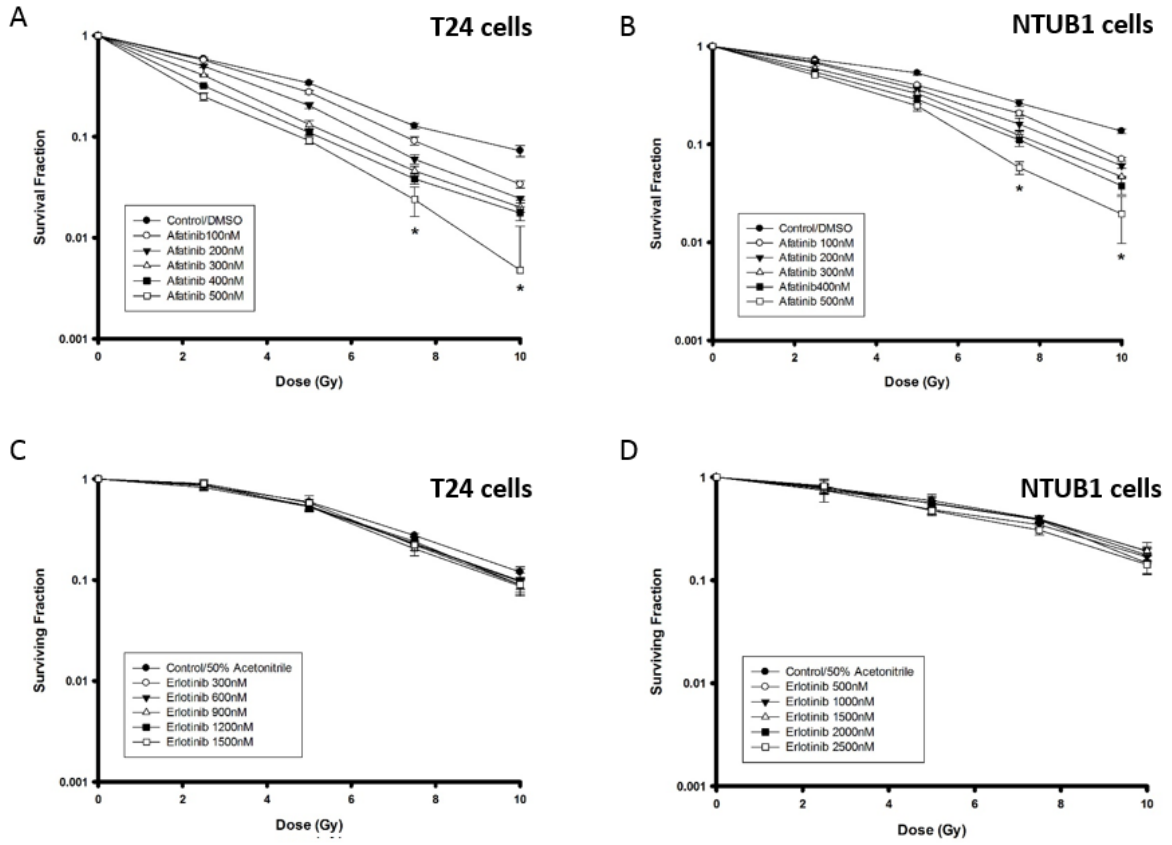

Figure 4-2. Clonogenic survival analysis shows a difference in the radiosensitizing effect of TKIs that inhibit EGFR/HER2 or EGFR alone. Afatinib, an EGFR/HER2 dual inhibitor, has a significant radiosensitizing effect and causes a dose-dependent decrease in clonogenic cell survival 7 days after initial treatment in both cell lines (A and B). Erlotinib, an EGFR TKI, fails to radiosensitize either cell line even at high-dose (C and D). The results are expressed as mean ± SD values from three independent experiments. (*, $p< 0.05$ when comparing with control group.)

4.3.3 Combined EGFR and HER2 tyrosine kinase inhibition promotes radiation-induced apoptosis

To understand whether drug-mediated enhancement of radiosensitivity was due to synchronization of the cell cycle, propidium iodide staining and flow cytometry were used to determine the cell cycle phase distribution of bladder cancer cells 24 hours after the treatment. As shown in Figure 4-3A, irradiation (compared with afatinib or erlotinib treatment alone) significantly increased the proportion of T24 and NTUB1 cells in sub-G1 phase, and treatment with 200 nM afatinib 30 min before irradiation (compared with irradiation alone or irradiation plus 200 nM erlotinib) significantly increased the percentage of T24 cells in sub-G1 phase. The effects on NTUB1 cells are similar (Fig. 4-3B). Irradiation also significantly increased the proportion of both cells in G2-M phase. Treatment with afatinib, but not erlotinib, abolished this effect. To confirm the biological significance of combined treatments in enhancing apoptosis, we performed fluorescence microscopic analysis of annexin V binding of both T24 and NTUB1 cells (Fig. 4-3C & 4-3D). The results showed the percentage of annexin V-binding cells increased dramatically after RT. The combined treatment of RT and afatinib, not the RT and erlotinib combination, further increased annexin V-positive cells. This indicated that radiation-induced apoptosis was enhanced by afatinib but not erlotinib. These data suggested that this EGFR/HER2 dual inhibitor (in contrast to EGFR inhibitor alone) increases radiation-induced apoptosis in both

T24 and NTUB1 cells.

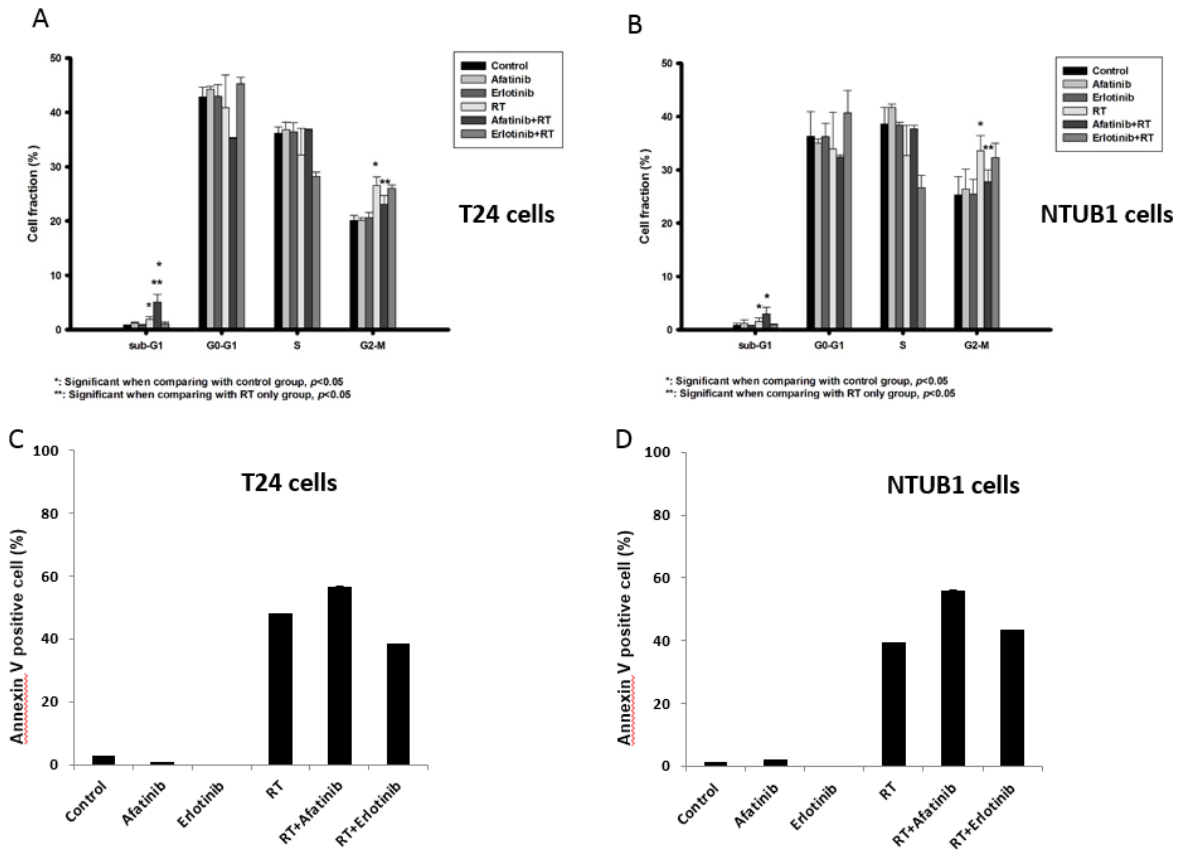

Figure 4-3. Tyrosine kinase blockade of both EGFR and HER2 by afatinib, not blockade of EGFR alone, promotes radiation-induced apoptosis. (A) Propidium iodide staining and flow cytometry analysis 24 hours after the treatment in T24 cells reveals that irradiation significantly increases the proportion of cells in sub-G1 phase, and pretreatment with afatinib, not erlotinib, further increases that proportion. Irradiation also increases the proportion of cells in G2-M phase, and pretreatment with afatinib, not erlotinib, abolishes this effect. The results are expressed as mean ± SD values from three independent experiments. (B) The effects on NTUB1 cells are similar. (C) Fluorescence microscopic analysis of annexin V binding in T24 cells showed that percentage of annexin V-binding cells increased

dramatically after RT, and the combined treatment of RT and afatinib further increased annexin V-positive cells. The results are expressed as mean ± SD values from three independent experiments. (D) Analysis of annexin V binding in NTUB1 cells.

4.3.4 Effects of combining irradiation and afatinib on cell apoptosis

We subsequently assessed the expression of apoptosis markers, cleaved forms of caspase 3 and PARP, in the two bladder cancer cell lines 24 hours after treatment. As shown in Fig. 4-4, afatinib pretreatment combined with irradiation (when compared with irradiation or afatinib treatment alone) resulted in greater expression of cleaved forms of caspase-3 and PARP in both NTUB1 and T24 cells. The result further validates the finding that afatinib increases radiation-induced cell apoptosis.

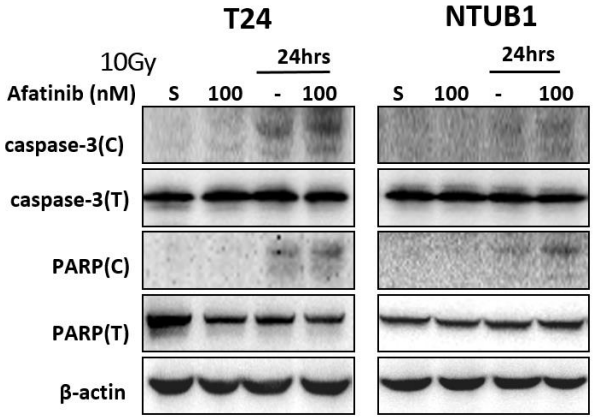

Figure 4-4. Western blots show that irradiation increases the expression of the apoptosis markers (cleaved forms of caspase 3 and PARP) which are further enhanced by afatinib pretreatment.

4.3.5 Combined EGFR and HER2 tyrosine kinase inhibition enhances radiation-induced DNA damage

Since radiation-induced DNA damage is a common cause leading to cancer cell apoptosis, we investigated whether tyrosine kinase inhibition by afatinib or erlotinib modulates the level of unrepaired DNA damage. We assessed the quantity of phosphorylated histone H2AX (γ-H2AX), which forms foci at DNA double-strand breaks and recruits double-strand break repair proteins. As shown in Fig. 4-5A to 4-5D, following 2.5 Gy of radiation, DNA damage in T24 and NTUB1 cells increased significantly as demonstrated by the mean number of γ-H2AX foci per cell 30 min after irradiation. Adding 1 h of 100 nM afatinib pretreatment (but not 100 nM erlotinib pretreatment) to irradiation resulted in an increased mean number of γ-H2AX foci per cell in both T24 and NTUB1 cells (25±0.7 vs 19±0.4 , $p<0.001$, and 26±0.6 vs 17±0.4, $p<0.001$, respectively). These results suggest that increased DNA damage may account for the enhancement in cytotoxicity caused by adding this EGFR/HER2 dual inhibitor to irradiation.

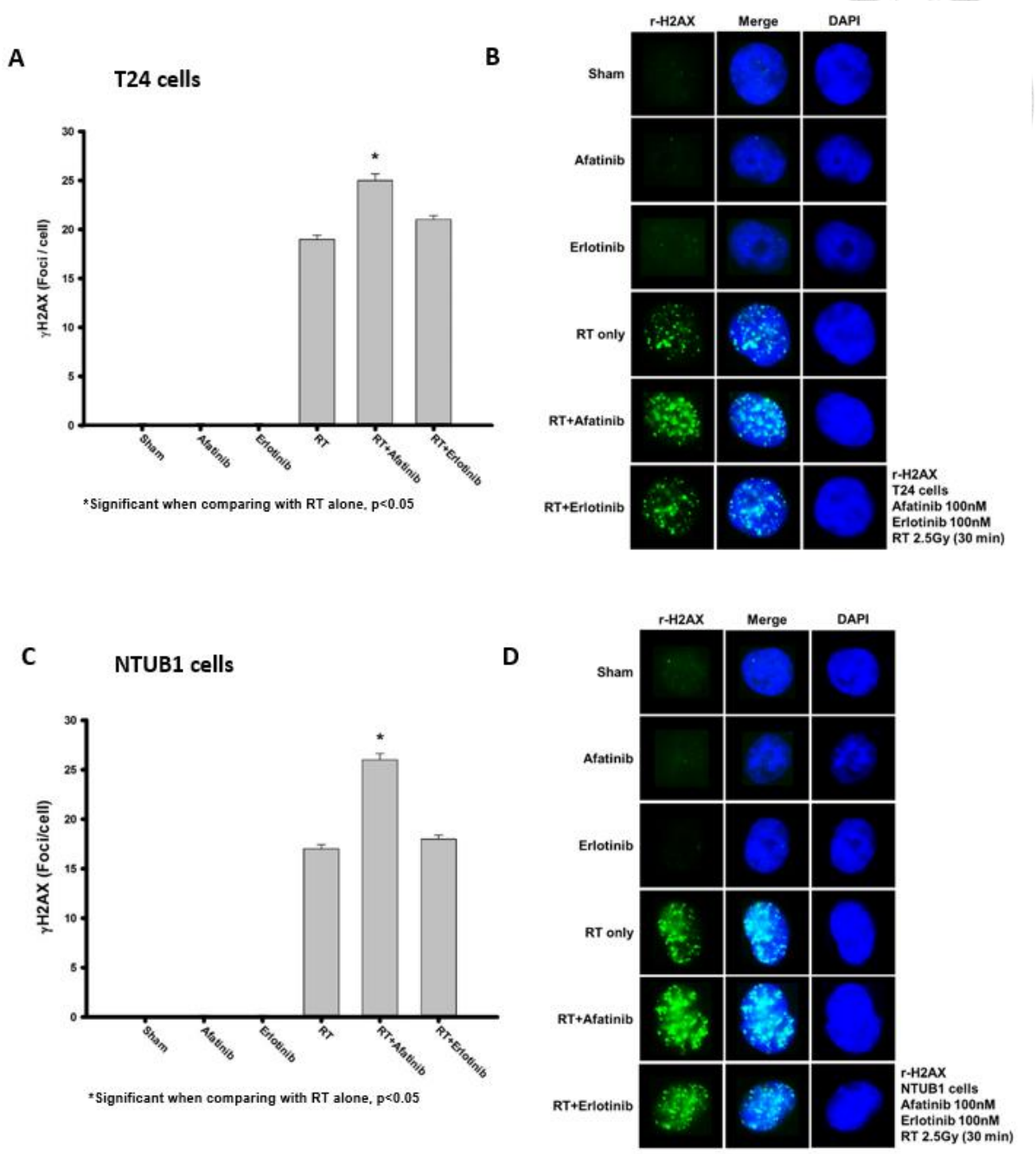

Figure 4-5. The EGFR/HER2 dual inhibitor afatinib, not the EGFR inhibitor erlotinib, promotes radiation-induced DNA damage. (A) The average number of γ-H2AX foci per cell increases 30 min after irradiation and is further enhanced by pretreatment with afatinib (but not with erlotinib) in T24 cells. (B) The representative images after different treatments in T24 cells. (C and D) The effects in NTUB1 cells are similar. The results are expressed as

mean ± SD values in a minimum of 150 cells per treatment group.

4.3.6 Concomitant *in vivo* EGFR and HER2 tyrosine kinase inhibition results in improved tumor control

We next sought to examine whether concomitant EGFR and HER2 tyrosine kinase inhibition enhances the *in vivo* effect of radiation on bladder cancer cells. As shown in Fig. 3-6A, 7-day oral treatment with afatinib in combination with RT on day 4 suppressed the growth of tumor xenografts to a greater extent than RT alone or erlotinib-RT combination. Afatinib itself had only a minor inhibitory effect on tumor growth. The tumor volume required 14.8, 16.3, 26.8, 34.9, 31.5, and 63.6 days, respectively, to reach 500 mm^3 in the sham treatment, erlotinib alone, afatinib alone, RT alone, erlotinib-RT combination, or afatinib-RT combination groups.

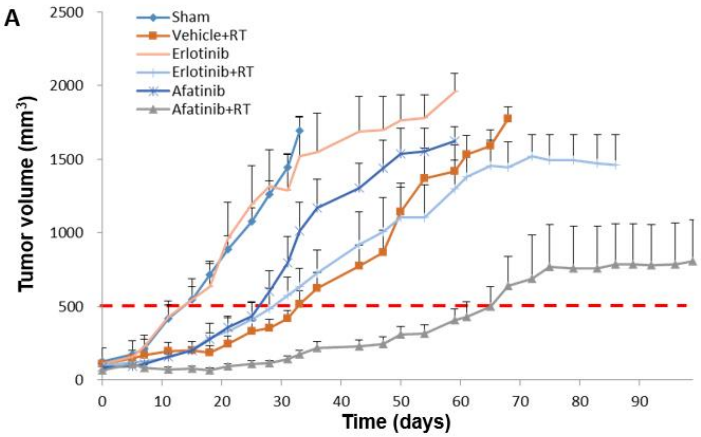

Figure 4-6A. Tumor volume of mouse xenograft model. Seven-day oral treatment of nude mice with afatinib in combination with RT on day 4 suppresses the growth of tumor xenografts to a greater extent than RT alone or the erlotinib-RT combination. The results are

expressed as mean ± SEM values from 6 mice in each group. Intersection of the dashed line with each curve indicates the number of days required to reach a tumor volume of 500 mm^2.

We then used 18F-FDG-micro-PET/CT to assess the effect of combining irradiation with dual blockade of EGFR and HER2 tyrosine kinases on glucose metabolism one week after the completion of drug treatment (day 14). The day-14 image (as compared to the pretreatment [day-0] image) showed that the metabolic tumor volume and level of glucose metabolism, representative of tumor viability, decreased after the afatinib-RT combination (Fig. 4-6B). In contrast, the metabolic tumor volume and level of glucose metabolism increased in mice treated with erlotinib-RT, while metabolic tumor volume increased even more in mice treated with sham, RT, erlotinib alone, or afatinib alone.

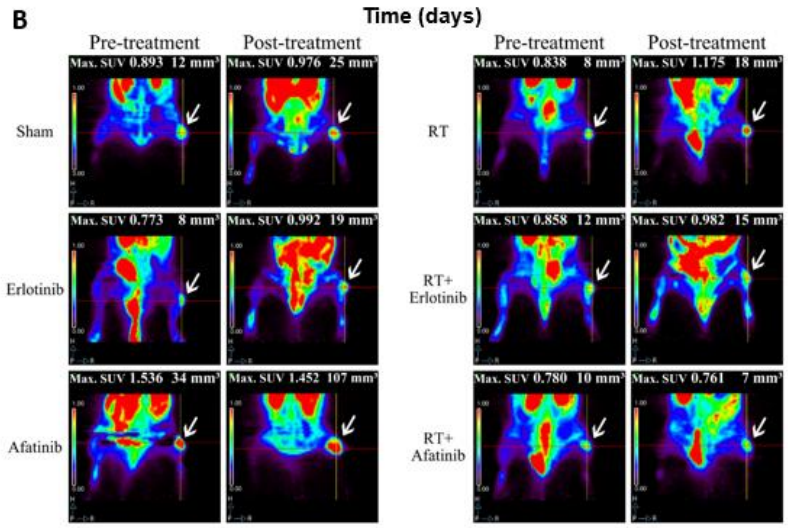

Figure 4-6B. Representative images of animal PET/CT before treatment (day 0) and 2 weeks after initial treatment (day 14). Arrows indicate the viable right thigh tumors. The SUV and

the viable volume of tumor are shown above the image. Tumors in mice receiving the afatinib-RT combination are the least viable.

The phosphorylation of EGFR, HER2, and Akt in mouse tumor xenografts was then assessed by Western blot analysis of lysates of tumor tissue harvested on day 3. As shown in Fig. 4-6C, RT induced the activation of these proteins, and only the RT-afatinib combination treatment effectively suppressed all these signals. Similarly, RT combined with afatinib (but not RT combined with erlotinib) induced an increase in the level of cleaved PARP expression on day 7.

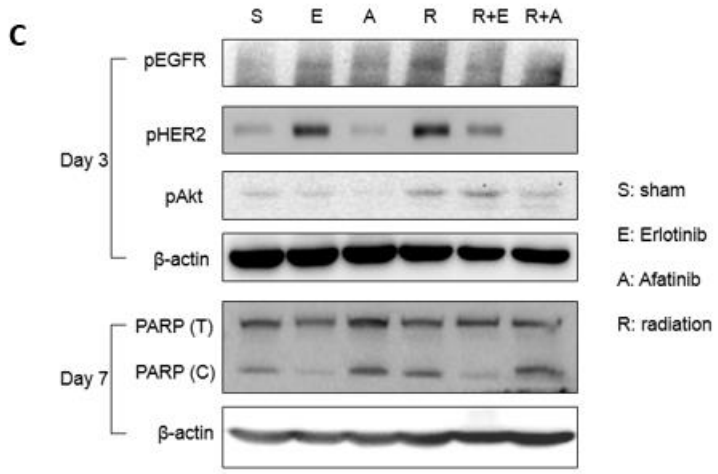

Figure 4-6C. Western blot analysis shows that the RT-afatinib combination (compared to the RT-erlotinib combination) effectively suppresses radiation-activated EGFR, HER2, and Akt signals and enhances cleaved PARP expression.

4.3.7 The superior radiosensitizing effect of afatinib over erlotinib is probably through

HER2 inhibition

Given the *in vitro* and *in vivo* superiority of the EGFR/HER2 dual tyrosine kinase inhibitor (afatinib) over the EGFR inhibitor (erlotinib) in radiosensitizing bladder cancer cells, it is reasonable to suggest that HER2 synergizes with EGFR to determine the level of radiosensitivity. To prove this hypothesis, we used a target-specific lentiviral vector plasmid to silence HER2 expression by RNA interference. The expression of HER2 in T24 and NTUB1 bladder cancer cells decreased dramatically after the transfection with this plasmid (Fig. 4-7A and 4-8A). Clonogenic assays showed that pretreatment with different concentrations of erlotinib had a radiosensitizing effect on T24 and NTUB1 cells transfected with HER2 shRNA (Fig. 4-7B and 4-8B) but not on T24 and NTUB1 cells transfected with control vector (Fig. 4-7C and 4-8C). Meanwhile, pretreatment with afatinib in control vector-transfected T24 and NTUB1 cells retained the radiosensitizing effect (Fig. 4-7D and 4-8D).

The number of γH2AX foci in T24 and NTUB1 cells transfected with HER2 shRNA was higher after treatment with the erlotinib-radiation combination than with radiation alone (Fig. 4-7E, 4-7F and Fig. 4-8E, 4-8F), indicating that by inhibiting HER2 gene expression, bladder cancer cells treated with EGFR tyrosine kinase inhibitor increased their vulnerability to radiation-induced DNA damage.

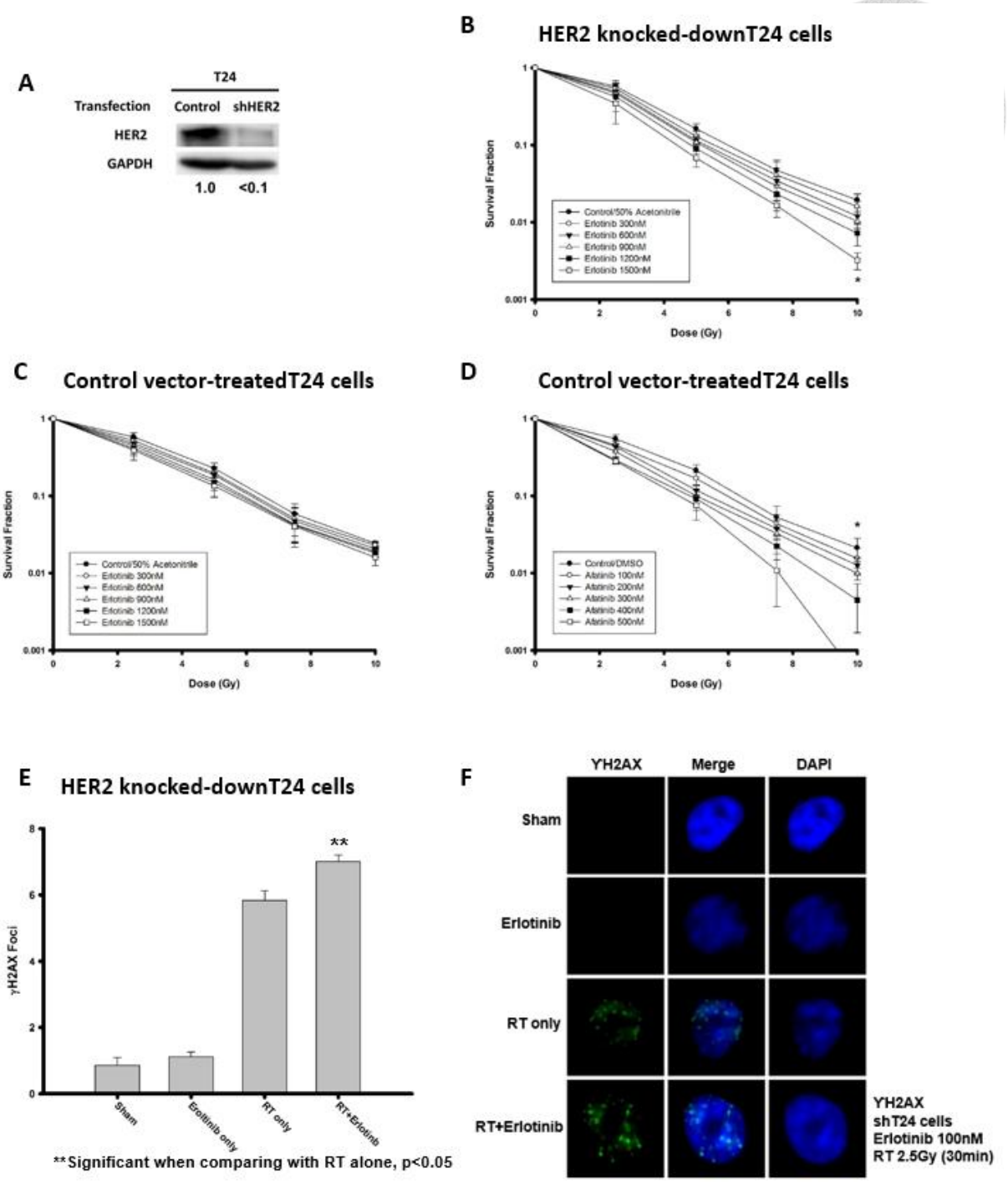

Figure 4-7. The radiosensitizing effect of erlotinib in HER2 knocked-down T24 cells emphasizes the synergism between EGFR and HER2 in determining radiosensitivity. (A) The expression of HER2 in T24 cells decreases dramatically after the transfection with the lentiviral vector plasmid for HER2 RNA interference. (B and C) Clonogenic survival analysis

shows the radiosensitizing effect of erlotinib in HER2 knocked-down cells but not in control vector-treated cells. (*, $p< 0.05$ when comparing with control group.) (D) Afatinib still has a radiosensitizing effect in control vector-treated T24 cells. (E) In T24 cells transfected with HER2 shRNA, the number of γ-H2AX foci per cell is greater in the erlotinib plus radiation group, indicating more radiation-induced DNA damage. (F) Representative images of γ-H2AX foci.

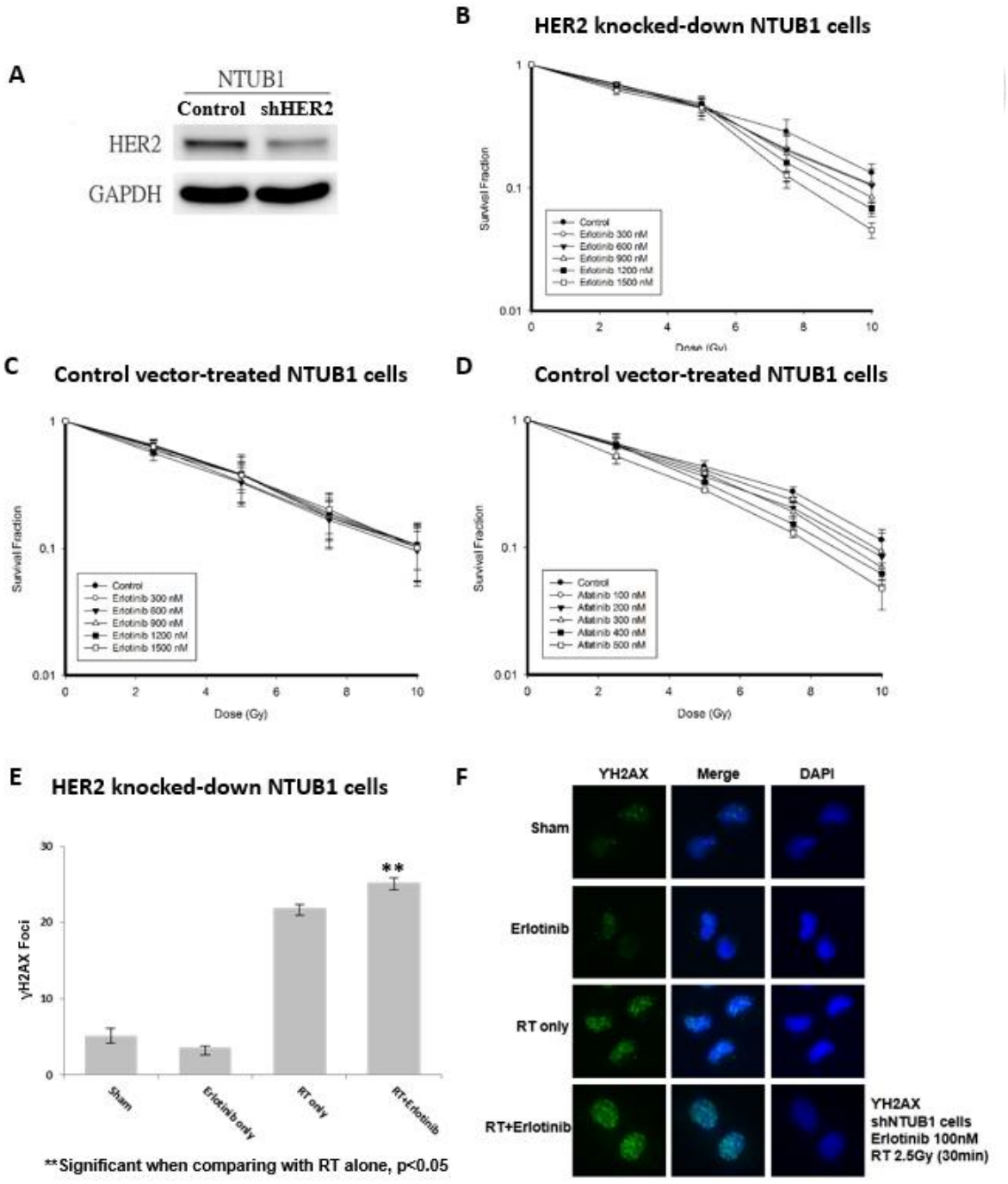

Figure 4-8. The radiosensitizing effect of erlotinib in HER2 knocked-down NTUB1 cells also emphasizes the synergism between EGFR and HER2 in determining radiosensitivity. (A) The expression of HER2 in NTUB1 cells decreases dramatically after the transfection with the lentiviral vector plasmid for HER2 RNA interference. (B and C) Clonogenic survival

analysis shows the radiosensitizing effect of erlotinib in HER2 knocked-down NTUB1 cells but not in control vector-treated cells. (D) Afatinib still has a radiosensitizing effect in control vector-treated NTUB1 cells. (E) In NTUB1 cells transfected with HER2 shRNA, the number of γ-H2AX foci per cell is greater in the erlotinib plus radiation group, indicating more radiation-induced DNA damage. (F) Representative images of γ-H2AX foci.

4.3.8 Receptor heterodimerization plays an important role in the radiosensitizing effect of afatinib.

First, to investigate the influence of radiation and/or afatinib on the dimer formation of EGFR or HER2, we performed western blotting of cell lysates treated with chemical cross-linking. As shown in Fig. 4-9A and 4-9B, T24 bladder cancer cells showed the increased dimer formation of both EGFR and HER2 at 60 minutes after radiation. However, the phenomenon was less prominent in T24 cells treated with the combination of radiation and afatinib (administered 30 minutes before radiation), suggesting the inhibition of afatinib in the radiation-induced dimer formation of both EGFR and HER2

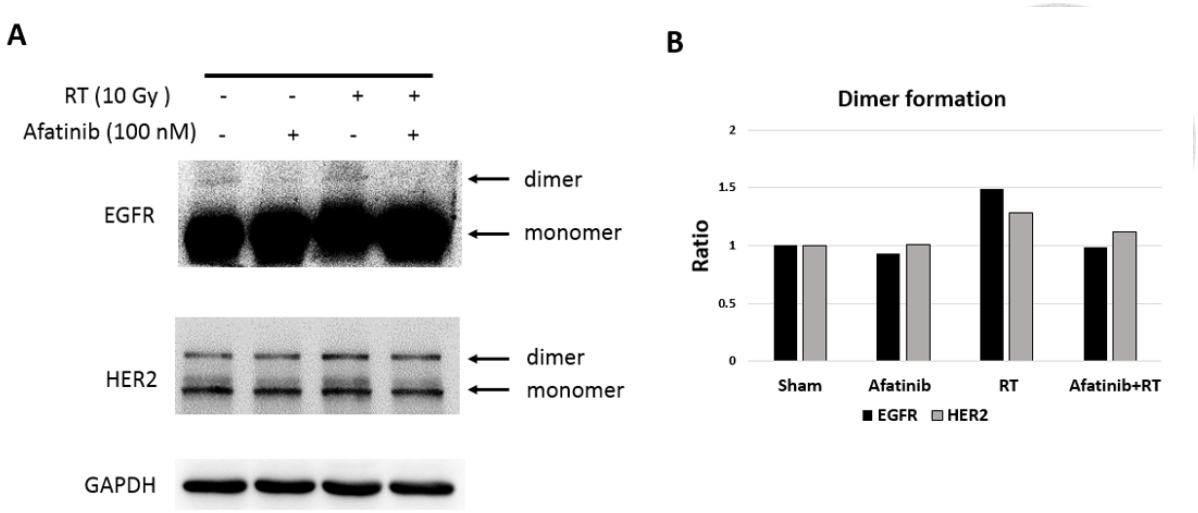

Figure 4-9A. Western blotting of T24 cell lysates treated with chemical cross-linking**.** Sixty minutes after sham, 10-Gy radiation (RT), 100 nM afatinib, or the combined treatment, the cells were washed with PBS three times and incubated for 60 min at room temperature with 5 mM Suberic acid bis(3-sulfo-N-hydroxysuccinimide ester) sodium salt in PBS, and the reaction was terminated using 20mM Tris-HCl, pH 8.5 for 15 min at room temperature. Subsequently, cells were washed with PBS and solubilized in lysis buffer for western blotting. The result showed the increased dimer formation of both EGFR and HER2 after RT. However, the phenomenon was less prominent in T24 cells treated with the combination of RT and afatinib. **Figure 4-9B** showed the ratios of EGFR or HER2 dimer formation after different treatment, when comparing with sham group.

To further examine the effect of radiation and/or afatinib on EGFR-HER2 heterodimer, immunoprecipitation analysis of T24 bladder cancer cells was performed. As shown in Fig. 4-9C, western blotting of cell lysate precipitated with EGFR antibody showed the increased

HER2 expression at 30 minutes after radiation, and the effect was decreased after the combined treatment of radiation and afatinib (administered 30 minutes before radiation). The results indicate that the level of heterodimerization between EGFR and HER2 was enhanced by radiation, but the enhancement was inhibited by the combined treatment of radiation and afatinib.

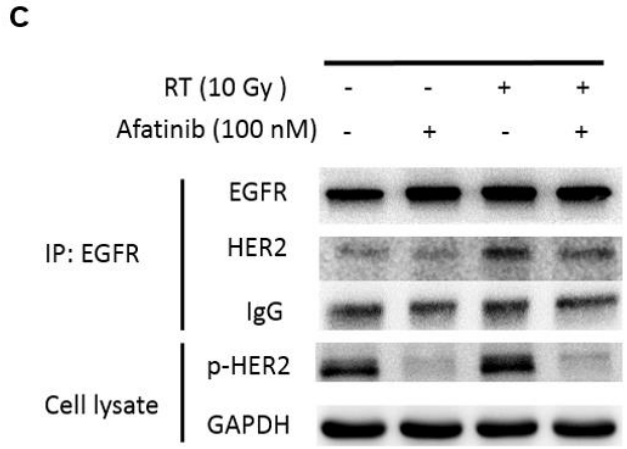

Figure 4-9C. Western blotting of T24 cell lysate precipitated with EGFR antibody. HER2 expression increased at 30 minutes after irradiation (RT), but the effect was decreased after the combined treatment of RT and afatinib. The results indicate that the level of heterodimerization between EGFR and HER2 was enhanced by RT but the enhancement was inhibited by the combined treatment of RT and afatinib.

CHAPTER FIVE: DISCUSSION

5.1 Pilot study

5.1.1 Significance

It is out of our expectation that lapatinib, an EGFR and HER2 dual inhibitor, didn't enhance radiation effect. The inhibitory effect of lapatinib is even weaker than erlotinib (an EGFR inhibitor) and trastuzumab (a HER2 inhibitor). This is probably because lapatinib is a reversible inhibitor, and the inhibitory effect may be reversed during the interval without drug treatment. To overcome this problem, we found a new generation EGFR inhibitor, afatinib (BIBW2992), with irreversible tyrosine kinase inhibition against EGFR and HER2 (Table 5-1). The activity of *in vitro* kinase inhibition by afatinib is also significantly stronger than lapatinib (Table 5-2).

	(WT EGFR) H1666	(L858R mutant) H3255	(L858R/T790M mutant) NCI 1975	Targets	Inhibition
BIBW2992	60	0.7	99	EGFR/HER2	irreversible
lapatinib	534	63	> 4000	EGFR/HER2	reversible
canertinib	198	1	101	EGFR/HER2	irreversible
gefitinib	157	5	> 4000	EGFR	reversible
erlotinib	110	40	> 4000	EGFR	reversible

Table 5-1. IC50 of anchorage independent cell (Li et al., 2008)

	BIBW2992	*Lapatinib*	*Canertinib*	*Gefitinib*
$EGFR^{wt}$	0.5	3	0.3	3
$EGFR^{L858R}$	0.4	8	0.4	0.8
$EGFR^{L858R/T790M}$	10	> 4000	26	1013
HER2	14	15	30	1830
β-InsRK	> 100 000	> 100 000	> 100 000	> 100 000
HGFR	13 000	> 20 000	> 20 000	> 20 000
c-SRC	> 4000	> 20 000	1480	> 10 000
VEGFR-2	> 100 000	> 100 000	24 900	> 100 000

Table 5-2 *In vitro* inhibitory activity of afatinib (Li et al., 2008)

5.1.2 Conclusion

In this pilot study we demonstrated the potential of EGFR or HER2 inhibitors to radiosensitize human bladder cancer cell lines. However, erlotinib (EGFR inhibitor) and trastuzumab (HER2 inhibitor) are not potent enough. Moreover, lapatinib, an EGFR/HER2 dual inhibitor, failed to show its radiosensitizing effect. Therefore in the following studies, we used afatinib, a new generation EGFR tyrosine kinase inhibitor with activity against HER2, to test our hypothesis.

5.2 Murine Bladder Cancer Model

5.2.1 Significance

We demonstrated in this study the *in vitro* and *in vivo* radiosensitising effect of afatinib (an irreversible EGFR/HER2 dual inhibitor) on MBT-2 murine bladder cancer model. The synergism with irradiation was likely mediated through enhancement of radiation-induced DNA damage and apoptosis. We also showed that afatinib suppressed radiation-activated EGFR and HER2 signaling. To our knowledge, the present study is the first to report that the combined blockade of EGFR and other Erb-B family receptor tyrosine kinases enhances the radiosensitivity of bladder cancer cells.

EGFR specific tyrosine kinase inhibitor like gefitinib (Colquhoun et al., 2007; Dominguez-Escrig et al., 2004; Maddineni, Sangar, Hendry, Margison, & Clarke, 2005) and erlotinib (Colquhoun & Mellon, 2004) has been reported to enhance the radiosensitivity of bladder cancer cells. Compared to the gefitinib, the radiosensitizing effect of afatinib in our study was superior, indicating the critical role of HER2 signaling pathway in determining the radiosensitivity of bladder cancer cells. Similarly, monoclonal antibody to HER2/neu receptor has been shown to modulate the repair of radiation-induced DNA damage and enhance the radiosensitivity of breast and esophageal cancer cells overexpressing HER2 (Pietras et al., 1999; Sato et al., 2005). Furthermore, the Radiation Therapy Oncology Group trial on bladder preservation demonstrated a significant association of HER2 staining of bladder

tumor tissue with complete response after tri-modality treatment (Chakravarti et al., 2005).

Lapatinib, a reversible EGFR/HER2 dual inhibitor, had been shown to have a radiosensitizing effect in certain breast cancer cells (Sambade, Camp, Kimple, Sartor, & Shields, 2009; Sambade et al., 2010) and patients (Abboud, Saghir, Salame, & Geara, 2010; Harrington et al., 2009). However, its effect was unsatisfactory in our pilot study. Compared to lapatinib, afatinib (an irreversible inhibitor of EGFR and HER2) was the more potent radiosensitizer (Li et al., 2008). One large randomized trial recently reported the clinical benefit of afatinib for patients with non-small-cell lung cancer unresponsive to EGFR inhibitors or chemotherapy (Miller et al., 2012). Furthermore, afatinib had been shown to enhance responses to an EGFR specific monoclonal antibody in bladder cancer cells resistant to this treatment (Quesnelle & Grandis, 2011).

Schütze et al. investigated radiosensitization by afatinib of human squamous cell carcinoma cells only marginally affected by radiation (Abraham, Pagano, Gomella, & Baffa, 2007). The design of the present study differed from their design in the *in vivo* dosing schedule of afatinib (10 mg/kg for 7 days in our study versus 20 mg/kg until reaching a tumor diameter of 15 mm in their study) and radiotherapy (15 Gy on day 4 in this study versus 20 Gy after the last afatinib dose in their study). Moreover, we measured not only the volume of the ectopic tumor but also its glucose metabolism by PET, as well as the corresponding expression of EGFR and HER2 immunohistochemically. We additionally showed that

afatinib enhanced radiation-induced DNA damage and apoptosis. The molecular response to irradiation combined with afatinib might vary between different malignant cells (Krause, Gurtner, Deuse, & Baumann, 2009; Toulany, Dittmann, Baumann, & Rodemann, 2005) and this variation might account for discrepancies between our study and the one by Schutze et al.

MBT-2 cells are poorly differentiated urothelial carcinoma and derived from a carcinogen-induced bladder tumor in C3H/He mice. The tumor resembles its human counterpart both grossly and histologically (Horiguchi et al., 2008). The advantage of the MBT-2 murine bladder cancer model in this study is the immune-competent system for the *in vivo* data with the integrated physiological response after irradiation (Blomgren, Edsmyr, von Stedingk, & Wasserman, 1986) and inhibition of Erb-B family receptor tyrosine kinases (Hamilton et al., 2012; Y. Yan et al., 2006).

In this study, radiosensitization by afatinib was mediated through enhanced irradiation-induced DNA damage as indicated by the increase in γ-H2AX foci. γ-H2AX is a marker of DNA double-strand breaks (Rogakou, Pilch, Orr, Ivanova, & Bonner, 1998). Increased numbers of γ-H2AX foci have been found in bronchial carcinoma and breast adenoma cells treated with a EGFR inhibitor for radiosensitization (Dittmann, Mayer, & Rodemann, 2005), and this increase has been correlated with dead or dying cells (Gonzalez, Barquinero, Lee, Garcia, & Casaco, 2012). Double-strand breaks of DNA are the principal lesions responsible for cell killing by ionizing radiation. The radiosensitivity and clonogenic survival of

irradiated bladder cancer cell lines have been previously assessed by alkaline comet assay, a method to detect DNA strand break damage (Moneef et al., 2003). Our findings of reduced clonogenic survival and significantly increased numbers of γ-H2AX foci after combined afatinib and irradiation imply that afatinib augments DNA damage. Given that DNA is the most important cellular target of ionizing radiation, afatinib is an ideal radiosensitizer for bladder cancer.

5.2.2 Limitation

Although we demonstrate that afatinib suppresses irradiation-activated EGFR and HER2 pathways, the full mechanism of enhanced radiosensitization of bladder cancer cells remains elusive. Sambade et al. reported that lapatinib mediates radiosensitization in breast cancer cells primarily by inhibiting the Raf > MEK > ERK mitogen-activated protein kinase cascade (Sambade et al., 2009). We showed that afatinib inhibits post-radiation Akt phosphorylation in response to EGFR and HER2 signaling. Nicolle et al. similarly found that growth and invasiveness inhibition by gefitinib in urothelial carcinoma cell lines involves strong phosphorylation of Akt/MAPK pathways in association with activation of EGFR (Nicolle et al., 2006). Further investigation is required to identify the pathways through which DNA damage and apoptosis signals are transduced.

5.2.3 Conclusion

In this murine bladder cancer model we demonstrated for the first time the *in vitro* and *in vivo* radiosensitizing activity of afatinib, an orally bioavailable EGFR/HER2 dual inhibitor. Afatinib likely mediates its effect on bladder cancer cells by suppressing radiation-activated EGFR and HER2 signals and thereby causing enhanced DNA damage and cell apoptosis. The greater potency of afatinib as a radiosensitizer compared with previously reported EGFR inhibitors underscores the importance of other Erb-B family receptor tyrosine kinases such as HER2, and indicates a new direction for future clinical trials in bladder cancer.

5.3 Human Bladder Cancer Model

5.3.1 Significance

In the human bladder cancer model, we tested T24 and NTUB1 cells with low baseline EGFR expression (Dominguez-Escrig et al., 2004), a lack of EGFR activating mutations, and low probability of benefiting from EGFR tyrosine kinase inhibition. It is not surprising that erlotinib failed to enhance their radiosensitivity. Notably, the *in vitro* and *in vivo* radiosensitizing effect of an EGFR/HER2 dual inhibitor was demonstrated to surpass that of an EGFR TKI. Interestingly, the EGFR TKI showed improved radiosensitization in T24 cells with down-regulated HER2 expression as compared to vector-treated cells. The results indicate that EGFR is an important determinant of radioresponse in bladder cancer cells, provided the associated pathways like HER2 are concomitantly blocked.

It has been proposed that EGFR-mediated radioprotection occurs in three phases: (a) an immediate early phase involving DNA damage repair, (b) a later phase of suppressed DNA damage-induced apoptosis, and (c) a repopulation phase in which tumor cells can recover from radiation-induced cell cycle arrest (D. J. Chen & Nirodi, 2007). We clearly showed the advantage of EGFR/HER2 blockade in enhancing radiation-induced DNA damage, promoting apoptosis, and inhibiting the recovery of cells from radiation. Our approach reversed all three phases of EGFR-mediated radioprotection, irrespective of baseline EGFR expression.

This study also underscores the importance of HER2 in EGFR-mediated radioprotection. It was shown that HER2 expression can be induced by radiation in breast cancer cells with a low basal level of HER2, and inhibition of HER2 resensitizes resistant cell lines to radiation (Cao et al., 2009). Toulany et al. reported that radiation, but not epidermal growth factor (EGF), enhances EGFR/HER2 heterodimerization and activates the downstream Akt signaling pathway (Toulany et al., 2010). Given that HER2 is a prognostic factor of bladder cancer progression (Gandour-Edwards et al., 2002; Kruger et al., 2002), some may wonder whether HER2 inhibition itself accounts for the difference in radiosensitizing activity (Liang et al., 2003; No, Choi, & Kim, 2009) between afatinib and erlotinib. Our results failed to support this idea because clonogenic survival after radiation alone is basically the same in HER2 down-regulated and control vector-treated T24 (Fig. 4-7B, 4-7C) and NTUB1 (Fig. 4-8B, 4-8C) cells. Interestingly, afatinib also overcomes resistance to cetuximab (an anti-EGFR monoclonal antibody) in T24 cells, probably through downregulation of 611-CTF (a C-terminal fragment of HER2) (Quesnelle & Grandis, 2011). Since 611-CTF is robustly expressed in T24 cells and perhaps afatinib could affect its expression, the role of 611-CTF in afatinib-enhanced radiosensitivity deserves further investigation.

Based on our RTK signaling antibody array data, afatinib inhibited both radiation-activated MAPK and Akt signals, but erlotinib inhibited MAPK signal only. It is reasonable to assume that Akt and downstream molecules play important roles in HER2-augmented

radioresistance by EGFR. Akt activation has been shown to affect the activation of the G2/M checkpoint induced by DNA damage and to enhance the survival of cells by blocking the function of proapoptotic proteins (Kandel et al., 2002; Manning & Cantley, 2007). In contrast to radiation alone or radiation plus erlotinib, radiation plus afatinib induced a significant decrease in cells in G2/M phase and an increase in cells in sub-G1 phase. This implies that Akt may be critical in modulating the cell cycle distribution and promoting apoptosis. Akt can also directly phosphorylate the DNA damage checkpoint kinase Chk1 on serine 280 and block checkpoint function (King, Skeen, Hay, & Shtivelman, 2004; Puc et al., 2005). However, whether Chk1 is correlated with increased DNA damage by radiation plus afatinib is not confirmed.

5.3.2 Limitation

The finding that the radiosensitizing effect of HER2 depletion plus erlotinib is not as good as the radiosensitizing effect of afatinib alone suggests that other factors may be involved, such as a higher affinity to EGFR (Li et al., 2008) and inhibition of HER3 (Dungo & Keating, 2013) or HER4 (Hirsh, 2011) by afatinib. In addition, since chemotherapeutic agents like cisplatin are widely used to enhance radiosensitivity in bladder cancer therapy (R. C. Chen, Shipley, Efstathiou, & Zietman, 2013; Kachnic et al., 1997), whether EGFR/HER dual inhibition can further improve the outcome of chemoradiation deserves more studies.

Finally, although we clearly demonstrated the *in vivo* benefit of afatinib to enhance radiosensitivity, the response of a subcutaneous tumor graft may differ from that of the orthotopic bladder tumor model (Dominguez-Escrig et al., 2004).

5.3.3 Conclusion

The radiosensitizing effects of different generations of clinically useful EGFR TKIs were compared for the first time. We show the inadequacy of EGFR inhibition alone and the advantage of concomitant blockade of radiation-activated EGFR and HER2 signaling to inhibit the *in vitro* and *in vivo* growth of bladder cancer cells. The radiosensitizing effect of an EGFR inhibitor was much higher in HER2 knocked-down than wild-type cells, therefore HER2 may play a synergistic role with EGFR in determining radiosensitivity. The regained radiosensitizing activity of erlotinib implies that with proper HER2 inhibition, EGFR tyrosine kinase is still a potential target to enhance radiotherapy effect in these seemingly unresponsive bladder cancer cells.

5.4 Unfinished Study: Mutant EGFR - Related Research

5.4.1 Planned experiments

We have proved that both T24 and NTUB1 human bladder cancer cells have wild-type EGFR. On the other hand, non-small cell lung cancer with the L858R or the ΔE746-E750 mutations in the tyrosine kinase domain of EGFR was reported to exhibit enhanced sensitivity to radiation, and this strongly correlated with dramatically diminished capacity to resolve radiation-induced DNA double strand breaks (Das et al., 2006). Moreover, non-small cell lung cancer patients with mutant EGFR patients treated with radiotherapy had a better clinical outcome than patients with wild-type EGFR in terms of locoregional control (Mak et al., 2011) or response to whole brain radiotherapy (Gow et al., 2008). It is also reported that the mutant EGFR in these non-small cell lung carcinomas failed to exhibit radiation-induced nuclear translocation or binding to DNA-PKcs (Das et al., 2007). Therefore it is plausible to hypothesize that EGFR mutations may confer sensitivity to radiation.

To introduce different forms of EGFRs into T24 cells, we used the pLenti6/directional TOPO cloning kit. Full-length fragment of wild-type EGFR was amplified from pcDNA3.1-EGFR-wt and cloned into pLenti6/directional TOPO vector according to the instructions of the manufacturer. The L858R mutation was introduced by using site-directed mutagenesis kit (Stratagene). We also planned to introduce the full length of E746-A750 del mutation from cDNA of HCC827 cell line and cloned into pLenti6/directional TOPO vector. Correct

sequences were confirmed by sequencing for all vectors. However, although we successfully introduced the plasmid with mutated EGFR to T24 bladder cancer cell, the majority of EGFR in T24 cells was the wild-type. After evaluation we abandoned the rest of the experiments.

5.4.2 Lessons learned from recent literature

Interestingly, Zhang et al. recently showed that afatinib increases radiosensitivity of non-small cell lung cancer cells with acquired EGFR T790M mutation (Zhang et al., 2015). Since the experimental design is similar to ours, it deserves description and comparison. In Fig. 5-1 the authors showed the change of EGFR/Akt/ERK pathways in lung cancer cells after irradiation and afatinib. The phosphorylation levels of EGFR, Akt and ERK increased after irradiation in PC-9 (EGFR del E746-A750), PC-9-GR (PC-9 with gefitinib resistance) and H1975 cells (EGFR L858R and T790M mutation). Pretreatment with afatinib remarkable blocked basal level of the phosphorylations of EGFR and ERK proteins, and caused delays of irradiation-induced phosphorylation of Akt in these cells.

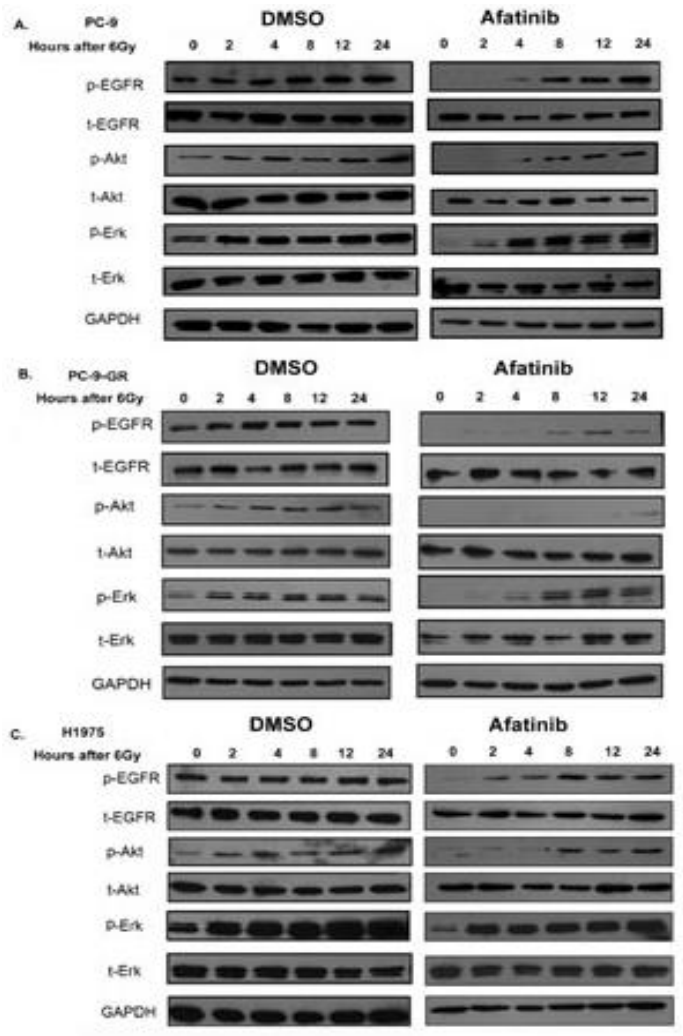

Figure 5-1. Effects of afatinib on protein phosphorylation after irradiation or afatinib pretreatment+irradiation in PC-9 (A), PC-9-GR (B) and H1975 (C) cells (Zhang et al., 2015)

But afatinib did not cause changes of the basal levels for phosphorated EGFR, Akt and ERK proteins in H460 (wild-type EGFR) cells which has a low baseline of these proteins (Fig. 5-2). Although we don't have the data about EGFR-mutated bladder cancer cells, the result confirmed our previous observation in Fig. 3-2, Fig 3-3 and Fig 4-1 that afatinib inhibits certain radiation-activated signaling pathways.

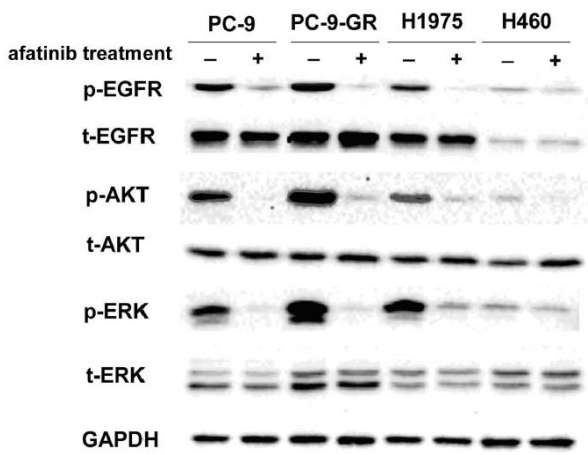

Figure 5-2. The effects of afatinib on protein phosphorylations in lung cancer cells (Zhang et al., 2015)

The authors also demonstrated the clonogenic assay in PC-9, PC-9-GR, H1975 and H460 cells after the treatment of radiation and/or afatinib (Fig. 5-3). PC-9-GR cells which acquired T790M mutation in addition to the original deletion in exon 19 (del E746-A750) demonstrated the radiosensitizing effect of afatinib. Similar to our experimental design, the authors showed that afatinib treatment lead to increased apoptosis (by flow cytometry) and suppressed DNA damage repair (by γ-H2AX foci) in irradiated PC-9-GR cells, and enhanced tumor growth inhibition when combined with irradiation in PC-9-GR xenografts. However, the exact mechanism why lung cancer cells with different EGFR mutation have different susceptibility to afatinib-induced radiosensitization was not addressed

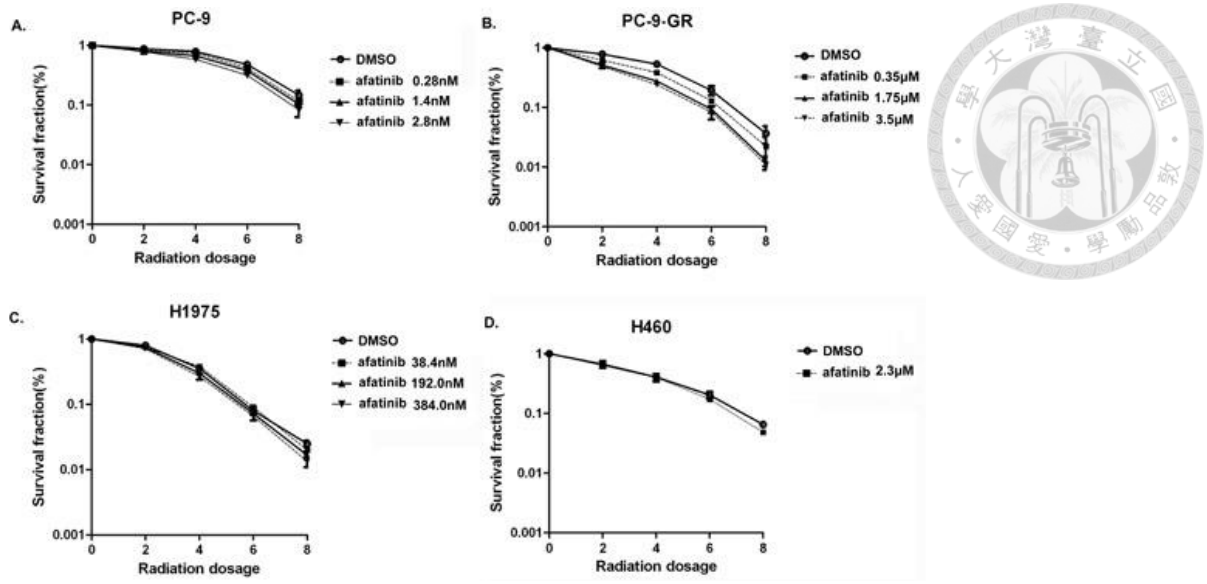

Figure 5-3. Effect of afatinib on clonogenic survival in irradiated lung cancer cells (Zhang et al., 2015)

It is also a pity that the expression of baseline and radiation-induced HER2 phosphorylation was not compared in this study. Although H460 cell as well as T24 and NTUB1 bladder cancer cells in our study has wild-type EGFR, the extent of HER2 activation after irradiation may explain the difference in radiosensitizing effect of afatinib. More mechanistic studies are still awaited.

CHAPTER SIX: PROSPECT

6.1 Radiosensitizing Activity of Afatinib and Microenvironment

Till now our study of the radiosensitizing effects of new-generation EGFR inhibitor focus on the factors influence cell growth, like DNA damage and apoptosis. On the other hand, the interaction between cancer cells and microenvironment also determine the potential to invasion and metastasis, therefore it is very important to the radiation effect. Rapidly accumulating evidence suggests that radiation exposure also promotes cancer metastatic ability through epithelial-mesenchymal transition (EMT), which has a central role in cancer metastasis and has become the subject of intense investigation (Kawamoto et al., 2012; Liu et al., 2014; S. Yan et al., 2013). However, the signaling molecular mechanisms underlying radiation-induced EMT remain obscure (Cui et al., 2015), and there is virtually no report regarding this field in bladder cancer research. Since the failure pattern of radiotherapy in bladder cancer patients includes local recurrence and distant metastasis, the influence of how new-generation EGFR TKIs like afatinib on these phenomena will be of great significance.

Till now we have some preliminary result. As shown in Fig. 6-1, Boyden chamber invasion assay revealed that after 2.5Gy irradiation, there is an increase of cell invasion in T24 and 5637 bladder cancer cells. After pretreatment with afatinib, the radiation-enhanced invasion was decreased, but the pretreatment with erlotinib did not show this effect.

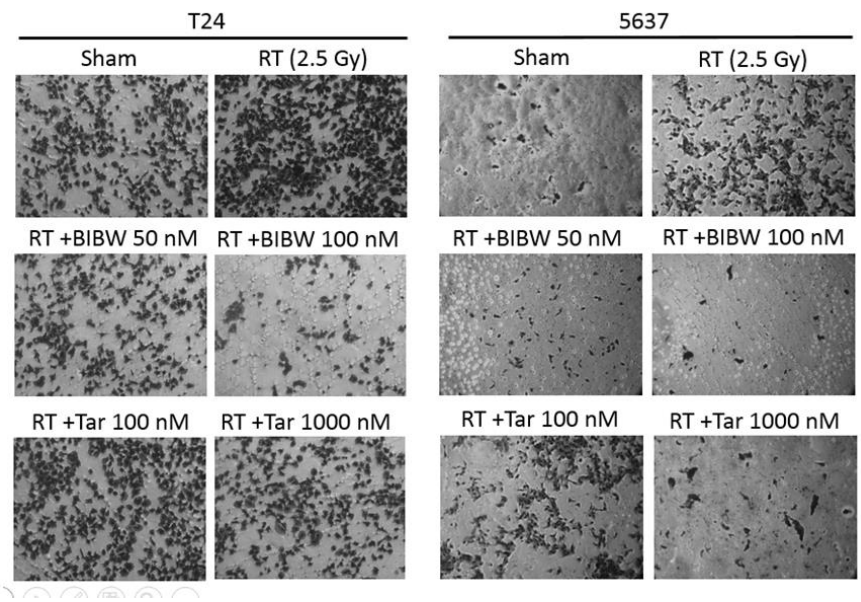

Figure 6-1. Invasion assay of T24 and 5637 bladder cancer cells treated with irradiation +/- afatinib (BIBW) or erlotinib (Tar)

Then we tested the expression of phosphorylated EGFR and HER2 as well as MMP-9 and MMP-2 in T24 and 5637 bladder cancer cells. As shown in Fig. 6-2, after irradiation the expression of p-EGFR, p-HER2 and MMP-9 was increased, and the effect was decreased after the pretreatment with afatinib. However the pretreatment with erlotinib did not show this effect.

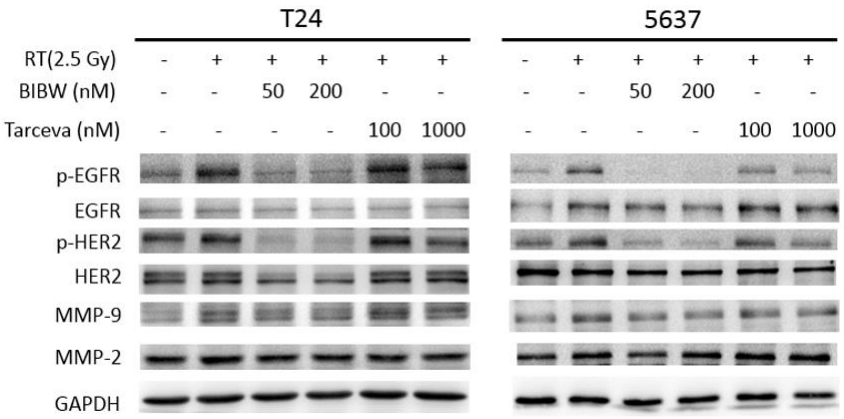

Figure 6-2. Western blot of T24 and 5637 bladder cancer cells treated with irradiation +/- afatinib (BIBW) or erlotinib (Tarceva)

In addition, enzyme activity in culture media using zymography was also tested. The result showed that after irradiation the activity of MMP-9 was increased, and the effect was decreased after the pretreatment with afatinib. The influence of erlotinib pretreatment is more variable in T24 and 5637 cells. In contrary, there was no obvious change in MMP-2 activity (Fig. 6-3).

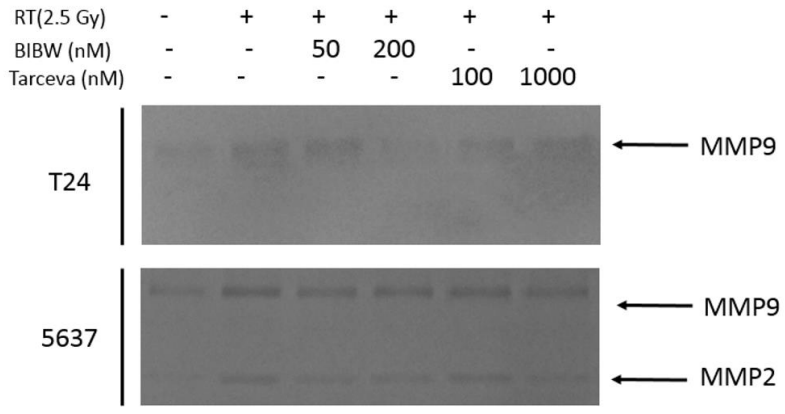

Figure 6-3. Gelatin zymography of culture media from T24 and 5637 bladder cancer cells treated with irradiation +/- afatinib (BIBW) or erlotinib (Tarceva)

Currently mechanistic and animal studies are ongoing to find out more ways to improve the treatment outcome of bladder cancer radiotherapy.

6.2 Clinical Application

Since radiotherapy for localized muscle-invasive bladder cancer is associated with a relatively high rate of local recurrence, much effort was put to enhance the efficacy of radiotherapy by concurrent chemotherapy. For a long time, cisplatin was considered as the drug of choice in chemoradiotherapy in bladder cancer, although only one randomized trial has compared these two approaches in bladder cancer (Coppin et al., 1996). In that study, 99 patients were randomly assigned to undergo radiotherapy with or without cisplatin, followed by elective cystectomy or further radiotherapy. The concurrent cisplatin group had improved pelvic control of locally advanced bladder cancer with preoperative or definitive radiation, but has not been shown to improve overall survival. The concern of potential toxicities is also a problem.

In the Bladder Cancer 2001 (BC2001) trial, the investigators tested if the concurrent use of a non-platinum regimen, fluorouracil and mitomycin C would be more efficacious than radiotherapy alone in 360 bladder cancer patients. At 2 years, rates of locoregional disease - free survival were 67% in the chemoradiotherapy group and 54% in the radiotherapy group. Five-year rates of overall survival were 48% in the chemoradiotherapy group and 35% in the radiotherapy group (James et al., 2012). Grade 3 or 4 adverse events were slightly more common in the chemoradiotherapy group than in the radiotherapy group during treatment (36.0% vs. 27.5%, P=0.07) The fluorouracil and mitomycin C combination is obviously not

optimal because patient in study group still have 8.5% increase of high-grade toxicities (although statistically insignificant).

Despite our preclinical data is very inspiring, the potential enhancement of toxicities by concurrent use of afatinib and radiation is also a big concern. According to the data in phase I study, the mean Cmax of afatinib after 4-week use of 50mg once daily dose in 5 patients was 66.8(ng/mL) (Murakami et al., 2012) which is equivalent to 138 nM. This concentration is below most concentration we used in this study. For example, in clonogenic assay we used 200-1000nM in murine bladder cancer model and 100-500nM in human bladder cancer model. If future clinical trial of concurrent administration of afatinib and radiotherapy is planned, the dose must be carefully titrated to ensure the safety in bladder cancer patients.

In addition, although in animal study we didn't observe severe diarrhea or weight loss in mice, the potential additive gastrointestinal toxicity is also important. In LUX-Lung 3 phase III clinical trial of afatinib or cisplatin plus pemetrexed in patients with metastatic lung adenocarcinoma with EGFR mutations, 95.2% of patients receiving afatinib have diarrhea. (14.4% ≥ Grade 3), compared with 15.3% all grade diarrhea in chemotherapy group (Sequist et al., 2013) Similarly, In LUX-Lung 6 phase III clinical trial of afatinib versus cisplatin plus gemcitabine for first-line treatment of Asian patients with advanced non-small-cell lung cancer harboring EGFR mutations, 88.3% of patients receiving afatinib have diarrhea. (5.4%

Grade 3), compared with 10.6% all grade diarrhea in chemotherapy group (Y. L. Wu et al., 2014). On the other hand, in Bladder Cancer 2001 trial of radiotherapy with or without chemotherapy in muscle-invasive bladder cancer, 9.6% in chemoradiotherapy group and 2.7% in radiotherapy group have Grade 3-5 gastrointestinal toxicity (James et al., 2012), indicating chemotherapeutic agents as radiosensitizers may enhance the already existed risk of diarrhea. Therefor the handling of potential gastrointestinal toxicity is very important for further radiosensitizing studies.

Despite the challenges in designing clinical trial of afatinib as radiosensitizer in bladder cancer, my PhD study provided many precious data for future preclinical and clinical studies. In 2015 ASCO Annual meeting, Powles *et al.* reported a phase II/III, double-blind, randomized trial comparing maintenance lapatinib versus placebo after first line chemotherapy in HER1/2 positive metastatic bladder cancer patients. (abstr 4505) Although maintenance lapatinib does not improve outcomes in EGFR or HER2 positive bladder cancer patients, this is the first personalized randomized trial in metastatic urothelial carcinoma. It also showed that EGFR and HER2 are still considered important pathways in bladder cancer therapy, but the selection of appropriate patients may be required to achieve good result.

Just like lapatinib failed to demonstrate radiosensitizing activity in our screening study but afatinib successes, we need more well-designed study to examine the clinical potential of new generation EGFR inhibitor like afatinib in bladder cancer treatment.

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APPENDIX

Publication during Ph.D training

Ph.D dissertation-related

1. Lin CC, Hsu CH, Huang CY, Keng HY, **Tsai YC**, Huang KH, Cheng AL, Pu YS.: Gemcitabine and ifosfamide as a second-line treatment for cisplatin-refractory metastatic urothelial carcinoma: a phase II study. *Anticancer Drugs*. 2007 Apr; 18(4):487-91.
2. Lin CC, Hsu CH, Huang CY, **Tsai YC**, Huang KH, Cheng AL, Pu YS. Prognostic factors for metastatic urothelial carcinoma treated with cisplatin and 5-fluorouracil-based regimens. *Urology*. 2007 Mar; 69(3):479-84.
3. Chen CH, Shun CT, Huang KH, Huang CY, **Tsai YC**, Yu HJ, Pu YS. Stopping smoking might reduce tumour recurrence in nonmuscle-invasive bladder cancer. *BJU Int*. 2007 Aug;100(2):281-6.
4. Lin CC, Hsu CH, Cheng JC, Huang CY, **Tsai YC**, Hsu FM, Huang KH, Cheng AL, Pu YS. Induction cisplatin and fluorouracil-based chemotherapy followed by concurrent chemoradiation for muscle-invasive bladder cancer. *Int J Radiat Oncol Biol Phys*. 2009 Oct 1;75(2):442-8.
5. **Tsai YC**, Yeh CH, Tzen KY, Ho PY, Tuan TF, Pu YS, Cheng AL, Cheng JC. Targeting epidermal growth factor receptor/human epidermal growth factor receptor 2 signaling

pathway by a dual receptor tyrosine kinase inhibitor afatinib for radiosensitisation in murine bladder carcinoma. *Eur J Cancer*. 2013 Apr;49(6):1458-66

6. Hsu I, Yeh CR, Slavin S, Miyamoto H, Netto GJ, **Tsai YC**, Muyan M, Wu XR, Messing EM, Guancial EA, Yeh S. Estrogen receptor alpha prevents bladder cancer via INPP4B inhibited akt pathway in vitro and in vivo. *Oncotarget*. 2014 Sep 15;5(17):7917-35
7. **Tsai YC**, Ho PY, Tzen KY, Tuan TF, Liu WL, Cheng AL, Pu YS, Cheng JC. Synergistic Blockade of EGFR and HER2 by New Generation EGFR Tyrosine Kinase Inhibitor Enhances Radiation Effect in Bladder Cancer Cells. *Mol Cancer Ther*. 2015 Mar; 14(3):810-820
8. Kuo KL, Ho IL, Shih TH, Wu JT, Lin WC, **Tsai YC**, Chang HC, Chou CT, Hsu CH, Hsieh JT, Chang SC, Pu YS, Huang KH. MLN4924, a novel protein neddylation inhibitor, suppresses proliferation and migration of human urothelial carcinoma: In vitro and In vivo study. *Cancer Lett*. 2015 Jul 28;363(2):127-36

II. non-Ph.D dissertation-related

1. Lin CC, Yeh KH, Yang CH, Hsu C, **Tsai YC**, Hsu WL, Cheng AL, Hsu CH. Multifractionated paclitaxel and cisplatin combined with 5-fluorouracil and leucovorin in patients with metastatic or recurrent esophageal squamous cell carcinoma. *Anticancer Drugs*. 2007 Jul;18(6):703-8.
2. **Tsai YC**, Pu YS, Cheng AL. Targeted therapy for renal cell carcinoma. *Formosan Journal of Medicine*. 2008 Jan;12(1):67-74
3. Hsu FM, Lin CC, Lee JM, Chang YL, Hsu CH, **Tsai YC**, Lee YC, Cheng JC. Improved local control by surgery and paclitaxel-based chemoradiation for esophageal squamous cell carcinoma: results of a retrospective non-randomized study. *J Surg Oncol*. 2008 Jul 1;98(1):34-41.
4. Hsu FM, Lee YC, Lee JM, Hsu CH, Lin CC, **Tsai YC**, Wu JK, Cheng JC. Association of clinical and dosimetric factors with postoperative pulmonary complications in esophageal cancer patients receiving intensity-modulated radiation therapy and concurrent chemotherapy followed by thoracic esophagectomy. *Ann Surg Oncol*. 2009 Jun;16(6):1669-77.
5. Hour TC, Kuo YZ, Liu GY, Kang WY, Huang CY, **Tsai YC**, Wu WJ, Huang SP, Pu YS.: Downregulation of ABCD1 in human renal cell carcinoma. *Int J Biol Markers*. 2009 Jul-Sep;24(3):171-8.

6. Hsu FM, Lee JM, Huang PM, Lin CC, Hsu CH, **Tsai YC**, Lee YC, Chia-Hsien Cheng J.: Retrospective analysis of outcome differences in preoperative concurrent chemoradiation with or without elective nodal irradiation for esophageal squamous cell carcinoma. *Int J Radiat Oncol Biol Phys*. 2011 Nov 15;81(4):e593-9.

7. Yu S, Yeh CR, Niu Y, Chang HC, **Tsai YC**, Moses HL, Shyr CR, Chang C, Yeh S.: Altered prostate epithelial development in mice lacking the androgen receptor in stromal fibroblasts. *Prostate*. 2012 Mar;72(4):437-49.

8. Xu D, Lin TH, Zhang C, **Tsai YC**, Li S, Zhang J, Yin M, Yeh S, Chang C. The selective inhibitory effect of a synthetic tanshinone derivative on prostate cancer cells. *Prostate*. 2012 May 15;72(7):803-16.

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10. Pu YS, Huang CY, Chen JY, Kang WY, Lin YC, Shiu YS, Chuang SJ, Yu HJ, Lai MK, **Tsai YC**, Wu WJ, Hour TC. Down-regulation of PKCζ in renal cell carcinoma and its clinicopathological implications. *J Biomed Sci*. 2012 Apr 5;19:39.

11. Tai HC, Chang AC, Yu HJ, Huang CY, **Tsai YC**, Lai YW, Sun HL, Tang CH, Wang SW. Osteoblast-derived Wnt-1-induced secreted protein 1 increases VCAM-1 expression and

enhances prostate cancer metastasis by down-regulating miR-126. *Oncotarget*. 2014 Sep 15;5(17):7589-98.