Fluoxetine Inhibits DNA Repair and NF- κ B-modulated Metastatic Potential in Non-small Cell Lung Cancer

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Abstract. *Background/Aim:* The aim of present study was to verify the effect of fluoxetine on DNA repair and metastatic potential in non-small cell lung cancer (NSCLC) *in vitro*. *Materials and Methods:* Highly metastatic NSCLC CL1-5-F4 cells were used in this study. Cells were treated with different concentrations of fluoxetine or QNZ (NF- κ B inhibitor) for 48 h. After treatment, cell viability, apoptotic signaling, NF- κ B activation, expression of DNA repair and metastasis-associated proteins, and cell migration/invasion were evaluated by (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, flow cytometry, NF- κ B reporter gene, western blotting, and cell migration/invasion assay, respectively. *Results:* Fluoxetine induced apoptosis and reduced cell viability, NF- κ B activation, expression of DNA repair and metastasis-associated proteins, and cell migration/invasion in CL1-5-F4 cells. Also, NF- κ B activation was the critical factor in fluoxetine-inhibited metastatic potential. *Conclusion:* Fluoxetine induced apoptosis and inhibited DNA repair and metastatic potential in NSCLC CL1-5-F4 cells.

Lung cancer is divided into two categories, small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC), and is the leading cause of cancer-related death worldwide (1). NSCLC accounts for 85% of all lung cancers and includes adenocarcinoma (AC), squamous cell carcinoma (SCC), and

large cell carcinoma (LCC) (2). Chemotherapeutic agents that induce DNA damage and lead to cell apoptosis are used to treat lung cancer (3). Chemotherapeutic agent-induced DNA damage can be repaired by efficient DNA repair capacity (DRC) resulting in treatment failure. Effective DRC contributes to poor prognosis in NSCLC patients treated with chemotherapy, but is not associated with survival of patients who do not receive chemotherapy (4). Tumor metastasis is a major cause of death in patients with lung cancer (5). Furthermore, patients with metastatic NSCLC often have unsatisfactory response to chemotherapy, radiotherapy, or chemoradiotherapy and their median survival is less than 32 months (6). Therefore, development of new adjuvants which inhibit DNA repair and tumor metastasis may provide benefits for patients with NSCLC.

Antidepressants have a long history in the treatment of mood disorders. In addition, some studies indicated that antidepressants have anti-cancer potential in various cancers (7). Zingone *et al.*, found that lung cancer patients treated with antidepressants [dopamine reuptake inhibitors (NDRIs) or tricyclic antidepressants (TCA)] have a significantly better survival as compared to patients not treated with antidepressants (8). Imipramine, a tricyclic antidepressant, induces cell death by enhancing activation of stress pathways in a SCLC model *in vitro* and *in vivo* (9). Antidepressants may be used as potential adjuvants for lung cancer treatment. Fluoxetine, an antidepressant, belongs to selective serotonin reuptake inhibitors (SSRIs) which are widely used to treat depression and anxiety disorders. In addition, many studies have indicated that fluoxetine has anti-inflammatory and anti-cancer effects (10, 11). Fluoxetine ameliorates dextran sulfate sodium (DSS)-induced colitis through inhibition of NF- κ B signaling in mice (12). Helga *et al.* suggested that fluoxetine inhibited development of colon tumor *via* disruption of tumor metabolism (10). However, whether fluoxetine inhibits DNA repair and metastasis in NSCLC is ambiguous. Therefore, the

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Figure 1. *Continued*

effect of fluoxetine on DNA repair and metastatic potential in NSCLC CL1-5-F4 cells was investigated.

Materials and Methods

Chemicals. Fluoxetine and 3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), L-glutamine and penicillin-

streptomycin (PS) were obtained from Gibco/Life Technologies (Carlsbad, CA, USA). NF-κB inhibitor 4-N-[2-(4-phenoxyphenyl) ethyl] quinazoline-4, 6-diamine (QNZ or EVP4593) were bought from Selleckchem (Houston, TX, USA). JetPEI™ transfection reagent was bought from Polyplus Transfection (Sélestat, Bas-Rhin, France). D-luciferin was purchased from Promega (Madison, WI, USA). Hygromycin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Matrigel was obtained from Corning (Tewksbury, MA, USA). Primary antibodies against VEGF and MMP-9 were purchased from Merck Millipore (Billerica, MA,

Figure 1. Effect of fluoxetine on cell growth and expression of DNA repair-associated proteins in CL1-5-F4 cells. Cells were treated with different concentrations of fluoxetine for 48 h. (A) Cell viability was evaluated using the MTT assay. (B) Protein levels of MDC1, MGMT, and 14-3-3 sigma were determined by western blotting. (C) Detection of apoptosis was performed by using flow cytometry. * $p < 0.05$ as compared to 0 μ M fluoxetine.

USA). Primary antibodies against MMP-2 and uPA were obtained from OriGene Technologies (Rockville, MD, USA) and Abbiotec (San Diego, CA, USA), respectively. Primary antibodies against MDC1, MGMT, and 14-3-3 sigma were purchased from Biorbyt (South San Francisco, CA, USA), Thermo Fisher Scientific (Waltham, MA USA), and GeneTex (Irvine, CA, USA), respectively. Primary antibody against β -actin was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Secondary antibodies were bought from Jackson ImmunoResearch (West Grove, PA, USA). Annexin V-Fluorescein isothiocyanate (FITC) apoptosis detection kit was bought from BioVision (Milpitas, CA, USA).

Cell culture. Human lung adenocarcinoma cell line CL1-5-F4 was obtained from Dr. Chia-Lin Hsieh (Taipei Medical University, Taiwan) and used for this study. Cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) and Ham's F-12 Nutrient Mixture supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin. Cells were incubated at 37°C in a humidified incubator containing 5% CO₂ and 95% air (13).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. CL1-5-F4 cells were seeded into 96-well plates with a density of 2×10^4 cells/well and incubated overnight. Cells were treated with different concentrations of fluoxetine (0-40 μ M in 0.1% DMSO) or QNZ (0-1 μ M in 0.1% DMSO) for 48 h. Cell viability of CL1-5-F4 was then evaluated by MTT assay as described by Liu *et al.* (14).

Detection of apoptosis. CL1-5-F4 cells were seeded into 12-well plates with a density of 2×10^5 cells/well and incubated overnight. Cells were treated with different concentrations of fluoxetine (0, 20, and 40 μ M in 0.1% DMSO) for 48 h. After treatment, cells were harvested and washed twice by centrifugation and phosphate-buffered saline (PBS) and then re-suspended in 500 μ l PBS. Cell suspension was stained with 5 μ l of annexin-V-FITC and propidium iodide [PI] 50 mg/ml for 5 min in the dark. Distribution of annexin-V and PI was evaluated by using flow cytometry with FL1 and FL2 channels.

Western blotting assay. 3×10^6 CL1-5-F4 cells were seeded into 10 cm diameter dishes and incubated overnight. Cells were treated with 0,

Figure 2. Effect of both fluoxetine and QNZ (NF- κ B inhibitor) on NF- κ B activation in CL1-5-F4 cells. Cells were treated with different concentrations of (A) fluoxetine or (B) QNZ for 48 h. NF- κ B activation was investigated using an NF- κ B reporter gene assay. ** $p < 0.01$ as compared to 0 μM fluoxetine.

20, 40 μM fluoxetine or 0.25 μM QNZ for 48 h. Cell lysis buffer (50 mM Tris-HCl pH 8.0, 120 mM NaCl, 0.5% NP-40, and 1 mM phenylmethanesulfonyl fluoride) was used to extract total proteins from cells. Expression of MDSC1, MGMT, 14-3-3 sigma, VEGF, MMP-2, MMP-9, and uPA proteins was investigated by using western blotting assay as described by Wang *et al.* (15). Protein expression was finally visualized by ChemiDoc MP Imaging System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The intensity of protein bands was quantified using Image Lab (Bio-Rad Laboratories Inc.).

Plasmid transfection. NF- κ B-luciferase2 vector (pNF- κ B/luc2) was bought from Promega (Madison, WI, USA). One million CL1-5-F4 cells were seeded into 10 cm diameter dishes and incubated overnight. Cells were transfected with pNF- κ B/luc2 by using jetPEI (Polyplus transfection, New York, NY, USA) transfection reagent as previously described (16).

NF- κ B reporter gene assay. CL1-5-F4 cells transfected with pNF- κ B/luc2 were seeded into 96-well plates with a density of 2×10^4 cells/well and incubated overnight. Cells were treated with different concentrations of fluoxetine (0-50 μM in 0.1% DMSO) or QNZ (0-1 μM in 0.1% DMSO) for 48 h. After treatments, 100 μl of 15 D-luciferin solution (500 μM D-luciferin in 100 μl PBS) were added into each well and incubated for 1 min. Photon emission from cells was acquired for 1 min by IVIS100 Imaging System (Xenogen, Alameda, CA, USA) and photons per second were quantified by using Living Image software (Xenogen). Relative NF- κ B activity was corrected with cell viability as previously described (17).

Cell migration assay. Transwell inserts of 8 μm pore size were obtained from Corning (Tewksbury, MA, USA). 3×10^6 CL1-5-F4 cells were seeded into 10 cm diameter dishes and incubated overnight. Cells were treated with 40 μM fluoxetine or 0.25 μM

Figure 3. Effect of both fluoxetine and QNZ (NF- κ B inhibitor) on the expression of metastasis associated proteins in CL1-5-F4 cells. Cells were treated with (A) 40 μM fluoxetine or (B) 0.25 μM QNZ for 48 h. Protein levels of MMP-2, MMP-9, uPA, and VEGF were evaluated by western blotting. ** $p < 0.01$ as compared to 0 μM fluoxetine or QNZ.

QNZ for 48 h. After treatment, cell viability was rapidly determined with trypan blue, and then migration ability of 1×10^6 viable cells was investigated as previously described (18). Migrated cells in were photographed under a light microscope at 100 $\times$ and cell number was quantified with the ImageJ software (National Institutes of Health, Bethesda, MD, USA).

Cell invasion assay. Fifty microliter matrigel solution (25 μl matrigel in 25 μl serum-free medium) was added to transwell inserts of 8 μm pore size and incubated overnight. Three million CL1-5-F4 cells were seeded into 10 cm diameter dishes and incubated overnight. Cells were treated with 40 μM fluoxetine or 0.25 μM QNZ for 48 h. After treatment, cell viability was rapidly determined

with trypan blue, and then invasion ability of 1×10^6 viable cells was investigated as described by Pan *et al.* (19). Invaded cells were photographed under a light microscope at 100 $\times$ and the number of invaded cells was quantified with ImageJ software (National Institutes of Health).

Statistical analysis. Parametric data were presented as the mean $\pm$ standard deviation. Student *t*-test was used to determine the significance of means difference between two groups. *p*-Value less than 0.05 was defined as statistically significant. Excel 2017 (Microsoft, Redmond, WA, USA) was used for statistical analyses in this study.

Results

Fluoxetine diminished cell viability, expression of DNA repair-associated proteins and induced apoptosis in CL1-5-F4 cells. Effect of fluoxetine on cell growth and expression of DNA repair-associated proteins was evaluated by MTT assay, flow cytometry, and western blotting assay. Figure 1A indicates that fluoxetine significantly reduced cell viability of CL1-5-F4 cells by 7%-53% as compared to control (vehicle treatment). After 48 h treatment, the half maximal inhibitory concentration (IC₅₀) of fluoxetine was about 40 μ M. Fluoxetine significantly inhibited expression of DNA repair-associated proteins [mediator of DNA damage checkpoint 1 (MDC1), O⁶-methylguanine-DNA methyltransferase (MGMT), and 14-3-3 sigma] by 30-90% as compared to control (Figure 1B). Additionally, fluoxetine significantly induced apoptosis by 18-27% as compared to control (Figure 1C).

Both fluoxetine and QNZ (NF- κ B inhibitor) suppressed NF- κ B activation of CL1-5-F4 cells. NF- κ B activation is required for tumor metastasis (14). After treatment with fluoxetine or QNZ for 48 h, NF- κ B activation was evaluated using an NF reporter gene assay. Fluoxetine significantly inhibited NF- κ B activation of CL1-5-F4 cells by 23%-96% at 48 h as compared to control (Figure 2A). In addition, NF- κ B activation was also inhibited by (0.25-1.0 μ M) QNZ treatment for 48 h (Figure 2B).

Both fluoxetine and QNZ (NF- κ B inhibitor) inhibited expression of metastasis-associated proteins in CL1-5-F4 cells. Effect of both fluoxetine and QNZ on expression of metastasis-associated proteins was determined by western blotting assay. The protein levels of MMP-2, -9, uPA, and VEGF were significantly decreased after treatment with 40 μ M fluoxetine or 0.25 μ M QNZ for 48 h by 70-90% and 65-82% respectively, as compared to control (Figure 3A and B).

Both fluoxetine and QNZ (NF- κ B inhibitor) reduced cell migration and invasion in CL1-5-F4 cells. Cell migration and invasion assay was used to investigate whether fluoxetine and QNZ can inhibit migration and invasion of CL1-5-F4 cells. Forty μ M fluoxetine significantly inhibited

the number of migrated and invaded cells as compared to control (Figure 4A and B). QNZ significantly reduced cell migration and invasion as compared to control (Figure 4C and D).

Discussion

Acquired resistance to chemotherapeutic agents in NSCLC can be mediated by DNA repair mechanisms. Aberrant expression of DNA repair-associated proteins correlates with poor prognosis in patients with NSCLC (3-4). 14-3-3 sigma protein enhances non-homologous end joining (NHEJ) repair of radiation-induced DNA double strand breaks leading to inhibition of radiation-induced G₂/M phase arrest and apoptosis (20). Cetintas *et al.*, found that increased expression of 14-3-3 sigma diminished cisplatin efficacy in NSCLC *in vivo* (21). O⁶-methylguanine-DNA methyltransferase (MGMT), a DNA repair protein, reduced alkylating agents-induced cytotoxicity by removing alkyl adducts from the O⁶ position of guanine in DNA (22). Brabender *et al.*, indicated that MGMT expression is associated with chemotherapy response and outcome in patients with NSCLC (23). Mediator of DNA damage checkpoint protein 1 (MDC1), a protein involved in DNA strand break repair, regulates cell resistance to chemotherapy and radiotherapy in breast and nasopharyngeal cancer (24-25). Fluoxetine has been shown to induce cell death in NSCLC A549 cells (26). However, whether fluoxetine inhibits DNA repair potential was not examined. The results presented here showed that fluoxetine induced apoptosis and inhibited expression of DNA repair-associated proteins MDC-1, MGMT, and 14-3-3 sigma in NSCLC CL1-5-F4 cells.

Many breast or lung cancer patients have symptomatic brain metastases (27, 28). Shapovalov *et al.* showed that fluoxetine promotes breast cancer metastasis to brain in a murine model. They suggested that increased numbers of brain metastases by fluoxetine were accompanied by enhanced permeability of the blood-brain barrier, changes of pro-inflammatory cytokines in the brain, and glial activation (27). CL1-5-F4, a highly metastatic lung adenocarcinoma cell line, was derived from lung metastases of severe combined immunodeficient mice inoculated with CL1-5 cells (29). In this study, this highly metastatic cell line was used to verify the anti-metastatic effect of fluoxetine. Contrary to its effect in breast cancer, fluoxetine significantly reduced migration and invasion abilities of NSCLC CL1-5-F4 cells (Figure 4A and B). Expression of metastasis-associated proteins MMP-2, -9, uPA, and VEGF contributes to tumor metastasis. High expression of metastasis-associated proteins was observed in patients with metastatic NSCLC (6-9). Moreover, protein levels of MMP-2, -9, uPA, and VEGF were significantly diminished by 40 μ M fluoxetine treatment for 48 h in CL1-5-F4 cells (Figure 3A).

Figure 4. Effect of both fluoxetine and QNZ (NF- κ B inhibitor) on cell migration and invasion of CL1-5-F4 cells. Cells were treated with 40 μM fluoxetine or 0.25 μM QNZ for 48 h. After treatment, migration and invasion ability were assayed by cell migration and invasion assays. (A-B) fluoxetine treatment. (C-D) QNZ treatment. ** $p < 0.01$ as compared to 0 μM fluoxetine or QNZ.

Figure 5. The potential anticancer properties of fluoxetine include induction of apoptosis, inhibition of DNA repair, and suppression of NF- κ B-modulated metastatic potential in NSCLC CL1-5-F4 cells.

Freire-Garabal *et al.* demonstrated that fluoxetine reversed development of lung metastasis by operative stress in rats. They hypothesized that fluoxetine reversed adverse effects of surgery through regulation of the immune response (30). Anti-cancer effects of antidepressants include initiation of antitumor immunity, promotion of apoptosis, and disruption of intracellular signal transduction resulting in inhibition of tumor growth (7). Mirtazapine, a noradrenergic and specific serotonergic antidepressant, has been demonstrated to inhibit tumor growth by up-regulating expression of anticancer cytokines interleukin-12 (IL-12) and interferon-gamma (INF γ) that trigger CD4⁺ and CD8⁺ T-cell infiltration within cancer tissue in colorectal cancer *in vivo* (31). Stepulak *et al.* showed that fluoxetine not only reduced phosphorylation of extracellular signal regulated kinase 1 and 2 (ERK1/2) and c-Myc but also up-regulated expression of p21(waf1) and p53 genes leading to suppression of tumor growth in NSCLC A549 cells (26).

Nuclear factor-kappaB (NF- κ B), a transcription factor, modulates immunity, inflammation, and tumorigenesis by

regulating the expression of NF- κ B target gene-encoding proteins (32). Positive NF- κ B expression was found in patients with poorly or moderately differentiated NSCLC and correlated to unfavorable prognosis (33). Furthermore, active NF- κ B signaling induces overexpression of metastasis-associated proteins such as MMP-2, MMP-9, uPA, and VEGF and acts as a target for lung cancer prevention and therapy (32). Inhibition of NF- κ B activation may disrupt the metastatic mechanism of NSCLC. Zhao *et al.* also demonstrated that knockdown of NF- κ B expression attenuated the metastatic potential in NSCLC *in vitro* (34). The results in that study demonstrated that both fluoxetine and QNZ (NF- κ B inhibitor) reduced NF- κ B activation, expression of metastasis-associated proteins (MMP-2, MMP-9, uPA, and VEGF), and cell migration/invasion in CL1-5-F4 cells (Figure 3A and B).

In conclusion, fluoxetine inhibited DNA repair and NF- κ B-modulated metastatic potential of NSCLC CL1-5-F4 cells (Figure 5). Fluoxetine may be used as a potential

adjuvant treatment which can offer additional benefits to NSCLC patients.

Conflicts of Interest

The Authors declare that they have no competing interests regarding this study.

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