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Article

Combinatorial treatment by siNOTCH and retinoic acid decreases A172 brain cancer cell growth

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SUMMARY

Treatments inhibiting Notch signaling pathways have been explored by researchers as a new approach for the treatment of glioblastoma tumors, which is a fast-growing and aggressive brain tumor. Recently, retinoic acid (RA) therapy, which inhibits Notch signaling, has shown a promising effect on inhibiting glioblastoma progression. RA, which is a metabolite of vitamin A, is very important in embryonic cellular development, which includes the regulation of multiple developmental processes, such as brain neurogenesis. However, high doses of RA treatment caused many side effects such as headaches, nausea, redness around the injection site, or allergic reactions. Therefore, we hypothesized that a combination treatment of RA and siRNA targeting *NOTCH1* (siNOTCH1), the essential gene that activates Notch signaling, would effectively inhibit brain cancer cell proliferation. The aim of the study was to determine whether inhibiting *NOTCH1* would inhibit the growth of brain cancer cells by cell viability assay. We found that the combination treatment of siNOTCH1 and RA in low concentration effectively decreased the *NOTCH1* expression level compared to the individual treatments. However, the combination treatment condition significantly decreased the number of live brain cancer cells only at a low concentration of RA. We anticipate that this novel combination treatment can provide a solution to the side effects of chemotherapy.

INTRODUCTION

Gliomas are some of the most common types of tumors in adults (1). Glioblastoma, a type of glioma, is one of the most common and lethal types of brain tumors (2). It causes headaches, nausea, and seizures. Glioblastoma occurs when a DNA mutation causes the uncontrollable replication of cancerous cells (3). The median age of patients diagnosed with glioblastoma is 62 years, and the median survival time for patients with glioblastoma is approximately 14.6 months (4).

Like most brain tumors, glioblastoma is hard to treat. The growth of glioblastoma within normal brain tissue renders these tumors challenging to remove through surgical procedures (5). As a result, glioblastoma can rarely be entirely removed through surgery, even when surgical removal of part of the tumor is possible (6). In addition, chemotherapy and radiation therapy, which are standard treatment methods for glioblastoma, cause serious side effects since they can harm both normal and cancerous cells (7). Even with chemotherapy and radiation therapy, there is no definite cure for glioblastoma, and patients with access to treatment still have a short life expectancy (8).

Researchers have begun exploring treatments relating to Notch signaling pathways as an alternative approach to the treatment of glioblastoma (9). Notch signaling occurs when the transmembrane ligands trigger a Notch protein extracellular domain to release the Notch intracellular domain (10). The Notch intracellular domain then translocates to the cellular nucleus, activating specific Notch target genes (11). Notch signaling regulates processes such as cell proliferation, cell fate, cell differentiation, and cell apoptosis (12).

Recent research indicates a strong connection between Notch signaling and glioblastoma tumorigenesis and growth (9). High levels of *NOTCH1* and *NOTCH2* transcripts have been observed in glioblastoma (13). NOTCH1 also activates the expression of *EGFR*, which is commonly overexpressed in glioblastoma (14). A previous study showed that NOTCH1 is a prognostic factor that promotes glioma cell survival through the NF-κB(p65) pathway (15). Since Notch signaling upregulates genes expressed in glioblastoma, we hypothesized that inhibition of Notch signaling may provide treatment for glioblastoma.

Several recent clinical trials have explored RNA interference (RNAi) in cancer treatment (16). RNAi involves microRNAs (miRNAs) or small interfering RNAs (siRNAs) in a process in which double-stranded RNA (dsRNA) molecules are cleaved by the enzyme Dicer into short fragments, which are then unwound into a passenger strand and a guide strand (17). The guide strand is incorporated into an RNA-induced silencing complex (RISC) while the passenger strand is degraded (18). The RISC is coded to bind to a particular messenger RNA (mRNA), which the RISC deactivates, thereby shutting down the translation of the protein corresponding to the mRNA (19).

Recent studies have examined the use of siRNA targeting *NOTCH1* in cancer treatment (19). In particular, the Notch signaling pathway directly regulates the expression of the c-MYC proto-oncogene, which has been observed to promote proliferation and inhibit apoptosis in many cancer cells (20). High c-MYC proto-oncogene expression has been observed in Grade III and Grade IV glioblastoma tumors (21). These studies have raised the possibility of siRNA targeting *NOTCH1* genes as an approach to cancer treatment (20, 21). The strong correlation between the NOTCH1-regulated c-MYC proto-oncogene and advanced glioblastoma recommends research on the use of siRNA targeting NOTCH1 pathway expression as a treatment for glioblastoma.

All-trans-retinoic acid (RA) therapy focused on Notch

genes has shown potential in treating glioblastoma (22). RA, a metabolite of vitamin A, is a small molecule that plays a crucial role in embryonic cellular development, including the regulation of numerous developmental processes such as neurogenesis (23). Unlike other protein factors that trigger intracellular pathways by interacting with cell surface receptors, RA directly enters the cellular nucleus, where it serves as a ligand for receptors that bind with DNA, regulating transcription (24). In that way, RA causes differentiation in gene expression and has been observed to cause a reduction in the level of NOTCH1-regulated c-MYC mRNA (25). A study showed that NOTCH1 was decreased after RA treatment in embryonic stem cell large cells (26). Therefore, we speculated that RA treatment may inhibit NOTCH1 expression level in glioblastoma cells.

The response of stem-like glioblastoma cells (GMB-SCs) to RA treatment has shown promising results in laboratory testing (27). Laboratory-grown GMB-SCs showed rapid changes in morphology and arrested growth in response to RA treatment (27). Among the pathways positively impacted by RA treatment in laboratory testing, Notch signaling pathways have been most responsive (27). Specifically, RA treatment has resulted in the downregulation of Notch pathways in GMB-SC, demonstrating the promising potential for glioblastoma treatment through inhibition of glioblastoma tumor propagation (27). However, high doses of RA induced side effects such as dry skin, cheilosis and nosebleeds, and hair loss (28). We hypothesized that adding RA with siRNA targeting *NOTCH1* (siNOTCH1) transfection in brain cancer cells may lead to cancer cell death. Therefore, we aim to find out the effect of a combination treatment of RA with siRNA on brain cancer cells.

RESULTS

We analyzed the effect of RA, siNOTCH1, and a combination of RA + siNOTCH1 on *NOTCH1* expression level in A172 brain cancer cells. To investigate the effect of RA, we incubated three different concentrations of RA (6.67 μM, 13.34 μM, and 20 μM) on A172 because a previous study showed that 20 μM RA may cause cell damage (29). Since previous research showed 20 nM of siNOTCH1 resulted in significant knockdown of *NOTCH1* expression, we used 20 nM siNOTCH1 final concentration for transfection (30). Lastly, we tested three different conditions of combination treatment (siNOTCH1 + RA), for which we used the concentrations of individual treatments of RA (6.67 μM, 13.34 μM, and 20 μM), and a constant siNOTCH1 concentration (20 nM).

Unexpectedly, 6.67 μM RA treatment resulted in a significantly increased expression level of *NOTCH1* as compared to the no treatment control (p = 0.0015, one-way ANOVA, **Figure 1**). Interestingly, only the combination treatment of siNOTCH1 + 6.67 μM RA significantly decreased *NOTCH1* expression level (p = 0.0011, one-way ANOVA, **Figure 1**).

Next, we analyzed how RA, siNOTCH1, and a combination of RA + siNOTCH1 treatment affect the A172 cell viability to determine if treatment would decrease the number of brain cancer cells. We prepared four conditions: no treatment for the negative control, RA (6.67 μM), siNOTCH1, and combination treatment (siNOTCH1 + 6.67 μM RA). As a readout for survival, we quantified the live cells.

RA treatment significantly decreased the number of

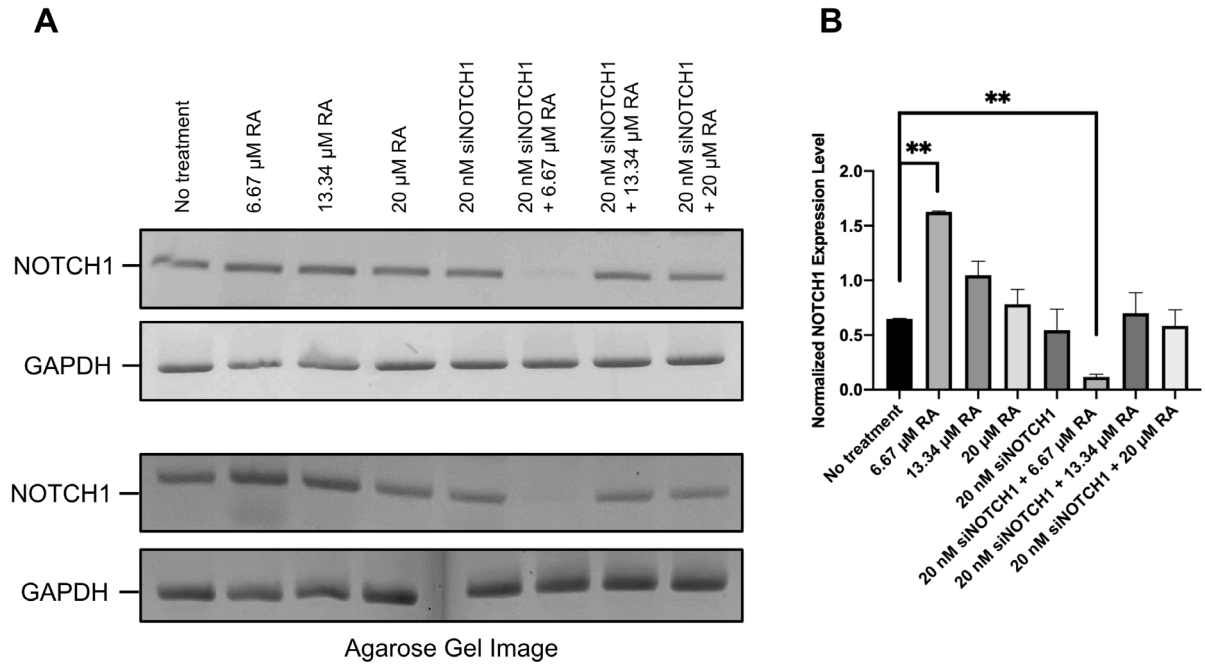

Figure 1: Combination treatment of siRNA targeting *NOTCH1* (siNOTCH1) and RA decreased the expression level of *NOTCH1*. A172 brain cancer cells were grown under either control condition (no treatment), in RA (6.67 μM, 13.34 μM, 20 μM), in 20 nM siNOTCH1, and in 20 nM siNOTCH1 + RA (6.67 μM, 13.34 μM, 20 μM) for 24 hours. (A) The agarose gel image showing the amplified DNA from *NOTCH1* and *GAPDH* after RT-PCR. Two technical replicates of agarose gel image are presented. (B) Mean ± standard deviation (SD) normalized *NOTCH1* expression level (n=1). GAPDH gene expression was used to normalize *NOTCH1* expression. One-way ANOVA with Tukey's post hoc test, $^{**}p < 0.01$.

live cells compared to no treatment (p = 0.0395, one-way ANOVA, **Figure 2**). siNOTCH1 also significantly decreased the cell viability compared to no treatment (p = 0.0395, one-way ANOVA, **Figure 2**). The combination treatment of siNOTCH1 + 6.67 μM RA significantly decreased the number of viable cells, supporting our hypothesis (p < 0.0001, one-way ANOVA, **Figure 2**). Compared to individual treatment of RA, the combination treatment significantly induced cell death (p < 0.03, one-way ANOVA, **Figure 2**). Also compared to individual treatment of siNOTCH, the combination treatment significantly induced cell death (p < 0.0001, one-way ANOVA, **Figure 2**).

DISCUSSION

Our analysis of *NOTCH1* expression in brain cancer cells revealed that the combination treatment containing siNOTCH1 + 6.67 μM RA decreased *NOTCH1* expression level. Cell imaging showed that RA (6.67 μM), siNOTCH1, and the combination treatment all significantly increased cell death. The combination treatment significantly induced brain cancer cell death than either individual treatment of RA or siNOTCH1 (**Figure 2**).

Since there are some acknowledged limitations in this study, future directions with the new experiments should be performed. First, we only tested one kind of glioblastoma cell line, which does not represent all human brain cancer. Therefore, more brain cancer cell lines should be used to confirm our results. Second, we investigated the cell viability of brain cancer cells incubating for only 24 hours. More treatment time points should be tested to verify the result. Third, even though cancer development is a multi-step process including cell migration, invasion, and proliferation, we only focused here on cell viability. Fourth, we only performed an *in vitro* experiment in a culture dish outside a living organism. Our *in vitro* results must be verified with a mouse model *in vivo* experiment. Fifth, we did not investigate how combination treatment affects the downstream NOTCH1 signaling pathway. Sixth, siRNA can have an off-target effect, which may degrade mRNA produced from other genes. Testing several siNOTCH1s that are designed to target different positions in *NOTCH1* transcript may confirm the specificity of siRNA. Seventh, we only analyzed two technical replicates. Therefore, more biological replicates are needed to confirm our result. Also, we only analyzed the cell survival but not the proliferation of the brain cancer cells. Additional experiments are needed to expand the effect of the combination treatment of RA and siNOTCH1. In addition, our study focused on *NOTCH1* gene expression only. Therefore, NOTCH-associated proteins may affect cancer cell proliferation after combination treatment. Lastly, our experiment lacks negative control siRNA. Therefore, screening the siRNA targeting specificity used in this experiment should also be tested in the future.

Our results indicate that RA treatment increased *NOTCH1* expression compared to no treatment, while 20 nM siNOTCH1 treatment did not significantly affect *NOTCH1* expression. A previous study showed that RA promotes the efficient delivery of transgenes to mouse skin (30). Another study also showed that RA pretreatment on mouse skin increased the in vivo transfection of liposome-DNA mixtures to hair follicle cells (31). Therefore, we speculated that the siNOTCH1 + 6.67 μM RA may increase the transfection efficiency of siRNA. However, it is still unclear how RA increased the transfection efficiency.

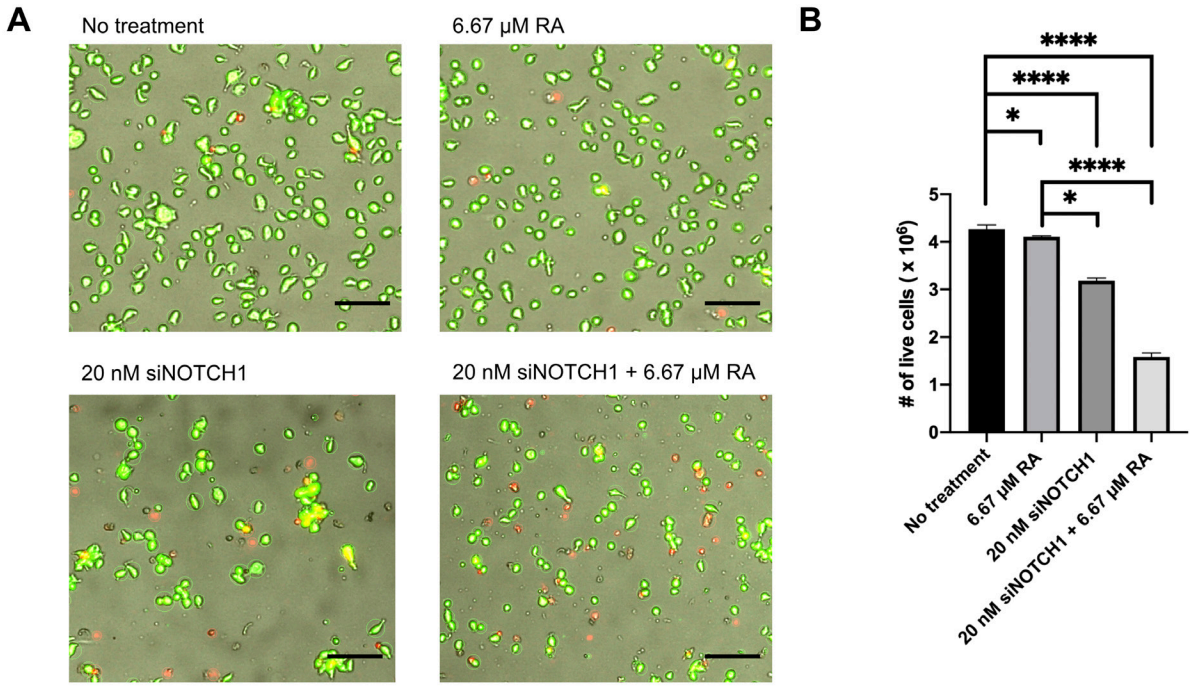

Figure 2: Combination treatment of siRNA targeting *NOTCH1* (siNOTCH1) and RA decreased the number of living cells. A172 brain cancer cells were grown under either control condition (no treatment), in RA (6.67 μM), in 20 nM siNOTCH1, and in 20 nM siNOTCH1 + RA (6.67 μM) for 72 hours. (A) Cell image showing the live cells (green fluorescent cells) and the dead cells (red fluorescent cells). (B) Mean ± SD of number of live cells (n=1). One-way ANOVA with Tukey's post hoc test, *p < 0.05, ****p < 0.0001. Scale bar = 50 μm.

Additionally, previous research also showed that EGF, Herg, and RA alone up-regulated *NOTCH1* transcript. However, combination treatment of EGF + RA and Herg + RA suppressed the induction of NOTCH1 expression level in human breast cancer SKBR3 cells (32). Therefore, RA treatment alone and combination treatment may show different effects on NOTCH1 expression levels in various cell types.

This is the first study investigating a combination treatment of RA and siNOTCH1. RA treatment has had side effects since it targets all human cells and causes harm to normal, healthy cells (28). Previous research indicated that RA decreased the NOTCH1 expression level, which blocks the NOTCH1 signaling pathway in cancer cells (23). Therefore, combination treatment with siNOTCH1, which targets NOTCH1 specifically, allows for a smaller dosage of RA to be used, inhibiting NOTCH1 signaling, which may reduce side effects. This combination treatment effectively induced brain cancer cell death and reduced the expression of *NOTCH1*. Unexpectedly, we found that RA combination treatment with siNOTCH1 may enhance the siNOTCH1 transfection efficiency only at a single low concentration of RA. Additionally, RA differentiates the cancerous cells into normal cells, meaning the RA and siNOTCH1 combination treatment can induce not only cell death but also has the chance of being able to decrease cell proliferation (33). Thus, RA and siNOTCH1 combination treatment can be used as a novel strategy for brain cancer treatment.

MATERIALS AND METHODS

A172 cell culture and maintenance

A172 cells were purchased from Korea Cell Line Bank. A172 cells were maintained in RPMI 1640 cell media (Gibco) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin (Gibco). 0.25 % Trypsin-EDTA solution (Gibco) was used to detach the cells from the cell culture plate. Every three days, fresh supplemented RPMI 1640 media was provided to the A172 cells to maintain the cell line in a healthy state. The cells were maintained in a 5% CO2 incubator. For every experiment, cells were counted using a LunaFL (LogosBio) automatic cell counter machine.

RA treatment and siRNA transfection

Eight experimental conditions were prepared with two technical replicates on A172 cells: no treatment samples (negative control), RA (6.67 μM), RA (13.34 μM), RA (20 μM), siRNA, co-treatment of RA (6.67 μM) and siNOTCH1 (20 nM), co-treatment of RA (13.34 μM) and siNOTCH1 (20 nM), and co-treatment of RA (20 μM) and siNOTCH1 (20 nM). One of the biological replicates was used for RNA extraction, and the other was for cell proliferation. The negative control sample, which only contained the media and cells, was prepared in both groups. The pre-designed siNOTCH1 (Cat # 4851-1) was purchased from Bioneer. We prepared three different concentrations of RA (Sigma-Aldrich): 6.67 μM, 13.34 μM, 20 μM. For siRNA transfection, 50,000 cells / well were added in 6-well culture plate. After RNAimax transfection reagent (Invitrogen) was mixed with the siNOTCH1 in Opti-MEM® I Medium (Invitrogen), the mixtures were incubated for 10 minutes. Then, the mixtures were added to the each well of the cell culture plates to make the final RNA concentration of 20 nM. The RNA extraction samples were incubated for 24 hours, and the cell viability samples were incubated for 72 hours.

Total RNA extraction from A172 cells

AccuPrep® Universal RNA Extraction Kit (Bioneer, K-3140) was used to purify total RNA from cells. Cell culture plates were washed with Trypsin-EDTA twice. Detached cells were spun down and resuspended in cell lysis buffer and 1% beta-mercaptoethanol. Then, RNA purification was performed as described in the manufacturer's protocol. Then, the purified RNA samples were stored for 24 hours at -20 °C.

cDNA synthesis from extracted RNA

The total RNA (2 μg) was quantified using the Nanodrop 2000 (Thermo Fisher) and used for cDNA synthesis using TOPscriptTM Reverse Transcriptase (Enzynomics). For each RNA preparation, reverse transcription was performed in a 20 μL reaction mixture using 12-mer Oligo (dT) primer. The eight PCR reactions were placed in a thermal cycler (BioRad) set to run at 50 °C for 1 hour, then 95 °C for 5 minutes, and then remained at 4 °C for storage.

Amplification of NOTCH1 and Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) by Polymerase Chain Reaction

Pre-mixed solutions for general PCR were purchased from Bioneer. For each PCR reaction, PCR was performed in a 20 μL reaction mixture with 50 nM of forward, 50 nM of reverse primers, and 100 ng of cDNA. The following primer sequence was used in this study. For NOTCH1, F: GAGGCGTGGCAGACTATGC, R: CTTGTACTCCGTCAGCGTGA was used. For GAPDH, F: GGAGCGAGATCCCTCCAAAAT, R: GGCTGTTGTCATACTTCTCATGG was used. All primer sequences were listed in 5' to 3'. The following PCR condition was used to amplify the target genes: 95 oC for 5 minutes, 35 cycles of 95 °C for 20 seconds, 58 °C for 30 seconds, and 72 °C for 20 seconds with a final extension at 72 °C for 5 minutes.

Agarose gel electrophoresis

PCR products were run on a 1.5% agarose gel stained with 2.5 μL of Redsafe (Intron). The gel was run in 1x Tris-acetate-EDTA (TAE) buffer for 20 min at 100 V. The gel was imaged using a blue light illuminator (SMOBIO) in a dark room.

Quantification of band intensity using Image J

ImageJ (ver.1.53k15) was used to quantify the band intensity by densitometry. The "Analyze" and "Gel" functions were used to select the area by drawing a rectangle. The "plot lane" function was used to measure the selected area of the band intensity. The background intensity was subtracted by drawing the line from the plot. The tracing tool was used to quantify the background-subtracted area. The band intensity of *NOTCH1* was divided by *GAPDH* for normalization.

Quantification of live cells

Luna-FL (Logos Bio), an automated fluorescence cell counter, was used to quantify the viable cells from each sample. The cells were stained with Acridine Orange (AO)/ Propidium Iodide (PI). AO stains all cells (green fluorescent cells), and PI stains dead cells only (red fluorescent cells).

If AO and PI exist together, AO fluorescence is suppressed by PI. After the cells were injected on the sample loading slide provided by Logos Bio, the integrated analysis software provides the total number of viable cells and the percentage of green fluorescent-positive cells.

Statistical analysis

All statistical tests were performed using GraphPad Prism (v. 8). One-way ANOVA with a Tukey's post hoc test was used to analyze the statistical significance. The statistically significant threshold was $p < 0.05$.

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