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# Effects of *Setaria italica* on Gap Junction-Mediated Intercellular Communication for the Development of Cancer Chemopreventive Agents

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**Abstract** – Inhibition of gap junction-mediated intercellular communication (GJIC) has been considered as an important factor in the tumor promotion phase of carcinogenesis. Recovery effects of natural products on gap junctional intercellular communication are measured by scrape-loading and dye transfer method using Lucifer yellow after administration of phorbol-12-myristate-13-acetate (PMA) on WB F344 cells. Among tested natural products, the hexane fraction and subfractions (F-01 and F-04) of *Setaria italica* were relatively effective for recovery of GJIC. The hexane fraction of *Setaria italica* ($EC_{25}$, 12.14 μg/ml) and subfractions (F-01: $EC_{50}$, 10.74 μg/ml; $EC_{25}$, 1.58 μg/ml, F-04: $EC_{50}$, 11.03 μg/ml; $EC_{25}$, 3.12 μg/ml) revealed dose-dependent recovery effects on GJIC. Our data show GJIC activity measurement by Lucifer yellow spread on cells can be an effective tool for the screening of natural products with possible cancer chemopreventive effects.

**Key words** – GJIC, *Setaria italica*, scrape-loading and dye transfer, Lucifer yellow, cancer chemoprevention.

## Introduction

Gap junctions are one type of plasma membrane junctional complex and consist of clusters of hundreds of particles approximately 10 nm in diameter that span the plasma membranes of adjacent, junction-forming cells. The channels are formed by connexons which are in turn comprised of six protein subunits of connexins (Loewenstein, 1981). The gap junction is present as a plaque-like element, variable in size and form in regions of contact between communicating cells (Hertzberg *et al.*, 1981). This junction has been correlated with the capacity for intercellular transfer of fluorescent dyes, labeled cations, nucleotides, amino acids and other substances. Several studies indicate that molecules with a molecular weight of less than approximately 1,200 dalton can pass from cell to cell through mammalian gap junctions (Lawrence *et al.*, 1978).

Gap junction-mediated intercellular communication (GJIC) has been considered as an important determinant for cell growth and differentiation (Hertzberg *et al.*, 1981). Cell culture studies have clearly shown that cellular growth and phenotype are influenced by contact-dependent and non-contact-dependent interactions with neighboring cells. These interactions include (1) the release of modulating factors (*e.g.*, growth factors) into the extracellular fluid that influence neighboring cells, (2) responses of cells to components on neighboring cell plasma membranes and within the extracellular matrix, and (3) direct signalling between cells through gap junctional

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channels. Data accumulated over the past three decades suggests that cell growth and phenotype are controlled, in part, by the cell-to-cell exchange of growth regulatory factors through gap junctions. Because the loss of gap junctions or impairment of their permeability has been observed in many neoplastic cells and cells treated with growth promoting carcinogens and other agents, the loss of GJIC appears to be an important event in the conversion of a normal cell into a neoplastic one. Inhibition of GJIC by various chemicals has been postulated to be a factor in the tumor promotion phase of carcinogenesis (Yotti *et al.*, 1979).

In this study, measurement of GJIC activity was achieved by using Lucifer yellow (M.W. 457.2) dye. Lucifer yellow dyes are intensely fluorescent 4-aminonaphthalimides which are readily visible in living cells at concentrations and levels of illumination at which they are nontoxic (Stewart, 1981). This dye spreads quickly throughout the body and the injected cell, but does not cross the intact cell membrane (Stewart, 1978). Lucifer yellow transfer to contiguous cells occurs through membrane gap junction (El-Fouly *et al.*, 1987). So this dye transfer phenomenon can be used as a marker of gap junction activity in relation to carcinogenesis process. GJIC recovery by natural products after cancer promoting agent administration on cells measured by scrape-loading and dye transfer method (El-Fouly *et al.*, 1987).

We examined the GJIC recovery effects from two hundreds of natural products. All natural products were partitioned into *n*-hexane, ethyl acetate, and water layers for the measurement of GJIC recovery effects. Here, we report possible cancer chemopreventive activity from *n*-hexane fraction of *Setaria italica* by GJIC recovery measurement after tumor promoting agent application on the WB F344 rat liver cell.

## Experimental

**Plant extracts** – Plant materials were purchased from a herb market in Seoul and voucher specimens have been deposited at Herbarium of Natural Products Research Institute, Seoul National University, Seoul, Korea. Dried plants were sliced, and then extracted three times with methanol at room temperature. The extracts were concentrated under reduced pressure at 40°C and the concentrated methanol extracts were partitioned into *n*-hexane, ethyl acetate, and water layers. The *n*-hexane, ethyl acetate, and water extracts of natural products were used for the measurement of GJIC activity. If necessary, the extracts were fractionated with column chromatography method.

**Assay** – Scrape-loading and dye transfer assay (El-Fouly *et al.*, 1987) was carried out. WB F344 rat liver cells were grown to 95% confluency on 6-well plates (Falcon, NJ, USA) in Dulbecco's modified Eagle's media (Gibco, USA) supplemented with 10% fetal bovine serum (Gibco, USA) and 50 μg/ml gentamicin sulfate (Gibco, USA) at 37°C in humidified air containing 5% $CO_2$. Cells were treated with a tumor promotor phorbol-12-myristate-13-acetate (PMA, Sigma) (Yotti *et al.*, 1979). After 3-hour incubation at 37°C and 5% $CO_2$, a plant extract was added (20 μg/ml). After this, incubation at 37°C and 5% $CO_2$ for an hour was done.

Cells were rinsed with phosphate buffered saline (PBS, pH 7.4) before the addition of Lucifer yellow CH (Sigma Co., USA) dye. One milliliter of 0.05% Lucifer yellow CH dissolved in PBS were added to the cells and scraped the cell layer on the plate using a scalpel (No. 21) at room temperature. The dye solution was left on the cells for 3 minutes, then discarded and the plates were rinsed with PBS to remove background fluorescence. Then the cells were fixed with 10% buffered formalin for 5 minutes. One milliliter of PBS was replaced and cells were examined under a fluorescence microscope (Carl Zeiss). Control plates were set by exposing the cells with PMA and Lucifer yellow without plant extracts. When an extract shows significant dose-dependant effect increase, $ED_{25}$ or $ED_{50}$ was estimated.

## Results and Discussion

We examined possible cancer chemopreventive activity from two hundreds of natural products by GJIC recovery measurement after tumor promoting agent application. In this study, measurement of GJIC activity was achieved by using Lucifer yellow dye. Because Lucifer yellow transfer to contiguous cells occurs through membrane gap junction (El-Fouly *et al.*, 1987), this dye transfer phenomenon can be used as a marker of gap junction activity. In communication-competent cells, the dye transmission occurred within minutes after loading. Among tested natural product extracts, the highest recovery effects on GJIC after PMA administration were seen in *Setaria ital-*

**Table 1.** Recovery effects on GJIC by the extracts of *Setaria italica*

| extracts | % of GJIC recovery effect[a] at 20 μg/ml | $ED_{50}$ (μg/ml) | $ED_{25}$ (μg/ml) |
|---|---|---|---|
| *n*-hexane | 35.6 ± 11.1 | >20 | 12.14 |
| ethyl acetate | 13.0 ± 3.6 | >20 | >20 |
| water | 13.3 ± 1.5 | >20 | >20 |

[a]Recovery effect of an extract was represented as following: % of recovery effect = [(No. of dye transferred cells on test plate - No. of dye transferred cells on control plate) / No. of dye transferred cells of control plate]×100. Data were represented as mean ± standard deviation (SD) (n = 3). $ED_{25}$ or $ED_{50}$ was estimated from simple linear regression equation.

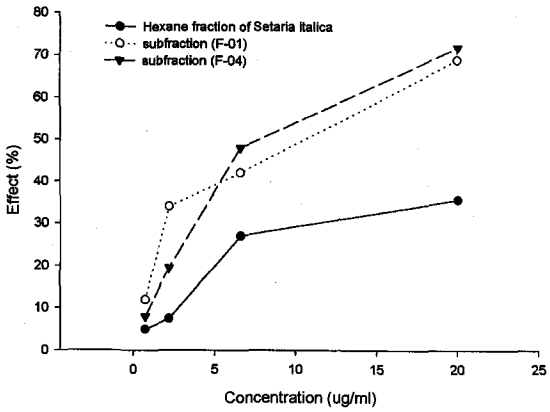


**Fig. 1.** Dose-dependant GJIC recovery effects of the hexane fraction and the subfractions (F-01 and F-04) of *Setaria italica.*

*ica.* While the *n*-hexane fraction of *Setaria italica* has strong recovery effect for GJIC, the ethyl acetate and water fractions of *Setaria italica* have weak recovery effect for GJIC (Table 1). The hexane fraction of *Setaria italica* ($EC_{25}$, 12.14 μg/ml) has dose-dependent recovery effects on GJIC (Fig. 1). The hexane fraction of *Setaria italica* was further fractionated with column chromatography method and subfractions were designated as F-01, F-02, F-03, and F-04. GJIC recovery effects of these subfraction were examined. While F-01 and F-04 have strong recovery effects for GJIC, F-02 and F-03 have weak recovery effect for GJIC (Table 2). Dose-dependent GJIC recovery effects of F-01 ($EC_{50}$, 10.74 μg/ml; $EC_{25}$, 1.58 μg/ml) and F-04 ($EC_{50}$, 11.03 μg/ml; $EC_{25}$, 3.12 μg/ml) were examined (Fig. 1). Fig. 2 shows GJIC recovery effect by the hexane fraction of *Setaria italica.* GJIC inhibition by PMA adminstration is recovered by treatment with the hexane fraction of *Setaria italica*, so Lucifer dye transfer is enhanced (Fig. 2B).

The intracellular molecular and biochemical changes that occur in dividing cells and that lead to neolpasia are being defined rapidly. Over the last two decades, numerous oncogenes, tumor suppressor genes, and cell cycle-regulated genes have discovered (Knudson, 1985; Weinberg, 1989). While the accumulation of genetic changes in a somatic cell is considered essential for the genesis of a cancer, it has become clear that not all carcinogens are genotoxic, suggesting that some carcinogens indirectly participate in the generation of genetic changes during carcinogenesis (Yamasaki *et al.*, 1996). GJIC is considered to play a crucial role in the maintenance of homeostasis, and in turn, aberrant GJIC is likely to be involved in carcinogenesis.

The restoration of GJIC in neoplastic cells has been achieved genetically by connexin gene transfection and pharmacologically by treatment with vitamins, hormones, and other agents. The enhancement of

**Table 2.** Recovery effects on GJIC by subfractions of the hexane fraction of *Setaria italica*

| Subfractions | % of GJIC recovery effect[a] at 20 μg/ml | $ED_{50}$ (μg/ml) | $ED_{25}$ (μg/ml) |
|---|---|---|---|
| F-01 | 68.9 ± 10.4 | 10.74 | 1.58 |
| F-02 | 19 ± 3.1 | >20 | >20 |
| F-03 | 31.3 ± 6.7 | >20 | >20 |
| F-04 | 71.8 ± 9.3 | 11.03 | 3.12 |

[a] Recovery effect of an extract was represented as following: % of recovery effect = [(No. of dye transferred cells on test plate - No. of dye transferred cells on control plate) / No. of dye transferred cells of control plate]×100. Data were represented as mean standard deviation (SD) (n = 3). $ED_{25}$ or $ED_{50}$ was estimated from simple linear regression equation.

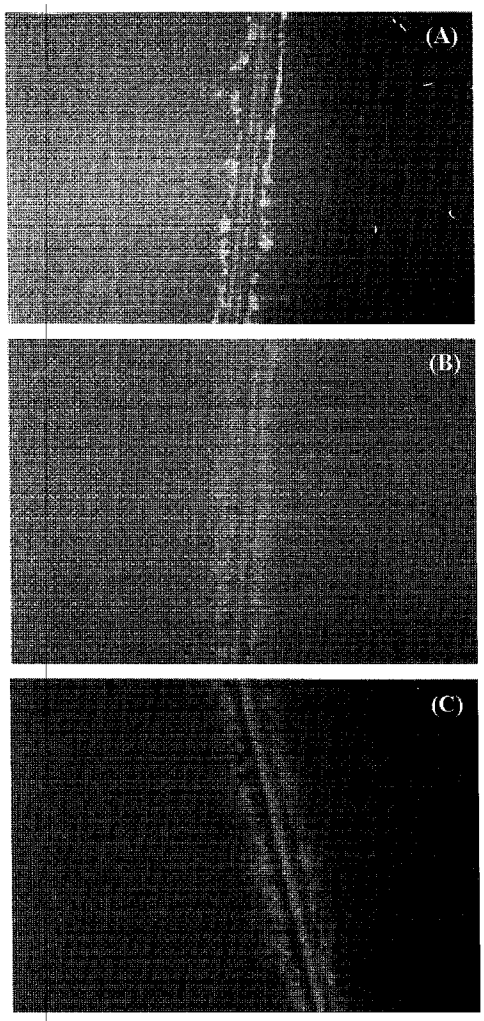


**Fig. 2.** GJIC recovery effect by the hexane fraction of *Setaria italica*. Cells were treated with PMA and incubated for 3 hours. And then natural product extract (20 μg/ml) was added and further incubated for one hour. After the addition of Lucifer yellow CH dye, dye transfer was measured by the method mentioned in Experimental. (A) GJIC is inhibited by administration of PMA and DMSO. (B) GJIC inhibition by PMA adminstration is recovered by treatment with the hexane fraction of *Setaria italica*, so Lucifer dye transfer is enhanced. (C) As negative control, cells were treated only with DMSO.

GJIC has resulted in reductions of neoplastic cell growth and tumorigenicity. Such results suggest that the stimulation of GJIC should be pursued as a potential anticancer therapeutic approach. GJIC recovery by natural products after cancer promoting agent administration on cells implied that natural product has anticancer activity.

In contrast to the effects of growth stimulatory and neoplastic agents on GJIC, many growth and cancer inhibitory agents increase GJIC and connexin expression in target cells. Retinoids, carotenoids, dexamethasone, and cyclic AMP agonists inhibit neoplastic transformation and/or tumor cell growth and increase connexin expression and gap junction formation in their target tissues (Mehta *et al.*, 1992; Zhang *et al.*, 1992). The hexane fraction and subfractions (F-01 and F-04) of *Setaria italica* have GJIC recovery effect but mechanism by which these recovery effects are caused is unclear.

Our research shows that GJIC activity measurement by Lucifer yellow spread on cells can be an effective tool for screening natural products having cancer chemopreventive activity. Furthermore, our research recommends further study on the pharmacological activity of the hexane fraction and subfractions (F-01 and F-04) of *Setaria italica*.

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