H-Ras oncogene counteracts the growth-inhibitory effect of genistein in T24 bladder carcinoma cells

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Among eight human bladder cancer cell lines we examined, only T24 cells were resistant to the growth inhibition effect of genistein, an isoflavone and potent anticancer drug. Since the T24 cell line was the only cell line known to overexpress oncogenic H-Rasval¹², we investigated the role of H-Rasval¹² in mediating drug resistance. Herein, we demonstrate that the phenotype of T24 cells could be dramatically reversed and became relatively susceptible to growth inhibition by genistein if the synthesis of H-Rasval¹² or its downstream effector c-Fos had been suppressed. The inhibition of Ras-mediated signalling with protein kinase inhibitors, such as PD98059 and U0126 which inhibited MEK and ERK, in T24 cells also rendered the identical phenotypic reversion. However, this reversion was not observed when an inhibitor was used to suppress the protein phosphorylation function of PI3 K or PKC. These results suggest that the signal mediated by H-Rasval¹² is predominantly responsible for the resistance of the cells to the anticancer drug genistein.

Keywords: H-Ras; bladder transitional cell carcinoma; genistein; microarray profiling gene expression pattern; drug resistance

The oncoprotein Ras, a 21 kDa guanine nucleotide-binding protein, is encoded by a member (Harvey-, Kirsten-, and Neural-ras) of the ras proto-oncogene family (Rayter et al., 1989; Maculuido et al., 2002). Ras transduces signals in discrete intracellular pathways (Marshall, 1995), with the route via raf-1, MEK, ERK, and finally to induce the expression of the immediate early gene c-fos for turning on a cascade of downstream genes as the most prominent pathway in regulating vital cellular processes (Rayter et al., 1989; Thorburn and Thorburn, 1994; Maculuido et al., 2002). Mutational activation transforms Ras into an oncogenic form, as demonstrated by the substitution of glycine by valine at codon 12 in Harvey (H)-Ras (H-RasG¹¹²), results in the loss of intrinsic GTPase function and therefore the protein is constitutively active, GTP-bound state and is continuously sending signals for cell growth (Rayter et al., 1989; Maculuido et al., 2002). Statistics reveal that 10–25% of all human malignancies in clinics were found to harbour a variety of Ras mutations (Barbacid, 1987; Bos, 1989), making Ras one of the most important targets to suppress tumour cell growth (Rinker-Schaeffer et al., 1993; Prendergast et al., 1996).

According to the recent surveys of the WHO (www.who.int/cancer/resources/incidences/en/), bladder cancer is rated within the five most common cancers in males in North America, Northern and Western Europe, and other developed countries. Transitional cell carcinoma (TCC) is a tumour that occurs mostly in the urinary bladder and has been linked to multiple and accumulated aberrations in oncogenes (e.g. H-ras mutations) and cancer-suppressor genes (e.g. p53 inactivation), as well as to the allelic loss of specific chromosomal loci (e.g. chromosomes 9q and 11p) (Habuchi et al., 1993; Linnenbach et al., 1993; Brandau and Bohle, 2001). Mutational activation of the H-ras oncogene was first reported (Reddy et al., 1982; Tabin et al., 1982) in human T24 TCC cell line that played an essential role in urothelial carcinogenesis. Molecular epidemiological studies conducted within different geographic regions or in different races and tumour stages/grades have revealed that up to 84% of bladder TCC carried activated H-Ras (Fontana et al., 1996; Hong et al., 1996; Vageli et al., 1996; Yu et al., 1996; Saito et al., 1997; Olderoy et al., 1998; Przybojewska et al., 2000). Therefore, oncogenic activation of H-Ras is an important tumorigenic factor for bladder tumours.

High soybean food consumption, which provides ingestion of a substantial amount of genistein, has been suggested to contribute to the relatively low rates of many cancers in Asian countries (Lee et al., 1991; Messina et al., 1994). Genistein, an isoflavone with a structure similar to oestrogen (a phyto-oestrogen), possesses potent inhibitory activities against growth factor-associated tyrosine protein kinases (Akiyama et al., 1987) and DNA topoisomerase II (Markovits et al., 1989). It is also an antioxidant (Naim et al., 1976) with the capability of suppressing angiogenesis...
and endothelial cell proliferation in vitro (Fotsis et al., 1993). Genistein is a compound with a variety of potential properties that mainly inhibit tumour growth in vitro and in vivo. In the mid-1990s, genistein was used for the first time to treat and efficaciously inhibit the growth of human bladder tumour cell lines (Lu et al., 1996). In subsequent experiments with bladder cell lines derived from superficial (RT4 cells) and invasive (T24 cells) tumour stages that had been transfected with the different genes to express the diverse expression levels of H-Ras and epidermal growth factor receptor (EGFR), this membrane protein has also been associated with tumorigenesis and is frequently clinically detected in bladder tumours (Dangles et al., 1997). Genistein and tyrophostin (an EGFR inhibitor) were both found to preferentially inhibit the motility and growth of bladder carcinoma cell lines that overexpressed EGFR (Theodorescu et al., 1998). Cancerous cells that harboured only mutated Ras but not overexpressed EGFR were less susceptible to inhibition by these drugs. The authors (Theodorescu et al., 1998) thus concluded that the upregulation of EGFR expression, but not oncogenic H-Ras, played a key role in developing tumour-invasive phenotype in T24 cells. In animal model studies, genistein has been consistently demonstrated to reverse the cancerous phenotype of mouse fibroblast (NIH3T3) cells transformed by v-H-Ras or H-Rasval 12 (Okura et al., 1998; Kuo et al., 1994). Zhou et al (1998) reported an in-depth study of the inhibitory effects of genistein on bladder tumorigenesis with both murine and human cell lines and in a mouse animal model (Zhou et al., 1998). The result of the study demonstrated that the compound inhibited the growth of two mouse and four human bladder cell lines, in a dose-dependent fashion, via cell cycle arrest at the G2/M phase and/or apoptosis. Importantly, mouse tumours derived from the transplantation of murine bladder cancer cells also responded to the inhibitory effects of genistein. A similar report (Su et al., 2000) published 2 years later also confirmed that genistein induced cell cycle arrest and inhibited CDC2 kinase activity that suppressed bladder tumour cell growth. These data suggest that the drug has potent anticancer effects both in vitro and in vivo, and, therefore, genistein has become one of the potential therapeutic compounds to treat patients with urinary bladder neoplasms.

Our research (Chen et al., 2001; Shieh and Li, 2004) has focused on gene expression alterations in human bladder tumour cells induced by genistein, in the hope to gain insight into the molecular mechanism(s) of growth inhibition mediated by this drug, and the identification of potential genes that may be further evaluated for possible therapeutic application. Herein, we demonstrate that among eight human bladder tumour cell lines (5637 (Fogh, 1978), BFTC905 (Tzeng et al., 1996), HT1197 (Rasheed et al., 1977), J82 (O’Toole et al., 1988), SCABER (Cheng et al., 1995), T24 (Reddy et al., 1982; Tabin et al., 1982), TSGH-8301 (Yeh et al., 1988), and TCCSUP (Nayak et al., 1977)) tested for the susceptibility of genistein-induced growth inhibition, only the activated H-ras-harbouring T24 cells were consistently resistant to this drug treatment. From this investigation, we concluded that the H-Rasval 12-induced signal transduction pathway was mainly responsible for the resistant phenotype of T24 bladder TCC cells to the anticancer drug.

**MATERIALS AND METHODS**

**Materials, cell cultures, and cell proliferation assay**

Molecular biology enzymes and reagents were purchased from Stratagene (La Jolla, CA, USA) unless otherwise specified. Standard chemicals and reagents were obtained from Sigma (St Louis, MO, USA). All reagents were used according to the recommendations of the manufacturers. Eight bladder tumour cell lines, including 5637, BFTC905, HT1197, J82, SCABER, T24, TSGH-8301, and TCCSUP, were all maintained in DMEM supplemented with 10% foetal bovine serum, 100 U ml⁻¹ penicillin, and 100 µg ml⁻¹ streptomycin (these tissue cultural reagents were from Gibco, Grand Island, NY, USA) under 5% CO₂ at 37°C. Primary bladder epithelial cells (BDEC) were obtained from BioWhittaker (San Diego, CA, USA) and maintained as recommended by the supplier. Cell proliferation assay with a tetrazolium compound and phenazine ethosulphate (CellTiter 96® AQueous One Solution Cell Proliferation Kit, Promega; Madison, WI, USA) was used to analyse the growth-inhibitory effect of genistein (Sigma) according to the protocol reported previously (Chen et al., 2001). Each 5000 bladder primary or tumour cells were seeded into wells of 96-well culture plates, followed by addition of genistein in the concentrations indicated in Figure 1. The cells were incubated for 72 h before applying the cell proliferation assays. The genistein stock solution (50 mM) was prepared with pure DMSO (Sigma) and was later diluted to the appropriate concentrations with DMEM at the time of use.

**cDNA microarray and antisense experiments**

The experiments of profiling gene expression patterns in different bladder tumour cells treated with 50 µM genistein were routinely performed by using self-produced cDNA microarrays and a hybridisation/detection protocol (Chen et al., 2001; Shieh and Li, 2004; w3.csmu.edu.tw/~chingli-Biochip/). Here, we showed the changes of egr-1 and c-fos expression levels in T24 and TSGH-8301 cells with respect to the time of induction. Several high-quality phosphorothioate oligodeoxynucleotides (ODNs) were synthesised (MDBio Inc.; Taipei, Taiwan, ROC) to block the synthesis of the target gene products. All these ODNs contained 14 phosphorothioate groups to ensure the best stability during transfection, as demonstrated by the anti-H-ras expression experiments reported previously (Monia et al., 1996). Except for the VDUP-1 gene (Han et al., 2003), we used the previously reported, as listed below, antisense and control ODNs in experiments targeted to the expressions of H-ras, c-fos, or egr-1 gene. They include anti-oncogenic-H-ras antisense and controls (antisense, 5'-CaGsAsCsAsGsGsGsGsGsGsGsCsGsCsCc-3'; control, 5'-GsAsGsGsTsGsGsGsGsGsGsGsGsGsGsAs-3').

| Genistein | Concentration (µM) | M genistein | scrambled, 5'-GsAsGsGsTsGsGsGsGsGsGsGsGsGsGsAs-3'; mutated, 5'-GsAsGsGsTsGsGsGsGsGsGsGsGsGsGsAs-3'; control, 5'-GsAsGsGsTsGsGsGsGsGsGsGsGsGsGsAs-3' |
|-----------|-------------------|------------|-------------------------------------------------|
| 25        | 0                 | 50         | 100                                             |

Figure 1. The susceptibilities of bladder TCC cell lines and BDEC to growth inhibition by genistein. The growth inhibition curves for BDEC and one bladder primary bladder tumour cells treated with 50 µM genistein were routinely performed by using self-produced cDNA microarrays and a hybridisation/detection protocol (Chen et al., 2001; Shieh and Li, 2004; w3.csmu.edu.tw/~chingli-Biochip/). Here, we showed the changes of egr-1 and c-fos expression levels in T24 and TSGH-8301 cells with respect to the time of induction.

**Figure 1** The susceptibilities of bladder TCC cell lines and BDEC to growth inhibition by genistein. The growth inhibition curves for BDEC and eight human bladder TCC cell lines are illustrated. To prevent confusion caused by drawing nine curves with error bars in a small plot, we divided the studied cell lines into three groups (panels A–C) according to their relative growth rates in the presence of increasing genistein concentrations. Panel A: insensitive cell lines include T24 (♀) and primary bladder epithelial cells (♂). Panel B: 5637 (♀), BFTC905 (♂), HT1197 (♂), and J82 (♀) were cell lines that were moderately inhibited. Panel C: relative sensitive cell lines include SCABER (♂), TCCSUP (♀), and TSGH-8301 (♀). These experiments were performed twice with duplicate samples.
GstScsGsCsAsGsGsGsGsGsGsGsG-3') (Sells et al., 1995), and anti-VDP1-1 gene antisense and sense (antisense, 5'-TsTsGsTsTGGACACTATCasCasAsT-3'; sense, 5'-AsTsGsTsTGGAT GTCCAsAsGsAsA-3') ODNs, where 's' indicates a phosphorothioate-capped moiety and they were used in cell proliferation assays or immunoblotting experiments. Approximately 6000 appropriate cells were seeded in the wells of a 96-well flat-bottom microtitre plate, followed by adding indicated amounts of an ODN premixed with ESCORT transfection reagent (used according to the instruction provided by Sigma) and incubating in a CO₂ incubator for 8 h at 37°C. The cells were further treated with 50 μm genistein for another 72 h before they were subjected to cell proliferation assays. As the controls for T24 cell proliferation assays under various conditions, BDEC and TCCSUP cells were also grown and passed through the identical procedure in parallel.

Reverse transcription–polymerase chain reaction and immunoblotting analyses

The expression levels of egr-1 and c-fos in TSGH-8301 and T24 cells treated without or with 50 μM genistein for the various time points were determined by semiquantitative reverse transcription–polymerase chain reaction (RT–PCR). The amplification primers used were: (1) β-actin: forward 5'-ATCATGTTTGA GACCTTCAA-3' and reverse 5'-CATCTTCGTGCAAGTCTCA-3'; (2) egr-1: forward 5'-CTGCGGCTCCTAGTTGTC-3' and reverse 5'-AGCAGCATCATCTCCTCCAG-3'; and (3) c-fos: forward 5'-AAG GGAATCCGAAGGAAA-3' and reverse 5'-GCCTGTAGTGG CTTGACGG-3'. The RT–PCR of these genes was performed at the cycles that the amplification of DNA molecules was in the exponential increasing stage. After many tests to correlate with the PCR product amounts and thermocycling numbers (data not shown), we used 20 amplification cycles for β-actin and 27 cycles for both egr-1 and c-fos genes. The RT–PCR products were visualised by agarose gel electrophoresis in the presence of ethidium bromide.

When performing Western blot analyses with ODN-treated cells, 5 x 10⁶ cells were placed in a six-well culture plate, followed by treating with ODNs, 5 μM each time, and the same amount of genistein, as in the protocol described above. After incubation and removal of culture supernatants, 50 μl of RIPA lysis buffer (Helfrich et al., 1994) was used to lyse and then collect cell extracts for SDS–PAGE. The entire immunoblotting procedure employed a commercial standardised technique (BM Chemiluminescence Western Blotting Kit, Roche Applied Science; Mannheim, Germany) with rabbit antibodies against human H-Ras (clone C-20), c-Fos (4), and Egr-1 (S80) proteins that were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Chemiluminescent detections of antibody–antigen complexes revealed the target proteins on X-ray film. The cell proliferation assays and Western blotting experiments were all performed in triplicate and identical results were obtained.

RESULTS

The effect of genistein on bladder tumour cell growth and gene expression

In order to extend our previous efforts to gain insights into the molecular mechanism for genistein-induced cell growth inhibition in bladder tumour cells (Chen et al., 2001; Shieh and Li, 2004), we tested the effect of the drug on eight bladder tumour cell lines (including 5637, BFTC905, HT1197, J82, ScaBER, T24, TSGH-8301, and TCCSUP) and BDEC. We divided the cell lines into three groups depending on their susceptibilities to the growth-inhibitory effect of genistein: T24 cell line and BDEC were relatively insensitive to the drug treatments that consistently maintained the growth rate above 75% of the respective mock-treated cells when the highest concentration (50 μM) of the drug was used (Figure 1A); cell lines HT1197, HT1376, J82, BFTC905, and 5637 moderately reduced their growth rates to 65–55% (Figure 1B); and ScaBER, TSGH-8301, and TCCSUP cells were always susceptible to the drug treatments as their growth rates were dramatically reduced to 30–35% at the genistein concentration of 50 μM. We also found that the J82 cell line had elevated growth rates at low genistein concentrations (Figure 1B), which might be due to the stimulatory effect of DMSO solvent (10%), a similar effect had been observed with HepG2 and Hep3B hepatocellular carcinoma cell lines (Li, unpublished data), and therefore the growth-inhibitory effect could be detected only when the highest concentration of genistein (50 μM) was used. Since BDEC was not a tumour cell line but was regarded as the normal cell control, the growth should not be greatly inhibited by the anticancer drug, and indeed, the growth rates were only slightly reduced and then maintained close to 80% at concentrations equal to or greater than 20 μM (Figure 1A). The cell proliferation assays demonstrated that T24 was the only TCC cell line that consistently resisted genistein, and the growth rates were always maintained at 90–95% during the drug treatments. Although the degree of the inhibition varied among cell lines, this result basically agreed with the previous results reported by Zhou et al. (1998).

In the previous experiments, we used a self-made Millennia Chip, version 1 cDNA microarrays to profile differential gene expression patterns in genistein-stimulated TCCSUP, TSGH-8301, and T24 bladder TCC cell lines (Chen et al., 2001; Shieh and Li, 2004). The most notable finding in these studies was the dramatic difference in the expression patterns of two immediate early genes egr-1 and c-fos. In the genistein-sensitive cell lines, TCCSUP and TSGH-8301, the genes revealed transient and inducible kinetics which peaked at 0.5 h post treatment of genistein just as were reported in many physiological conditions (Liu et al., 1991), whereas in genistein-resistant T24 cells both genes were constitutively expressed but could be induced to the higher levels by the drug, followed by declining to the lower levels. Figure 2A demonstrates the expression levels of egr-1 and c-fos in TSGH-8301 and T24 cells derived from cDNA microarray analyses, whereas Figure 2B confirms such gene expression profiles with RT–PCR. Since the overexpression of oncogenic H-Ras¹¹ Val has only been reported in T24 cells, we speculated that the oncogene may play a role in egr-1 and c-fos expression patterns in the cells, as reported previously (Stacey et al., 1987; Alexandropoulos et al., 1992). Therefore, we investigated the role of H-Ras¹¹ Val in mediating cellular signal transduction pathway in T24 tumour cell line that rendered the resistant phenotype against the growth-inhibition effect of genistein.

In vitro targeting gene expressions and the susceptibility to genistein inhibition

Since the most prominent signal transduction pathway regulated by Ras is Raf-1-mediated signalling which results in c-Fos induction (Maruta and Burgess, 1994), we examined the effect of H-Ras¹¹ Val or c-Fos expression on the susceptibility of T24 cell to genistein inhibition. Antisense phosphorothioate ODNs targeted to the expression of either gene was used to treat T24 cells, followed by assaying the cell proliferation rate in the presence of 50 μM genistein. The experiments detected that the levels of H-Ras¹¹ Val were moderately reduced in the cells treated with 5 μM of control ODN for 2 h (Figure 3A, lanes 5 and 6 versus lanes 1 and 2, respectively), whereas the levels fell dramatically to one-third or lower of the original level if the same amount of the anti-H-ras ODN was used for the same period of time (Figure 3A, lanes 3 and 4 versus lanes 1 and 2, respectively), as determined by immunoblotting. After 72 h incubation, the effect of anti-H-ras ODN on blocking H-Ras⁰⁰¹¹ synthesis was even more obvious (lanes 8 and 9 versus lanes...
Upon treatment of the cells with both anti-H-ras and genistein, T24 cell growth rate was further reduced to below 40% of the untreated cells and also in lanes 8–11. Since the anti-H-ras ODN plus genistein, as described previously (Chen et al., 1996), with increasing amounts of different ODNs, the growth rates of T24 cell line were determined in the presence or absence of 50 μM genistein. The plot reveals that only when the anti-H-ras, but not control, ODN (5 μM) is added, the growth of T24 cells is inhibited in the absence of genistein and the growth rate is further reduced to below 40% of the untreated cells when the drug is added, suggesting that the expression of H-Ras renders drug-resistant phenotype. The symbols used are: cells without any treatment, □; and cells treated with 0.5% DMSO solvent. ■, 50 μM genistein; ◆, the control ODN; ■, the control ODN supplemented with 50 μM genistein; ◼; the anti-H-ras antisense ODN; ◇; and the antisense ODN plus genistein, ▶.

Figure 3 Influence of H-Rasval12 overexpression on the growth of T24 cells responding to antisense oligonucleotide and genistein treatments. (A) T24 cells were treated with 5 μM of the indicated phosphorothioate ODN alone or the ODN and 50 μM genistein, followed by cell extract isolation and Western blotting with the anti-H-Ras antibody. The intensities of H-Rasval12 protein were quantified with a densitometer, and the Ratio line revealed the percentage of the protein produced (after normalising with the levels of actin protein) in the ODN or/and genistein-treated cells vs untreated T24 cells. The result revealed that the expression of H-Rasval12 was inhibited to a greater degree by treating with the antisense ODN than with the control ODN or genistein, and the effect was strikingly obvious after 72 h incubation. Under all experimental conditions, actin remained relatively unchanged. The control ODN was a 17-mer targeted to human immunodeficiency virus, which was used in parallel with anti-H-Rasval12 ODN, as described previously (Chen et al., 1996). (B) With increasing amounts of different ODNs, the growth rates of T24 cell line were determined in the presence or absence of 50 μM genistein. The plot reveals that only when the anti-H-ras, but not control, ODN (5 μM) is added, the growth of T24 cells is inhibited in the absence of genistein and the growth rate is further reduced to below 40% of the untreated cells when the drug is added, suggesting that the expression of H-Ras renders drug-resistant phenotype. The symbols used are: cells without any treatment, □; and cells treated with 0.5% DMSO solvent. ■, 50 μM genistein; ◆, the control ODN; ■, the control ODN supplemented with 50 μM genistein; ◼; the anti-H-ras antisense ODN; ◇; and the antisense ODN plus genistein, ▶.

Translational Therapeutics
pathway results in nuclear induction of c-Fos, a subunit of the transcription factor AP-1. We intended to test the drug sensitivity of T24 cells when the c-fos expression had been retarded in vivo by treating with the ODN that has been reported to be effective in blocking cellular c-Fos expression (Gibellini et al., 1997). We detected that when 5 μM anti-c-fos ODN was added to T24 cells, the levels of c-fos were dramatically decreased to half of the detected level in untreated cells at all time points (Figure 4A, lane 1 vs other lanes), and the drug-resistant phenotype of T24 cells was reversed (Figure 4B). In these experiments, we used a mutant anti-c-fos ODN, as a negative control, because it contained a sequence like the anit-c-fos ODN, but with four dispersed nucleotide substitutions that could interrupt the specific binding to its target c-fos and lost inhibitory capability. The use of the mutated ODN can thus demonstrate the specificity of antisense oligonucleotide in blocking the expression of c-Fos. As detected in Figure 4, the mutant anti-c-fos ODN and genistein together inhibited cell growth moderately (to about 60%), whereas 5 μM anti-c-fos ODN alone was able to reduce T24 cell growth rate to 40% of the mock-treated cells, which might be due to the efficient inhibition of c-Fos expression and activity that led to reduction in the growth rate. The growth rate of the T24 cell line was further decreased to 15% when both anti-c-fos ODN and genistein were employed simultaneously. Similar results were also observed when 10 μM of all ODNs was used to treat cells under identical conditions (data not shown), suggesting that the correlation between the treatment with antisense or mutated ODN and the drug-resistant phenotype of T24 cells is specific. We thus concluded that c-Fos expression also influenced the drug susceptibility of T24 cells, just as its upstream regulator H-Rasval12. Although Ras and c-Fos are in the same pathway, the inhibitory effect of c-fos ODN was greater than that of ras ODN, which may be represented by the differential efficiency of individual ODNs on inhibiting their targets, and that frequently depends on the nature and/or nucleotide sequences of the molecules.

The specificity of the above antisense experiments was further investigated. From the previous microarray hybridisation experiments, the egr-1 and VDUP-1 gene expressions were known to be significantly induced by genistein in TCCSUP and TSGH-8301 cells (Chen et al., 2001; Shieh and Li, 2004), and the former gene was constitutively overexpressed in T24 cells (Figure 2). Since alterations in gene expression patterns frequently indicate the involvement of the gene products in the biological/pathological processes of interest, we examined the role of egr-1 or VDUP-1 in the resistance of T24 cells to genistein, and the results were compared to those from the antisense ODN experiments (Figures 3 and 4) studying the functions of H-Rasval12 and c-Fos. Our experiments detected that, regardless of the egr-1 antisense or the scrambled control ODN used in cell cultures, the drug sensitivities of T24 cells remained unchanged, and the same results were also obtained from experiments using antisense or sense ODN targeted
functions of MEK and ERK. Figure 6A shows that T24 cells became genistein-sensitive as the growth rates were significantly decreased when 50 μM genistein and either PD85089 (20 μM) or U0126 (10 μM) are present (both P < 0.05 by t-test, two-tailed), and both inhibitory effects were in a dose-dependent manner. This result was completely in agreement with the previous (Figures 3 and 4) antisense experiments with the anti-H-ras and anti-c-fos ODNs. Protein kinase inhibitors H7 and LY294002, which block the signalling pathways associated to PKC and PI3 K, respectively, were also used in T24 cell proliferation assays with and without the presence of 50 μM genistein. We detected that, at H7 concentration equal to 10 μM or greater, the growth rates of T24 cells were decreased regardless of the presence or absence of the drug, suggesting that H7 did not influence the drug susceptibility, and the cytotoxicity was responsible for cell quiescence at the higher H7 concentrations (Figure 6B). The inhibitor LY294002 at the effective concentration of 20 μM could only slightly reduce the T24 cell growth rate from 95 to 75% of the mock-treated cells in the presence and absence of genistein. The results rule out the involvement of the PKC or PI3 K signal transduction pathway in the drug resistance of the T24 cells.

The Ras pathway is known to interact with the JNK pathway, as the Ras downstream effector c-Fos forms the transcription factor AP-1 with c-Jun, a nuclear protein regulated by the function of JNK (de Ruiter et al, 2000). Owing to this, we investigated the role of JNK in the susceptibility of T24 cells to genistein. Figure 6C demonstrates that the JNK inhibitor SP600125 itself exhibits a mild dose-dependent inhibitory effect on T24 cell growth, and the addition of 50 μM genistein potentiates the growth suppression further down to the significant lower level, about 50% of the mock-treated cells, at a concentration of 30 μM SP600125 used (P < 0.05 by t-test, two-tailed), and the level is similar to that by 20 μM PD98059 or 10 μM U0126 as demonstrated in Figure 6A. Since the JNK inhibitor alone partly inhibited cell growth, dual protein kinase inhibitors were tested. The dose-dependent growth inhibition was again observed if both inhibitors of the H-Ras (U0126) and genistein were used to dissect Ras-regulated signal transduction pathways. First, PD85089 and U0126 were employed to inhibit the signal transduced from H-Ras to c-Fos by blocking the kinase inhibitory effect must involve the H-RasVal12/c-Fos-mediated signal transduction pathway.
and JNK (SP600125) signalling pathways were used together. As illustrated in Figure 6C, treatment of T24 cells with 10 μM U0126 and increasing amounts of SP600125 (10–30 μM) renders the decreasing T24 cell growth rates down to 50% of untreated cells (all reached significant levels at P < 0.05). Under identical culture condition, addition of genistein further inhibited the growth rates significantly to approximately 20% of the original growth rate (all P < 0.05), which was comparable to those of the genistein-sensitive bladder TCC cell lines (Figure 1). These data suggest that signal transduction through the JNK pathway is also involved in the resistance of T24 cells to genistein.

DISCUSSION

In this study, we employed microarray technology, antisense oligonucleotide inhibition, and cell proliferation assays to identify and characterise genes that are playing key roles in the resistance of T24 bladder TCC cell lines to the anticancer drug genistein. Genistein is a phytosterogen that is present in high quantity in many traditional Asian diets containing soybean products (Messaia et al., 1994). The compound is regarded as a potential and ideal anticancer drug to treat patients with bladder tumours, because the compound is a natural product present in diet that has been proven to be effective in treatment of bladder TCC (Zhou et al., 1998; Su et al., 2000), as well as safe with minimal side effects and inexpensive. In 1996, the compound was subjected to the clinical chemoprevention trials for preventing breast cancer and acute lymphoblastic leukaemia (Quella et al., 2000; Wang, 2000). Although genistein has shown some promising cancer cell growth-inhibitory effects, many concerns, such as which function(s) is (are) actually playing an essential role in tumour suppression or does it involve the induction of gene expression, remain to be determined. We therefore performed a functional genomic study of genistein-induced gene expression alternation would be the most effective and also the most rapid means to identify the key gene(s) involving the cell growth regulatory pathway.

The anticancer effect of genistein has been mostly studied in breast tumours at the molecular level (El-Deiry et al., 1993; Harper et al., 1993), and the regulatory event was independent of the oestrogen receptor and the cancer suppressor p53 protein (Li et al., 1999; Xu and Loo, 2001). Bladder TCC has been shown to undergo apoptosis induced by genistein through the EGFR signalling pathway involving p21WAF1/CIP1 expression (Dangles et al., 1997). It has also been demonstrated that genistein exhibited dose-dependent (0–50 μM) growth inhibition of many murine and human bladder cancer cell lines, providing evidence of cell growth arrest at the G2/M phase followed by apoptosis in some cell lines (Zhou et al., 1998). In this study, we focused on the genistein growth inhibition of only human cell lines, and compared different gene expression patterns in human cell lines sensitive and resistant to the genistein treatments with cDNA microarrays. From the results, we are convinced that the overexpression of oncogenic H-Rasval12 and its control of the signal transduction pathway were solely responsible for the resistance of the T24 bladder TCC cell line to genistein growth inhibition. It is possible that genistein inhibits the growth signals from the tyrosine protein kinase-associated receptors, such as EGFR and PDGFR (platelet-derived growth factor receptor), to small G protein Ras and thus suppresses cell growth, just like the inhibitory model proposed for genistein prevention of phenylephrine-induced Ras activation (Thorburn and Thorburn, 1994). In such a situation, T24 H-Rasval12, which is downstream from the action site of genistein, constantly sends very strong cellular proliferative signals, resulting in the drug-resistant phenotype. H-Rasval12 is also responsible for maintaining the fast growth rate of T24 cells in routine tissue culture experiments, and blocking the protein or c-Fos synthesis with antisense ODN or interrupting the function of Ras downstream protein kinases by inhibitors frequently resulted in decreasing the growth rate, as we observed in many of our cell proliferation experiments (Figures 3B, 4B, and 6C). However, it is still unclear why other TCC cell lines such as TCCSUP and TSGH-8301 could be induced to transiently express c-Fos but remained susceptible to the inhibitory effect of genistein. Furthermore, our finding that the overexpression of the H-ras oncogene counteracts the anticancer effect of genistein in T24 cells is contrary to previous reports demonstrating that the drug was capable of (1) reversing the malignant phenotypes of mouse fibroblast (NIH3T3) cells transformed by v-H-Ras or H-Rasval12 (Okura et al., 1988; Kuo et al., 1994; Li, unpublished data) and (2) suppressing the Ras activity induced by phenylephrine in neonatal rat ventricular myocytes (Thorburn and Thorburn, 1994). This discrepancy may be due to differences in genetic and tumorigenic backgrounds, as suggested by a previous report (Hamad et al., 2002), and, certainly, the clinical behaviour of human bladder tumours responding to genistein treatment is likely to more closely resemble that of the T24 cell line which was derived from human bladder TCC.

In summary, this study provided data of cell proliferation experiments employing antisense ODNs and protein kinase inhibitors, which indicate that the resistance of T24 cells to the genistein growth-inhibitory effect is predominantly due to the activation of oncogenic H-ras that transduces the growth signal through the MEK/ERK pathway and interferes with the JNK signalling pathway.

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