Identification of Novel Genes Involved in the Synergistic Antitumor Effect of Caffeine in Osteosarcoma Cells Using cDNA Macroarray

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Abstract. Background: Caffeine enhances the cytotoxic effects of DNA-damaging agents. This study investigated genes involved in the synergistic effect of caffeine on osteosarcoma cells using gene-profiling analysis. Materials and Methods: Sensitivity to cisplatin and the synergistic effect of caffeine were evaluated in five osteosarcoma cell lines with different p53 gene status. Gene expression profiles were analyzed using cDNA macroarray and verified by real-time RT-PCR. Results: The cell lines were grouped into three types with different cytotoxic patterns. Comparison of profiling data from these groups identified twelve novel genes associated with the synergistic effect of caffeine. Real-time RT-PCR analyses verified up-regulation of two apoptosis-enhancing genes and down-regulation of two interferon-inducible genes related to the synergy of caffeine. Conclusion: These findings provide new insights into the molecular mechanisms of the synergistic effect of caffeine related to p53 gene status in osteosarcoma, providing candidates for an assay of responsiveness to caffeine-potentiated chemotherapy for osteosarcoma.

Caffeine, a methylxantine derivative, enhances both the growth inhibitory and cytotoxic effects of DNA-damaging agents, such as radiation, ultraviolet light and anticancer drugs on tumor cells. Some studies have suggested the importance of the DNA-repair inhibiting effect of caffeine in the augmentation of these effects (1-4). Caffeine is one of the inhibitors of ataxia telangiectasia mutated (ATM) and ATM-Rad3-related (ATR) kinases, which are master regulators of DNA damage-induced cell cycle checkpoints (5-7). Caffeine helps tumor cells overcome G1/S and G2/M checkpoint-responses to multiple genotoxic stresses before the completion of DNA repair, leading these cells to mitosis and cellular death (8-10).

We have previously reported the K2 protocol consisting of five courses of intra-arterial cisplatin (CDDP), doxorubicin and caffeine at 3-week intervals as preoperative neoadjuvant chemotherapy (11). While conventional multiagent chemotherapies yield a local tumor response of approximately 50 to 75% and a 5-year survival of 65 to 75% for osteosarcoma (12-14), the response rate of the K2 protocol was over 90%, and the 5-year survival rate was 90% (5-year event-free rate, 76%) for primary non-metastatic osteosarcoma (11). However, the exact molecular mechanism of the caffeine-induced augmentation of osteosarcoma chemotherapy remains unknown.

Alternations in p53 are known to occur in approximately 20-25% of osteosarcomas (15-17). In many malignancies, such as breast and colorectal cancer, abnormalities in p53 predict prognosis, as well as chemotherapy responsiveness (18, 19). However, previous studies of p53 expression in osteosarcoma have not demonstrated any significant association with patient outcome or other clinicopathological features (16, 20). And also the exact relationship between p53 status and the synergistic effect of caffeine in osteosarcoma remains unknown.

Recent applications of the cDNA array technique have facilitated the determination of the expression levels of thousands of genes in a single experiment. This technique has the potential to identify novel target genes that play key roles in mediating the synergistic effects of caffeine. Expression profiling analysis of genes associated with the synergistic effect of caffeine using cDNA array technique has not previously been reported. Therefore, in this study, the gene expression profiles of osteosarcoma cell lines with different
p53 status that showed different patterns of caffeine-induced synergy were compared using a cDNA macroarray of 1,176 human cancer-related genes. The analysis was then exploited to select genes associated with strong synergistic effects of caffeine on osteosarcoma cell lines.

Materials and Methods

Cell lines and cultures. Five human osteosarcoma cell lines were used: Saos2 and MG63, which show a deficiency of the p53 gene; HOS, which has a mutant p53 gene; OST (Osteosarcoma Takase), which was established in our department (21) and has two wild-type p53 alleles and OST/R, a CDDP-resistant subline of OST established by subcloning and maintenance in 0.7 mg/ml cisplatin for more than 3 months (22, 23). The first three cell lines were purchased from the American Type Culture Collection, Rockville, MD, USA. All the cells were cultured in RPMI-1640 medium (Gibco-Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Sigma, St. Louis, MO, USA) and 1% penicillin-streptomycin (Gibco-Invitrogen). The cultures were incubated at 37˚C in a 5% CO2 atmosphere with 100% humidity.

Cytotoxicity assay. To evaluate sensitivity to CDDP and the synergistic effect of caffeine in the osteosarcoma cells, exponentially growing cells of the five human osteosarcoma cell lines were transferred into 96-well microtiter plates at a density of 5x10^3 cells/well. After overnight incubation, the medium was aspirated and fresh media with different concentrations of CDDP (Nippon Kayaku, Tokyo, Japan) up to 100 μg/ml were added. The cells were treated with the drug for 1 h, then washed twice with PBS, followed by 72-h incubation in medium with or without 1 mM caffeine (Wako Chemicals, Osaka, Japan). The viability was determined at each drug concentration using the WST-1, a sodium salt of 4-[3-(4-iodomphenyl)-2-(4-nitrophenyl)-2H-5-tetrazol]-1,3-benzene disulfonate, viability dye assay (24). The cells were incubated with 10 μl of WST-1 for 1 h and absorbance at 450 nm was measured. The viability was expressed as the percentage of absorbance obtained in the treated wells relative to that in the untreated control wells. The significance was analyzed by repeated ANOVA. A p-value <0.05 was considered significant and the data are presented as the mean ± SEM of five independent experiments. The IC50, the concentration of CDDP required to inhibit cell survival by 50% with 1 mM caffeine compared with untreated controls, was calculated by dose-effect analysis.

Flow cytometry. Alterations in the cell cycle phase distributions in the osteosarcoma cells treated with CDDP combined with or without caffeine were analyzed using flow cytometry (25). The osteosarcoma cells were cultured in the presence of 5 or 20 μg/ml CDDP for 1 h respectively with or without the subsequent addition of 1 mM caffeine for 72 h. The cells were harvested by trypsinnization, washed twice with PBS, and fixed in cold 70% ethanol for 30 min. The fixed cells were washed twice with PBS and incubated in RNase A (Sigma) solution for 30 min. The cells were then washed with PBS, and resuspended in 1 ml of PBS containing 50 μg/ml propidium iodide (PI) (Sigma) for 15 min, and were analyzed by one-parameter flow cytometry (Becton Dickinson, San Jose, CA, USA).

TUNEL assay. To detect apoptosis, an Apop-Tag Peroxidase kit (Oncor, Gaithersburg, MD, USA) was used. The osteosarcoma cells were seeded at 5x10^3 cells per 100 mm dish. After 48 h, these cells were treated with 5 or 20 μg/ml CDDP for 1 h, washed twice with PBS, and incubated in medium with or without 1 mM caffeine for 72 h. Each sample was harvested, fixed in 70% ethanol and embedded in paraffin. The paraffin sections were de waxed, rehydrated in graded alcohols, washed with distilled water, and digested with proteinase K (Sigma). After the addition of an equilibration buffer, the sections were incubated in working-strength terminal deoxynucleotidyl transferase (TdT) enzyme containing deoxyuridine-5'-triphosphate-digoxigenin at 37˚C for 1 h. The reaction was stopped with a prewarmed stop/wash buffer. After washing in distilled water, an anti-digoxigenin antibody fragment carrying a conjugated peroxidase was applied for 30 min at room temperature in a humidified chamber. The localized peroxidase enzyme catalytically generated an intense signal from 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Sigma) (26). The apoptotic indices were calculated as the percentage of TUNEL positive cells relative to the total number of cells. The significance of the differences was calculated using the unpaired Student’s t-test. A p-value <0.05 was considered significant.

cDNA macroarray analysis. The osteosarcoma cells were exposed to CDDP with or without the subsequent addition of caffeine for 48 h. The total RNA was isolated using an RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s recommended protocol. The RNA (3-4 μg) was converted to cDNA and labeled with 32P-dATP using a Clontech cDNA array labeling kit (Clontech Laboratories Inc., Palo Alto, CA, USA). The Atlas Human cDNA Expression Array (Clontech) was used to characterize gene expression. The 32P-labeled cDNA was hybridized to a nylon membrane with immobilized cDNAs for different genes, each under strict transcriptional control. Hybridizations and washes were performed following the manufacturer’s recommended protocol with minor modifications. Hybridization images were obtained using a phosphoimager (BAS-5000 Bio-imaging Analyzer, Fuji, Kanagawa, Japan). The expressions of 1,176 genes were evaluated for each sample (Atlas Human Cancer 1.2K Array, Clontech). Gene expression analyses were performed using Atlas image analysis software (Clontech). The hybridization signals were normalized to those of the housekeeping genes including GAPDH. To select differentially expressed genes from the macroarray data, the cut-off limit was set as a three-fold change. A gene was regarded as significant if its expression changed at least three-fold for all three time-points.

Real-time RT-PCR. Based on previously published studies and database information, we focused on five genes which were reported to be involved in apoptosis or stress response. The expressions of these genes were also examined by real-time RT-PCR in the OST and OST/R cells. The total RNA was collected from the cell lines treated with 5 μg/ml CDDP for 1 h with or without subsequent addition of 1 mM caffeine for 0, 24 and 48 h, respectively. TaqMan MGB probes and primers (Assay-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA, USA) were used for analysis with the ABI Prism 7000 Sequence Detection System (Applied Biosystems). The following probes and primers were used for real-time PCR analysis: GADD153 (assay ID, Hs00358796_g1), E1AF (assay ID, Hs00383361_g1), LEU13 (assay ID, Hs01652522_g1), IFI27 (assay ID, Hs00271467_m1) and GIP3 (assay ID, Hs00242571_m1). The significance of differences was calculated using the unpaired Student’s t-test. A p-value <0.05 was considered significant. The data are presented as the mean ± SEM of three independent experiments.
Results

Cytotoxicity assay. Based on the cytotoxicity assay of sensitivity to CDDP and the synergistic effect of caffeine, the five osteosarcoma cell lines were grouped into three types with different cytotoxicity patterns (Figure 1). The type A group, including HOS and Saos2, were sensitive to CDDP but there was no significant difference in the lethal effects of CDDP with and without subsequent addition of caffeine. The type B group included OST and MG63. While the sensitivities to CDDP were low, the lethal effect was strongly augmented by the combination with caffeine. The type C group, the OST/R cells, was resistant to not only CDDP, but also CDDP + caffeine.

Flow cytometry. Hypodiploid subpopulations containing apoptotic cells were visible to the left of the G0/G1 cell cycle regions (sub-G1 peak) in the OST cells treated with CDDP combined with caffeine (Figure 2). When the OST cells were treated with CDDP alone, there was a slight change in the M3 component of the cell cycle phase compared to that in the control, but there was no significant increase in the hypodiploid subpopulation.

TUNEL assay. In the OST cells, the apoptotic indices increased following the exposure to CDDP with or without caffeine compared with the control. While the percentage of TUNEL-labeled cells was 7.0% when treated with CDDP alone, it was 46% when treated with the combination of CDDP and caffeine ($p<0.05$). In the HOS cells, the percentage of TUNEL-positive cells increased following exposure to CDDP with or without caffeine compared with the control. The percentage of TUNEL-positive cells was 23% when treated with CDDP alone, and 30% when treated with the combination of CDDP and caffeine, showing no significant difference between the two.

cDNA macroarray. To compare patterns of gene expression profiles based on the effects of adding caffeine, the HOS and OST cells were examined as representatives of type A and type B as defined in the cytotoxicity assay, respectively. The HOS cells were exposed to 20 μg/ml CDDP, and the OST cells exposed to 5 μg/ml CDDP for 1h with or without the subsequent addition of 1 mM caffeine for 48 h. The concentrations of CDDP were determined by the results of the cytotoxicity assay showing inhibition of cell survival by 50% 72 h after the addition of caffeine compared with the untreated controls. The cDNA array analyses showed eight genes that were up-regulated and four that were down-regulated only in the OST cells, but not in the HOS cells, by the subsequent addition of caffeine (Figure 3) (Table I).
Real-time RT-PCR. Two of the up-regulated genes, GADD153 (DNA-damage-inducible transcript 3) and E1AF (Ets variant gene 4), potentially involved in the enhancement of apoptosis and three of the down-regulated genes, LEU13 (interferon induced transmembrane protein 1, IFITM1), IFI27 (interferon, alpha-inducible protein 27) and G1P3 (interferon, alpha-inducible protein, IFI-6-16), known to be interferon (IFN)-inducible genes involved in resistance to anticancer drugs from the database were focused on. The expressions of these five genes were examined in detail by real-time RT-PCR.

Of these genes, up-regulation of GADD153 and E1AF at 48 h was confirmed by real-time RT-PCR in the OST cells, but not in the OST/R cells. Down-regulation of LEU13 and IFI27 was also confirmed (Figure 4). In the OST cells, the GADD153 gene was remarkably up-regulated 24 and 48 h after initiation of caffeine exposure compared with that in the cells in the absence of caffeine. In the OST/R cells, this gene was gradually up-regulated without caffeine after 24 and 48 h and there was no significant change induced by the addition of caffeine. The E1AF gene was also remarkably up-regulated 24 and 48 h after the initiation of caffeine exposure compared with that in the absence of caffeine in the OST cells. In the OST/R cells, this gene was up-regulated only 48 h after the initiation of caffeine compared with the absence of caffeine. However, the LEU13 gene was remarkably up-regulated without caffeine, but significantly down-regulated by the addition of caffeine after 48 h in the OST cells. In the OST/R cells, this gene was significantly up-regulated after 48 h with the addition of caffeine. The IFI27 gene was remarkably down-regulated 24 and 48 h after the initiation of caffeine exposure compared with the absence of caffeine in the OST cells. This gene was up-regulated after 24 h with caffeine exposure compared with that in the absence of caffeine in the OST/R cells. The G1P3 gene tended to be down-regulated 48 h after initiation of caffeine exposure compared with the absence of caffeine in the OST cells. There was no difference between the presence and absence of caffeine in the OST/R cells.

Following the addition of only 1mM caffeine for 24 and 48 h without CDDP, there were no apparent changes in the expressions of these five genes compared to those in the controls in any cell line (data not shown).

Discussion

In the present study using five osteosarcoma cell lines with varying p53 status simulating clinical osteosarcomas, the results of cytotoxic assays further supported the absence of a relationship between CDDP sensitivity and p53 status of osteosarcoma cells. Regarding the relationship between the synergistic effect of caffeine and p53 status, several earlier studies reported that caffeine abrogated G2/M arrest and induced radio-sensitization much more effectively in cells lacking normal p53 function. This has been demonstrated convincingly by studies examining the radio-sensitization of paired cell lines that differed in p53 status (27-29). Bache et al. reported that caffeine-mediated radio-sensitization of human sarcoma cell lines with a p53 mutation was linked to a complete prevention of irradiation induced G2/M arrest, but without induction of apoptosis (30, 31). These points are of particular interest because ATM and ATR appear to be essential upstream activators of p53, as well as of the p53-independent pathways that inhibit cell division cycle 2 (Cdc2). Clifford et al. found that p53 is still active in cells with wild-type p53 under the concentration of caffeine needed to abrogate the G2 arrest in cells lacking p53, and that a higher concentration of caffeine was needed to inactivate p53 (32). Yazlovitskaya et al. reported that caffeine inhibited cisplatin-induced ATR activation, but had only a modest effect on phosphorylation of p53 at serine 15, and on total p53 protein accumulation in an ovarian epithelial adenocarcinoma cell line (33). Little is known, however, concerning the relationship between p53 status and the synergistic effect of caffeine in osteosarcoma, either in vitro or in vivo. The p53 activation after DNA damage may occur by ATR and ATM-dependent or -independent mechanisms, or by a combination of both. The present study
did not show any correlation between synergistic effects of caffeine and p53 gene status.

The three cytotoxic patterns demonstrated in the present study can be applied to the clinical responses of osteosarcomas to our caffeine potentiated chemotherapy. For patients in the Type A, osteosarcoma cells are primarily sensitive to CDDP and clinical and histological responses are good with or without caffeine. For patients in the Type B, osteosarcoma cells are less sensitive to CDDP and responses are not good without caffeine. However, the subsequent addition of caffeine strongly sensitizes the cells to CDDP, leading to a good response. In patients in the Type C, osteosarcoma cells are resistant to CDDP and responses are not improved even with caffeine.

GADD153, a member of the CCAAT (cytidine-cytidine-adenosine-adenosine-thymidine) /enhancer-binding protein family of transcription factors, is transcriptionally activated and is highly expressed following treatment of cells with anticancer drugs including paclitaxel, CDDP, and \textit{N}-(4-hydroxyphenyl)retinamide (4HPR) (34-36). Several recent studies have suggested that the increased GADD153 gene expression caused by various inducers of apoptosis somehow triggers the critical early events leading to the initiation of apoptosis (36, 37). Furthermore, a good correlation between increases in GADD153 mRNA levels and the chemoresponse rates of CDDP in head and neck carcinomas has been shown (38), and the introduction of the GADD153 gene into gastric cancer cells increased their sensitivity to

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**Figure 3.** Scanned phosphoimages of cDNA macroarrays from the OST cells induced by CDDP with or without subsequent addition of caffeine. The images were aligned to show visually apparent changes in gene expressions. Circles indicate genes up-regulated by the addition of caffeine. Dashed circles indicate genes down-regulated by the addition of caffeine.
anticancer drugs (39). Thus, the sum of these findings suggests that the GADD153 gene is an important target gene for apoptotic cell death induced by anticancer agents. The induction of the GADD153 gene by anticancer agents has been reported to occur in a p53-independent fashion (40). In our study, expression of GADD153 was strongly enhanced by the use of caffeine in the OST cells. The increased GADD153 may induce tumor cells to apoptosis through a p53-independent pathway and contribute to the synergistic antitumor effect of caffeine.

The human polyomavirus enhancer activator 3 (PEA3) homologue, E1AF, shares a 94% homology with PEA3 and has an identical E twenty six (Ets) domain (41, 42). PEA3 overexpression has been reported in primary and metastatic lesions of mouse mammary carcinoma (43). PEA3 stimulated human epidermal growth factor receptor 2 (HER2/neu) gene transcription by binding at the Ets site of the HER2/neu promoter (44) and PEA3 protein expression predicted worse overall survival in breast carcinoma (45). Conversely, PEA3 expression suppressed HER2/neu expression in human breast and ovarian carcinoma (46) and mediated apoptosis in an HER2/neu overexpressing breast carcinoma cell line (47). Guerra-Vladusic et al. reported that constitutive expression of heregulin in SKBr3 cells induced up-regulation of PEA3 mRNA levels and down-regulation of erbB-2 and erbB-3 receptors tyrosine kinases, which promoted a decrease in extracellular signal-regulated kinase (ERK) and increase in c-Jun N-terminal kinase (JNK) activity, all of which resulted in cell growth inhibition and apoptosis (47). Menendez et al. reported the antitumoral actions of the anti-obesity drug orlistat in breast cancer cells (48). In that study, a significant accumulation of PEA3 was observed and HER2/neu promoter activity was inhibited in the orlistat-treated SK-Br3 cells. The HER2/neu promoter activities in the OST cells are not clear, but induction of E1AF was enhanced more significantly in the OST cells than in the OST/R cells by the addition of caffeine. Thus, increased E1AF expression may inhibit HER2/neu expression, leading to apoptosis.

In this study, several IFN-inducible genes tended to demonstrate the same expression patterns. Bani et al. reported that the expression of IFI27, G1P3 and IFITM1 were reduced 24 h after paclitaxel treatment of responsive ovarian carcinoma xenografts (49). These three genes were up-regulated in the tamoxifen-resistant mammary carcinoma xenograft model as compared with the sensitive model and up-regulated after treatment with tamoxifen (50). Tahara et al. also found that a gastric cancer cell line that does not express detectable G1P3 was sensitive to apoptosis induced by cycloheximide and 5-fluorouracil (51). These findings support the possible involvement of these IFN-inducible genes in resistance to anticancer agents, but the biological functions of these genes, including LEU13 and IFI27, remain unknown. G1P3 protein is known to be localized at the mitochondria and is thought to have an anti-apoptotic function through inhibition of the depolarization of the mitochondrial membrane potential and release of cytochrome c (51). In the present study, the OST cells were classified as Type B, which were less sensitive to CDDP. In addition to LEU13, IFI27 was up-regulated and G1P3 showed a tendency toward up-regulation after exposure to CDDP without caffeine. These findings were in accord with the previous reports (50). Although these three genes were up-regulated or tended toward up-regulation after the addition of caffeine in the OST-R cells, they were significantly down-regulated or tended toward down-regulation in the OST cells.

### Table I. Differentially expressed genes by exposure to CDDP with or without caffeine in the OST, but not in the HOS cells.

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<th>Accession no.</th>
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Figure 4. Expressions of five genes, GADD153 (DNA-damage-inducible transcript 3), E1AF (Ets variant gene 4), LEU13 (interferon induced transmembrane protein 1, IFITM1), IFI27 (interferon, alpha-inducible protein 27) and G1P3 (interferon(IFN) in the OST and OST/R cells exposed to CDDP with and without subsequent addition of caffeine examined by quantitative real time RT-PCR. The data were normalized using GAPDH. Control, untreated; CDDP 1h, treated with CDDP alone for 1h; ±caf 24 h and ±caf 48 h, open bars, treated with CDDP for 1 h without subsequent addition of caffeine for 24 h and 48 h and black bars, treated with CDDP for 1 h with subsequent addition of caffeine for 24 h and 48 h. Data are expressed as the mean ± SEM of three independent experiments. *p<0.05, **p<0.01.
These results indicated that some resistance mechanisms following chemotherapy by these IFN-inducible genes especially through anti-apoptotic functions were inhibited by the subsequent addition of caffeine.

In summary, five osteosarcoma cell lines were grouped into three types according to the sensitivity to CDDP and synergetic effect of caffeine. Gene expression profiling analysis for the OST cells suggested that several genes involved in apoptosis were up-regulated and those involved in resistant systems were down-regulated by caffeine in a time-dependent manner. Considering the cytotoxicity patterns of caffeine-potentiated chemotherapy in OST cells (Type B) with wild-type p53, early alterations in the expression levels of these five genes appear more likely to participate in signaling pathways that lead to the abrogation of G1/S or G2/M arrest and p53-dependent apoptosis by the addition of caffeine. Our findings contribute to a better understanding of the mechanisms underlying the molecular events induced by caffeine-potentiated chemotherapy and provide important clues for further investigation. Furthermore, novel molecular targets for the treatment of osteosarcoma and candidate genes for an assay of responsiveness to caffeine-potentiated chemotherapy in osteosarcoma patients have been identified.

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