Genes regulated by hepatocyte growth factor as targets to sensitize ovarian cancer cells to cisplatin

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Abstract

Advanced ovarian cancers are initially responsive to chemotherapy with platinum drugs but develop drug resistance in most cases. We showed recently that hepatocyte growth factor (HGF) enhances death of human ovarian cancer cell lines treated with cisplatin (CDDP) and that this effect is mediated by the p38 mitogen-activated protein kinase. In this work, we integrated genome-wide expression profiling, in silico data survey, and functional assays to identify transcripts regulated in SK-OV-3 ovarian cancer cells made more responsive to CDDP by HGF. Using oligonucleotide microarrays, we found that HGF pretreatment changes the transcriptional response to CDDP. Quantitative reverse transcription-PCR not only validated all the 15 most differentially expressed genes but also confirmed that they were primarily modulated by the combined treatment with HGF and CDDP and reversed by suppressing p38 mitogen-activated protein kinase activity. Among the differentially expressed genes, we focused functional analysis on two regulatory subunits of the protein phosphatase 2A, which were down-modulated by HGF plus CDDP. Decrease of each subunit by RNA interference made ovarian cancer cells more responsive to CDDP, mimicking the effect of HGF. In conclusion, we show that HGF and CDDP modulate transcription in ovarian cancer cells and that this transcriptional response is involved in apoptosis regulation. We also provide the proof-of-concept that the identified genes might be targeted to either increase the efficacy of chemotherapeutics or revert chemotherapy resistance. [Mol Cancer Ther 2006;5(5):1126–35]

Introduction

Drug resistance remains a major clinical challenge for cancer treatment. Patients suffering from advanced ovarian carcinoma are in most cases initially responsive to the combined cytoreductive and chemotherapy treatment. However, they later experience disease relapse due to eventual tumor recurrence and emergence of drug-resistant tumor cells. There are several mechanisms by which tumor cells resist to cytotoxic agents (1, 2). One is related to resistance to apoptosis, a type of cell death that is triggered in response to common chemotherapy and radiotherapy regimens. Evasion from apoptosis is also one of the fundamental hallmarks of cancer (3). A corollary of this is that if the apoptotic pathway inactivated in tumor development is the same as, or overlaps, that leading to cell death by drug, most cancers would be expected to be resistant to drug-induced apoptosis from their onset. Therefore, therapeutic interventions that can lower the threshold for apoptosis of tumor cells could become useful approaches to treat cancer when used either as a single agent or in combination with other therapeutic modalities (4).

In ovarian cancer, apoptosis is impaired by the anti-apoptotic action of several oncoproteins, which include growth factor receptors and other kinases that transduce signals from the membrane to the nucleus (5–7). We have shown recently (8) that the hepatocyte growth factor (HGF) sensitizes several ovarian carcinoma cell lines to cisplatin (CDDP) and Taxol, commonly used as first-line chemotherapy in advanced ovarian cancer. HGF increases cancer cell death at very low drug doses. This was a novel and surprising finding. HGF elicits a distinctive biological program known as “invasive growth” by orchestrating cell survival, proliferation, and motility through activation of its receptor, encoded by the MET oncogene, and of several downstream signaling pathways, such as the extracellular signal-regulated kinase 1/2 mitogen-activated protein kinases (MAPK) and phosphatidylinositol 3-kinase/AKT and the AKT substrate mammalian target of rapamycin (9, 10). Nevertheless, we have shown that activation of these survival pathways does not hinder the
ability of HGF to enhance drug-dependent apoptosis in ovarian cancer cells. In these cells, HGF simultaneously activates the p38 MAPK, which is further activated by drugs and leads to cell death (11).

As we have shown that in ovarian cancer cell lines HGF-dependent sensitization to drugs required long-term exposure (8), we hypothesized that HGF effect could be coupled to transcriptional regulation of apoptosis-related genes. To explore this possibility, we have studied the transcriptional targets of HGF and CDDP in an ovarian cancer cell line. The identified molecules were sought as regulators of ovarian cancer cell apoptotic death and can be prospectively viewed as targets for therapy. Here, we show the identification of transcripts modulated in cells committed to CDDP-induced apoptosis by HGF. Among them, we found subunits of protein phosphatase 2A (PP2A) that we targeted to enhance ovarian cancer cell response to the drug.

Materials and Methods

Flow Cytometry Analysis of Apoptosis Induction

Experiments were done on SK-OV-3 ovarian carcinoma cells, which showed resistance to platinum drugs (12). Exponentially growing cells were cultured for 48 hours in the presence of pure recombinant HGF (R&D Systems, Minneapolis, MN) at the concentration of 50 ng/mL or control medium. Apoptosis was then induced by adding fresh medium, with or without HGF, supplemented with 10 μmol/L CDDP. Flow cytometry recordings of several independent apoptotic changes were done by single-tube analysis as described (8, 13).

Microarray Sample Preparation

Total RNA was extracted and purified from SK-OV-3 cell lines at each time point using the Concert Cytoplasmic RNA Purification Reagent (Invitrogen, Carlsbad, CA) as suggested by the manufacturer. RNAs were then quantified and inspected by Bioanalyzer (Agilent Technologies, Waldbrum, Germany) analysis. cRNAs were generated and hybridized on 16 HGU133a Affymetrix (Santa Clara, CA) DNA chips according to the Affymetrix protocol. The chips were scanned with a specific scanner (Affymetrix) to generate digitized image data files.

Microarray Data Analysis

Two of the time-course experiments, done independently (TR5 and TR7), were analyzed. From digitized image data files of raw data, background-normalized image data (CEL files) were generated. Microarray quality control and statistical validation was done using Bioconductor (14). The presence of hybridization/construction artifacts was suggested by the manufacturer. RNAs were then quantified and inspected by Bioanalyzer (Agilent Technologies, Waldbrum, Germany) analysis. cRNAs were generated and hybridized on 16 HGU133a Affymetrix (Santa Clara, CA) DNA chips according to the Affymetrix protocol. The chips were scanned with a specific scanner (Affymetrix) to generate digitized image data files.

Coexpression Analysis

The coexpression analysis was done essentially as described (20). Briefly, we downloaded all the human cDNA microarray experiments deposited in the Stanford Microarray Database (4,129 experiments on May 2005) and identified the probes corresponding to the 15 top ranked differentially expressed genes. For each probe, we then calculated the Pearson correlation coefficient with each probe in the database normalized for the number of common experiments. The normalized Pearson coefficient was then used to rank all the probes in the database, and the top 1% of the ranked database was selected as the list of coexpressed probes. We chose a 1% cutoff as we showed previously that on average most of the biologically significant coexpression is concentrated in this interval (20). We thus obtained a total of 89 coexpression lists that were merged to evaluate the recurrence of probes representing the 334 differentially expressed genes. The observed frequency was then compared with that expected by chance, and an overrepresentation P was calculated (χ² test).

Quantitative Reverse Transcription-PCR

cDNA was synthesized from 1 μg RNA using MMLV-RT(H−) enzyme (Promega, Madison, WI). Quantitative reverse transcription-PCR (RT-PCR) was done on an ABI PRISM 7900HT Sequence Detection System (PE Biosystems, Foster City, CA) in 384-well plates assembled by Biorobot 8000 (Qiagen, Hilden, Germany) using a final volume of 20 μL. All quantitative RT-PCR (qPCR) mixtures contained 20 ng retrotranscribed RNA, 1× SYBR Green PCR Master Mix (2×, Applied, Foster City, CA), and 150 μmol/L of each target-specific primer. The primer sequences are reported in Supplementary Table S1B. The expression of each

6 http://www.tigr.org/software/

7 Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).
target genes was evaluated using a relative quantification approach (ΔΔCt method; ref. 21) with cyclophilin A as internal reference. The consistency of cyclophilin A (NM_021130) was assessed in a study aimed at defining the most stable expressed genes of seven putative reference genes. This study consisted of qPCR of the seven genes on the same samples (data not shown) followed by analysis with the genNorm software (22).

Short Hairpin RNA Stable Expression

SK-OV-3 and amphoteric phoenix retroviral producer cells were both from American Type Culture Collection (Manassas, VA). For production of retroviral stocks, phoenix cells were seeded at 4 × 10⁶ per 10-cm dish and transfected using CaPO₄ precipitation of 15 μg pRetroSuper-short hairpin RNA (shRNA) plasmid DNA. The following sequences were subcloned: PPP2R1B (NM_002716) AGGAGTTATTAGGGGGTTAA. Forty-eight hours after infection, cells were selected using 1 μg/mL puromycin; typically 95% to 100% of the cells were infected and drug resistant.

Results

HGF Sensitizes Ovarian Cancer Cells to CDDP-Dependent Apoptosis via the p38 MAPK

In response to CDDP, the SK-OV-3 ovarian cancer cells enter the apoptotic program as assessed using a multiparametric cytofluorimetric assay (8, 13), which simultaneously measures hallmarks of early, intermediate, and late phases of apoptosis: decrease of mitochondrial membrane potential, cell shrinkage, increase in granularity, exposure of phosphatidylserine at cell surface, and plasma membrane permeabilization to propidium iodide. We showed previously that SK-OV-3 cells express the receptor for the HGF encoded by the MET oncogene, which is activated by HGF (8, 23). As shown in Fig. 1A, a 48-hour long pretreatment with 50 ng/mL HGF caused a marked sensitization to the subsequent treatment with 10 μmol/L CDDP, with a net increase of the measured apoptotic features after 48 to 72 hours. Notably, this sensitization required pretreatment with HGF, as its coincubation with the drug was ineffective (Fig. 1A). These data confirmed what we observed previously (8) and established the time course of the increased death of SK-OV-3 cells caused by the combined treatment with HGF and CDDP.

We have also observed previously that either HGF or CDDP, alone or in combination, activate the p38 MAPK (11), which is activated by cell stress and is involved in cancer cell apoptosis induced by several chemotherapy agents, including platinum drugs and taxanes (24, 25).

We stably expressed a dominant-negative form of p38 MAPK (DN-p38) using a lentiviral vector carrying the double T180A/Y182F Flag-tagged p38 MAPK mutant (11). Lentiviral vectors drive the stable transgene random integration in cell genomic DNA, thus allowing repetitive studies of the bulk unselected cell population. We transduced SK-OV-3 and assessed using Western blot and fluorescence-activated cell sorting analysis with anti-Flag antibodies (data not shown) that ~90% of cells expressed the DN-p38. We showed previously that the DN-p38 MAPK abolished the wild-type p38 MAPK activation without interfering with other HGF-dependent signaling pathways (11). Remarkably, we found that the inhibition of the p38 MAPK pathway almost abolished apoptosis elicited by CDDP and enhanced by HGF in SK-OV-3 cells (Fig. 1B). These data indicate that p38 MAPK activation is required and has a central role in the induction and modulation of apoptosis driven by CDDP and enhanced by HGF in ovarian cancer cells. Thus, SK-OV-3 cells stably expressing the DN-p38 have been used as a model to establish a functional link between cell transcriptional response to HGF and CDDP and induction of apoptosis (see below).

HGF Modulates Transcription in Ovarian Cancer Cells Committed to CDDP-Dependent Apoptosis

As pretreatment is required to obtain maximal sensitization of ovarian carcinoma cells to CDDP, we assumed that...
HGF mainly enhances the apoptotic response by modulating gene transcription. Thus, we studied the expression profiles of ovarian carcinoma cells treated with HGF and CDDP alone or in combination. To perform genome-wide profiling, we first used microarray experiments and then validated the differentially expressed genes using real-time qPCR. As shown in Fig. 1, in both cells treated with CDDP alone or with CDDP and HGF, even the earliest apoptotic changes, such as the decrease of mitochondrial membrane potential, become manifest not earlier than after 48 hours of CDDP exposure (Fig. 1A). Therefore, time-course experiments were done, pretreating SK-OV-3 cells with HGF, exposing HGF-treated cells to CDDP, and preparing RNA after 6, 12, and 24 hours. Control cells were either exposed to control treatment or exposed to HGF alone or CDDP alone for 6, 12, and 24 hours. We analyzed the expression profiles of cells from three independent experiments after confirming apoptosis achievement by a sample aliquot of cells kept in culture for 48 and 72 hours; a representative control experiment is shown in Fig. 1A. Each of the three time-course experiments, called TR5, TR6, and TR7, included eight experimental points as follows: cells treated with control medium for 48 hours (0-hour CDDP), cells treated with HGF for 48 hours (HGF/0-hour CDDP), cells pretreated with control medium and thereafter with CDDP for 6 hours (6-hour CDDP), 12 hours (12-hour CDDP), and 24 hours (24-hour CDDP), and cells pretreated with HGF and thereafter with CDDP for 6 hours (HGF/6-hour CDDP), 12 hours (HGF/12-hour CDDP), and 24 hours (HGF/24-hour CDDP). mRNAs from cells at each of the above points were analyzed. Two of the three time-course experiments (TR5 and TR7) were analyzed using HG-U133a Affymetrix oligonucleotide microarrays. The third experiment (TR6) was added for the further validation of results with qPCR (see below).

Principal component analysis (26) was used to investigate the overall effects of HGF and CDDP in TR5 and TR7 experiments. This technique represents the global gene expression changes between samples as coordinates in a multidimensional space in which the axes are called the "principal components." In TR5 and TR7 experiments, the first and second components account for the greatest part of the variability within the data set (73.5%); thus, the graphical representation of the first two components (Fig. 2) is sufficient to show the differences between samples. Figure 2 shows that HGF pretreatment above 6 strongly influenced the overall gene expression variation, as experiments simply clustered based on this factor (above the dashed line in Fig. 2). Conversely, the effect of CDDP was time dependent, although the two experiments were only partially synchronized.

Therefore, we focused on the subsequent statistical analysis on the identification of probe sets differentially expressed in cells undergoing CDDP-dependent apoptosis as a consequence of the HGF pretreatment. Using linear modeling analysis (18), we identified 406 probe sets associated to the HGF pretreatment. Unsupervised hierarchical clustering of these 406 probe sets (Fig. 3A), corresponding to 334 genes, again showed HGF effect on CDDP-regulated transcripts in both TR5 and TR7 experiments. Subclustering related to the timing of CDDP effect can be also observed (Fig. 3A), more evidently in one cluster (blue bar in Fig. 3A).

For qPCR validation, we selected the 18 probe sets, of 406, which were characterized by the largest differential expression (>2-fold change in both TR5 and TR7 experiments; Fig. 3B). The 15 genes corresponding to these probe sets are listed in Fig. 3B and described in detail in Supplementary Table S1A. Differential expression of all these 15 transcripts was confirmed by qPCR not only in the TR5 and TR7 experiments but also in the TR6 experiment similarly carried out (see above) but not analyzed with microarrays. The qPCR validation of each of the 15 genes is shown in Fig. 4 and Supplementary Figs. S1 and S2. It is noteworthy that all the 15 top ranked differentially expressed transcripts showed a quantitative regulation, which paralleled that found in microarrays. However, by comparing the fold change in expression measured using microarray analysis (Fig. 3B) and that measured using qPCR (calculated by the equation $\Delta C_T$; Fig. 4; Supplementary Figs. S1 and S2), the differential expression came out to be two to four times higher using qPCR probably due to the higher sensitivity of the latter technique. qPCR also confirmed the consistency of transcript regulation in all the three time-course experiments (Fig. 4; Supplementary Figs. S1 and S2).

Furthermore, qPCR allowed a better classification of the 15 top ranked differentially expressed genes. Some were mainly regulated by the combined treatment with HGF and CDDP (e.g., RAMP and RRM2; Fig. 4). Others were already modulated by HGF alone (e.g., MAT2A and EIF5; Fig. 4); in the latter case, in fact, consistent differences in expression were already measured after 48 hours HGF treatment (i.e., before the addition of CDDP; 0-hour point in Fig. 4). Using qPCR, we confirmed that 12 of 15 top ranked genes were down-modulated by CDDP plus HGF (Figs. 3 and 4; Supplementary Figs. S1 and S2). Two of the 15 (CGB and GMDS; Fig. 4; Supplementary Fig. S1) were down-modulated by CDDP but reversed by HGF. Only 1 of 15 genes (KRT14; Fig. 4) was consistently and strongly up-regulated by both HGF alone and HGF plus CDDP. Supplementary Table S2 lists all the genes regulated >2-fold by HGF alone after 48 hours of treatment. This list included the above-mentioned MAT2A and EIF5 and the other genes (KRT14, ASNS, CGB, FBX09, SLCL352, LCMT2, and PHDLA1), which we validated as regulated by HGF in qPCR (Fig. 4; Supplementary Figs. S1 and S2).

Transcriptional Regulation by HGF and CDDP Relies on p38 MAPK Activation

As p38 MAPK regulates several transcription factors (27–30), we asked if HGF and CDDP transcription modulation requires p38 MAPK activation, which mediates their apoptotic effect. The bulk unselected SK-OV-3 cell population, stably expressing the DN-p38, was treated with either CDDP alone or CDDP and HGF, and the 15 top ranked transcripts were quantified using qPCR at each point time after treatment. The actual apoptotic response in the time-course experiments was confirmed on a
sample aliquot of cells kept in culture for 72 hours (Fig. 1B). As shown in Fig. 4 and Supplementary Figs. S1 and S2,7 all the 15 transcripts were differently regulated in cells where p38 MAPK was inactive. In most cases, inactivation of the p38 MAPK reverted the transcript regulation by HGF and CDDP. These data show that the HGF-dependent transcriptional regulation of ovarian cancer cells committed to CDDP-induced apoptosis mostly relies on p38 MAPK activation.

Identification of Genes Coregulated with the Transcriptional Targets of HGF and CDDP

The finding that transcription of 15 genes was consistently modulated by HGF and CDDP and reverted by DN-p38 raised the interesting possibility that they could be part of a wider transcriptional response functionally related to p38-dependent apoptosis. Furthermore, we noticed that the degree of differential expression of the 15 genes assessed using microarray analysis was remarkably lower than that measured with qPCR. Altogether, data suggested that a wider transcriptional response could include other genes possibly underestimated by the microarray analysis. If this was the case, the genes belonging to the group of the 15 top ranked differentially expressed genes, as well as many of the other genes modulated to a lesser extent by the HGF pretreatment, would be expected to undergo significant coregulation under unrelated experimental conditions.

To test this hypothesis, and to identify other genes possibly belonging to the same transcriptional module, we decided to perform a coexpression analysis using a collection of 4,129 cDNA microarray experiments deposited in the Stanford Microarray Database.

In particular, for each of the above 15 genes, we obtained coexpression ranked lists containing the 1% probes of the database showing the highest expression profile similarity (see Materials and Methods; see ref. 20). Consistent with our hypothesis, in these lists, the recurrence of the 15 top ranked genes was much higher than expected by chance (cumulative \( P = 2.7 \times 10^{-6} \), \( \chi^2 \) test), thus ruling out that the expression regulation observed in our settings was merely due to cell death.

We then calculated an overrepresentation \( P \) for each gene in the obtained coexpression lists and used this number as an independent filter to select those genes that, among the 334 differentially expressed genes, were above all recurrent (threshold \( P < 0.01 \)). The selected 94 genes, listed in Supplementary Table S3,7 are the most likely to be important in the functional response induced by the HGF and CDDP combined treatment.

Functional Evaluation of Genes Differentially Expressed in Ovarian Cancer Cells Committed to Apoptosis

We reasoned that genes regulated by HGF and CDDP in cells undergoing apoptosis could be directly targeted to...
modulate ovarian cancer cell response to CDDP. Among
the 94 differentially expressed genes that are also recur-
rently coexpressed with the top ranked 15 (Supplementary
Table S3),7 we focused on two regulatory subunits
(PPP2R1B and PPP2R5E) of the PP2A (described in
Supplementary Table S1A).7 Indeed, their unexpected role
as survival phosphatases had also been suggested by
MacKeigan et al. (31), who carried out a genome-wide
screening of kinases and phosphatases using a small
interfering RNA library. qPCR analysis confirmed that
these PP2A subunits were down-modulated by HGF and
CDDP and that down-modulation was reversed in cells
expressing DN-p38 (Supplementary Fig. S2).7

Therefore, we investigated if down-modulation of either
the PPP2R1B or the PPP2R5E subunit of the PP2A was alone
able to commit ovarian cancer cells to CDDP-mediated cell
death. shRNAs specific for either PPP2R1B or PPP2R5E
were stably expressed in SK-OV-3 cells by retroviral vectors.

After selection and propagation in culture, the bulk
population showed a mean 60% and 70% reduction of the
PPP2R5E and PPP2R1B subunit transcript, respectively, as
assessed with qPCR (data not shown). Figure 5 shows that
lessening this subunit expression did not induce cell death
per se but sensitized ovarian cancer cells to CDDP-mediated
apoptosis. Data show that cells expressing the shRNAs
specific to these PP2A subunits are stably ready to respond
to CDDP. Remarkably, phosphatase subunit knocking
down increased the number of cells in each apoptotic
phase, either early (cells displaying depolarized mitochon-
dria), intermediate (Annexin V–positive cells), or late
(propidium iodide–positive cells), although the intensity
of Annexin V staining was lowered with respect to control
conditions. The latter data indicate that apoptosis signaling
was boosted from its very early steps, but the propagation
of plasma membrane lipid scrambling could not be enhanced
by the absence of phosphatase activity.

**Figure 3.** HGF regulates the differential expression of 334 genes in ovarian cancer cells sensitized to CDDP-dependent apoptosis. Microarray data clustering shows the HGF- and time-dependent transcriptional response of ovarian cancer cells undergoing apoptosis in response to CDDP. SK-OV-3 cells were either not treated or treated with HGF for 48 h before exposure to CDDP for the indicated hours (0, 6, 12, and 24 h). Cells not treated with HGF are indicated as CDDP, whereas cells pretreated with HGF are indicated as HGF/CDDP. All eight experimental points of the TR5 experiment were examined, whereas only six of these conditions of the TR7 experiment were evaluated, as the 6-h treatment with CDDP was not taken into consideration (see Materials and Methods). **A,** gene-oriented unsupervised hierarchical clustering of the 406 probe sets, corresponding to 334 genes, associated to the HGF

**E.G.,** Entrez Gene.
Discussion
In this work, we combined different approaches to identify genes whose expression mediate the ability of HGF to sensitize ovarian cancer cells to drug-induced apoptosis. The aim was to find genes whose modulation was per se able to predispose cells to better respond to drugs. We here provide proof-of-concepts that we have been able to identify genes that might be targeted to increase the response of ovarian cancer cells to CDDP.

We combined *in vitro* and *in silico* analyses to identify the transcripts modulated in cells committed by HGF to respond to CDDP and relying on the p38 MAPK activation as we have shown previously that p38 MAPK mediates HGF and CDDP combined effects (11). The association between each of the most differentially expressed genes and cell death regulation is confirmed by the description reported in Supplementary Table S1A. Genes modulated only in response to the combined treatment with HGF and CDDP, but not by the single treatment, are particularly interesting as they could be better related to HGF-mediated sensitization to CDDP. In fact, this is a distinctive response of ovarian cancer cells as shown in several ovarian cancer cell lines (8, 11). In this group, those above all down-modulated were PANK3, RAMP, RRM2, and TDG. Most of them have been already associated to cell proliferation and apoptosis and even to cancer cell resistance to chemotherapy as described in detail in Supplementary Table S1A (32–35). Among the most differentially expressed genes, we identified a second group regulated by HGF alone and further modulated, in most cases, after cell treatment with CDDP. This group includes MAT2A, ASNS, EIF5, FBX09, LCMT2, SLC35E2, and PHDLA1, which have been associated to cell proliferation and survival, as described in Supplementary Table S1A (36, 37).

![Graphs showing gene expression regulation](image)

**Figure 4.** p38 MAPK activity mediates gene expression regulation by HGF in ovarian cancer cells undergoing CDDP-dependent apoptosis. qPCR validation of 6 of the 15 most differentially expressed genes identified with microarray analysis (the other 9 genes are shown in Supplementary Figs. S1 and S2; available online at http://mct.aacrjournals.org). Expression variations of the genes were measured in wild-type (wt) SK-OV-3 cells pretreated for 48 h either with pure recombinant HGF (■) or with control medium (○) and then cultured with 10 μmol/L CDDP for the indicated times (0, 6, 12, and 24 h). Transcripts were also measured in SK-OV-3 cells stably expressing the DN-p38 MAPK transgene (DN-p38) and similarly treated. ▲, control medium; ▲, + HGF. Points, mean expression variations measured at each time point using mRNAs prepared from three experiments carried out independently; bars, SD. The mean fold change in expression of the target gene in treated cells at each time point versus untreated cells at time 0 was calculated using the formula: \( \Delta C_T = -\frac{(C_{T, target} - C_{T,cyclophilin, Amana})_{time 0}}{(C_{T, target} - C_{T,cyclophilin, Amana})_{time 0}} \).
Data suggest that each of the 15 top ranked genes is pertinent to apoptosis mediated by HGF and CDDP. In fact, the regulation of their expression was reverted by the DN-p38 MAPK, which abolished the HGF-enhanced apoptotic response to CDDP (11). This establishes a functional link between genes regulated by HGF and ovarian cancer cell sensitization to CDDP and allowed the identification of the regulated genes as suitable functional targets. As expected, because each of the 15 top ranked genes responded differently to HGF and CDDP, the reversion induced by the DN-p38 MAPK resulted in different outcome. This further supports the idea that the 15 top ranked genes belong to different transcriptional modules.

The qPCR validation not only fully confirmed the reliability of microarray experiments but also showed that microarray analysis led to underestimate the degree of expression regulation. Thus, we assumed that some of the 334 genes identified by this analysis as modestly differentially expressed might be concurrently and consistently regulated in cells undergoing death. More importantly, we could have missed genes either linked to more physiologic cellular functions than response to drug or involved in important biological processes or more targetable, such as those associated to enzymatic activities. The coexpression test, set up to use the huge number of microarray experiments available online, helped us to refine the selection of HGF-regulated genes. Among the genes that passed this filter, we found two subunits of the PP2A, which were down-modulated in cells treated with HGF and CDDP in a p38 MAPK-dependent way. Repression of these genes sensitized cells to CDDP, mimicking HGF effect and confirming their role as survival phosphatases, in agreement with the results reported in a wide, small interfering RNA–based, screen of human kinases and phosphatases (31).

PP2A is a family of abundantly expressed serine/threonine phosphatases implicated in a multitude of cellular functions (38). The PP2A core structure consists of a catalytic C and a scaffolding A subunits. The core A/C dimer can recruit a third regulatory B subunit. Each PP2A subunit has at least two isoforms. The differential association of all these subunits gives rise to an extensive subset of oligomeric A/B/C holoenzymes, allowing the fine-tuning of PP2A activity and substrate specificity (39). Several lines of evidence concur in classifying the PP2A complex as a tumor suppressor protein. Conversely, as other authors (31), we found that the PPP2R1B and PPP2R5E subunits are “survival” proteins. This is in line with the discovery that many other phosphatases protect cells from death (31) and with other reports that highlight the complexity of the PP2A-regulated activity (40). The PPP2R1B gene down-modulated by HGF and CDDP encodes the A/PR65 PP2A scaffolding subunit. It has been shown recently (41) that whereas suppression of the endogenous PPP2R1A (the α isoform of the same subunit) by 50% leads to cell transformation, further suppression resulted in cell cycle arrest and apoptosis. These findings are also consistent with the demonstration that in rat cells (42) and Droso phila S2 cells (43) a minimal level of PP2A scaffolding A subunit is required for cell survival. Furthermore, we also found the down-modulated PPP2R5E gene, which encodes the B56ε subunit. As each specific B subunit might change PP2A enzymatic activity or even direct each holoenzyme PP2A complex to distinct intracellular location (44), it is not surprising that the reduction of the B56ε subunit alone might sensitize cells to CDDP-induced apoptosis. Interestingly, it has been shown that another noncatalytic PP2A subunit is required to repress apoptosis in murine cells (45).

Here, we describe the transcriptional response of ovarian cancer cells made more susceptible to CDDP-mediated death by pretreatment with HGF. The genes regulated by HGF and the pathways in which they are involved might be envisaged as prospective therapeutic targets. We provide a proof-of-concept, as we made an ovarian cancer cell line more susceptible to CDDP by suppressing the
PP2A subunits, which we found down-modulated by HGF and CDDP in that cell line. Therefore, this work proposes a valuable protocol to identify potential targets that should be validated in several additional settings and possibly tailored to ovarian cancer subsets. We found that most of the genes that we considered most likely to be important in the apoptotic response induced by the HGF and CDDP combined treatment are not reported in the signatures that have been proposed to classify either chemoresistant or chemosensitive ovarian cancers (12, 35, 46–48). This was not surprising, as the steady-state expression level of the identified genes is not expected to mark ovarian cancer cells sensitivity to CDDP. Nevertheless, HGF-regulated genes are of potential value to modify response to CDDP, the most active chemotherapeutic in ovarian cancer treatment. The potential use of low-dose chemotherapy in chemotherapy-sensitive cancers, like ovarian carcinomas, is important, as lower dosage is easily attainable and less likely to cause toxicity in patients. In addition, in ovarian cancer, chemotherapy resistance is the major cause of secondary failure of conventional chemotherapy. Therefore, the discovery of novel pathways and molecules involved in the apoptotic response to drugs is valuable, provided that they are targetable either to kill cells or to revert chemotherapy resistance.

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