analyzed with FACSCalibur and CellQuest software (BD Biosciences). The statistical analysis was performed with ANOVA.

Western blot analysis

Cells were treated with IFNs and the cell lysate was subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. The protein was transferred to a nylon filter and was hybridized with antibodies against p53 (Lab Vision, Fremont, CA, USA), phosphorylated p53 at serine (Ser) 15, Bax, cyclin E, caspase-3, cleaved caspase-3, caspase-8, cleaved caspase-8, caspase-9, cleaved caspase-9, FADD (Cell Signaling, Beverly, MA, USA), p21 (Santa Cruz Biotech, Santa Cruz, CA, USA), p27 (BD Biosciences), or actin (Sigma-Aldrich, St Louis, MO, USA). The membranes were developed with the ECL system (GE Healthcare, Buckinghamshire, UK).

Results

Expression of IFN receptor complexes

We examined receptor expressions of type I and type III IFNs in 5 kinds of human mesothelioma and Met-5A cells with RT-PCR analyses (Figure 1). The type I IFN receptor complex consists of IFNAR-1 and IFNAR-2 molecules and all the cells expressed both receptor genes. In contrast, all the mesothelioma expressed only one of the heterodimeric type III receptor genes, the *IL-10Rβ*, but not the *IL-28Rα*. Immortalized Met-5A cells expressed both of the type III receptor genes. These data collectively suggested that the mesothelioma cells could respond to type I IFNs but not to type III IFNs, whereas Met-5 cells could response to both IFNs.

Growth inhibitory action of IFNs on mesothelioma

We examined cytotoxic activities induced by IFNs with the panel of mesothelioma and Met-5A cells. IFN-α and IFN-β suppressed the viability in a dose-dependent manner, and the suppressive activities of IFN-β were greater with than those of IFN-α (Figure 2A). We confirmed the suppressive actions with Bonferroni multiple testing correction (Table S1). In contrast, IFN-λ1 did not produce growth inhibitory effects in mesothelioma cells, whereas human esophageal carcinoma T.Tn cells, which were positive for both IL-28Rα and IL-10β [17], were sensitive to IFN-λ1 (Figure 2B & Table S1). Growth of Met-5A cells was minimally suppressed with IFN-λ1 at a high dose. The differential susceptibility to type III IFNs, as found in T.Tn and Met-5A cells, suggested that sensitivity to IFN-λ1 was dependent on cell types even though they expressed type III receptor complexes [17]. IFN-α or -β-mediated growth inhibition was also demonstrated by counting live cells (Figure 2C & Table S1). The growth retardation caused by the same unit was greater with IFN-β than IFN-α in any of cells tested. These data demonstrated that type I but not type III IFNs suppressed proliferations of mesothelioma and that IFN-β had the inhibitory activities greater than IFN-α.

Type I IFNs-mediated cell cycle changes

We compared cell cycle changes induced by I IFN-α and -β on mesothelioma and Met-5A cells (Table 1, representative data in Figure 3A). Type I IFNs-treated cells showed increased S- and G2/M-phase populations, and subsequently augmented sub-G1-phase fractions although these cell cycle alterations were dependent on respective cells used. The time-course data suggested that the increase at S-phase preceded that of G2/M-phase. The cell cycle changes, increased S- and G2/M-phase populations and then sub-G1 fractions, were greater with IFN-β treatments than with IFN-α treatments. Cell cycle changes of Met-5A were similar to mesothelioma cells except that Met-5A cells did not show increased sub-G1 populations. We also stained IFNs-treated cells with annexin V or PI, and found that the cells became annexin V-positive but not PI-positive (Table S2). These data suggested that mesothelioma cells were subjected to apoptosis with type I IFNs.

We also examined up-regulated expression levels of class I molecules of the major histocompatibility complex (MHC) in mesothelioma cells (Figure 3B). IFN-α and -β increased the expression in NCI-H2052 and NCI-H226 cells, and the increased levels were greater with IFN-β than with IFN-α in both cells. In contrast, IFN-λ1 did not change the class I expression levels in these cells (data not shown). Compared activities of IFN-α and IFN-β on cell cycle changes and up-regulated expression of MHC class I molecules indicated that IFN-β had stronger biological actions than IFN-α.

Activation of p53 and apoptotic pathways

We further investigated possible p53 activation induced by the type I IFNs in 2 representative mesothelioma cells (Figure 4A & 4B). Cell cycle analyses after IFN-β treatments showed that NCI-H2052 cells increased S- and G2/M-phase fractions but less significantly augmented sub-G1 population, whereas NCI-H28 cells increased S-phase and then sub-G1 fractions (Table 1). The IFN-β treatment induced up-regulated p53 levels and the phosphorylation at Ser 15 residues, a marker of the p53 activation, in both NCI-H2052 and NCI-H28 cells, but these changes were undetectable or minimal in IFN-α-treated cells. IFN-β-treated NCI-H2052 and NCI-H28 cells showed up-regulated levels of p21 and Bax expression. The increased p21 expression was however undetectable in IFN-α-treated cells, and the up-regulated Bax expression was less significant in IFN-α-treated cells than in IFN-β-treated cells. Expression levels of p27 increased in NCI-H28 cells with either IFN-α or IFN-β treatment but those in NCI-H2052 cells remained unchanged.

We examined activations of caspases in IFNs-treated cells (Figure 4C & 4D). Cleavage of caspase-3 was augmented by IFN-β treatments in NCI-H2052 and NCI-H28 cells. Expression levels of cleaved and uncleaved caspase-8 were up-regulated in IFN-β-treated NCI-H28 cells and less significantly in IFN-β-treated NCI-H2052 cells. IFN-α treatments increased caspase-3 cleavage and slightly augmented caspase-8 cleavage only in NCI-H28 cells. Both IFN-β and IFN-α did not induce caspase-9 cleavage. Expression levels of FADD, an upstream molecule of the extrinsic pathway, increased in IFN-β-treated NCI-H28 and NCI-H2052 cells, and in IFN-α-treated
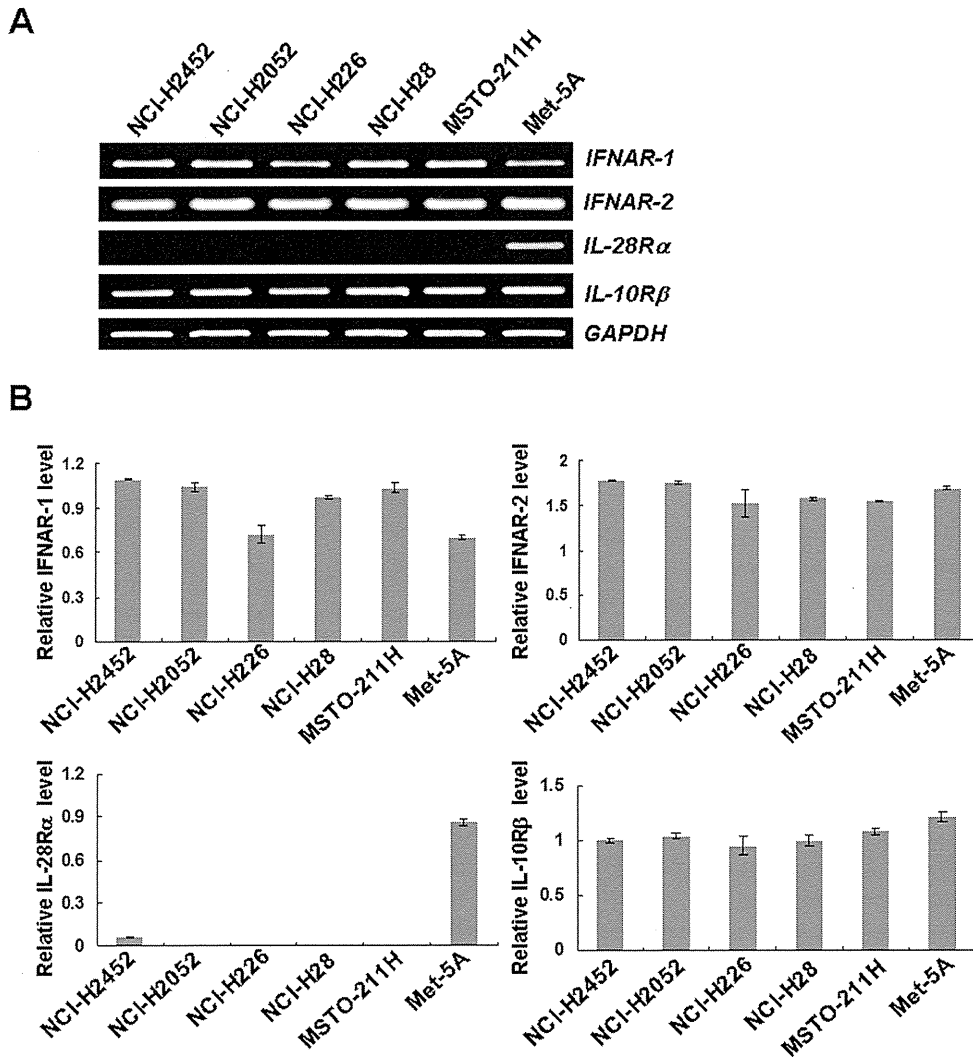
Figure 1. Type I and III IFNs receptors expression. (A) Expressions of *IFNAR-1*, *IFNAR-2*, *IL-28Rα* and *IL-10Rβ* genes were analyzed with RT-PCR. Representative data of 3 independent experiments and GAPDH is shown as a control. (B) Expression levels of *IFNAR-1*, *IFNAR-2*, *IL-28Rα* and *IL-10Rβ* genes were quantified with the ImageJ software (http://rsb.info.nih.gov/ij/features.html) and the respective relative intensities were determined based on the corresponding GAPDH expression level. Average intensities with SEs are shown (n=3).

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NCI-H28 cells. These data showed that the activation was greater with IFN-β than with IFN-α and suggested that IFNs activated the extrinsic apoptotic pathway in mesothelioma. Expression levels of cyclin E, associated with G1- to S-phase progression [18,19], were also up-regulated in IFN-β-treated NCI-H28 and NCI-H2052 cells and in IFN-α-treated NCI-H2052 cells (Figure 4C & 4D).

Combinatory effects of IFN-β and anti-cancer agents

We tested a possible combinatory cytotoxic activity produced by IFN-β and anti-cancer reagents, CDDP and PEM.

Mesothelioma cells were treated with IFN-β and with CDDP or PEM at various concentrations, and the combinatory effects were examined by calculating CI values (Figure 5). The CI values at various Fa points showed that IFN-β and CDDP were synergistic in the inhibitory activity in most of the cells except NCI-H2452 cells at above 0.3 Fa points and NCI-H28 cells at above 0.4 Fa points (Figure 5A). Combination of IFN-β and PEM also produced synergistic effects in all the cells at Fa points between 0.3 and 0.7 except NCI-H226 cells at above 0.6 Fa points (Figure 5B). We also examined percentages of sub-G1-phase fractions induced by the combinatory treatment of

A

B

C
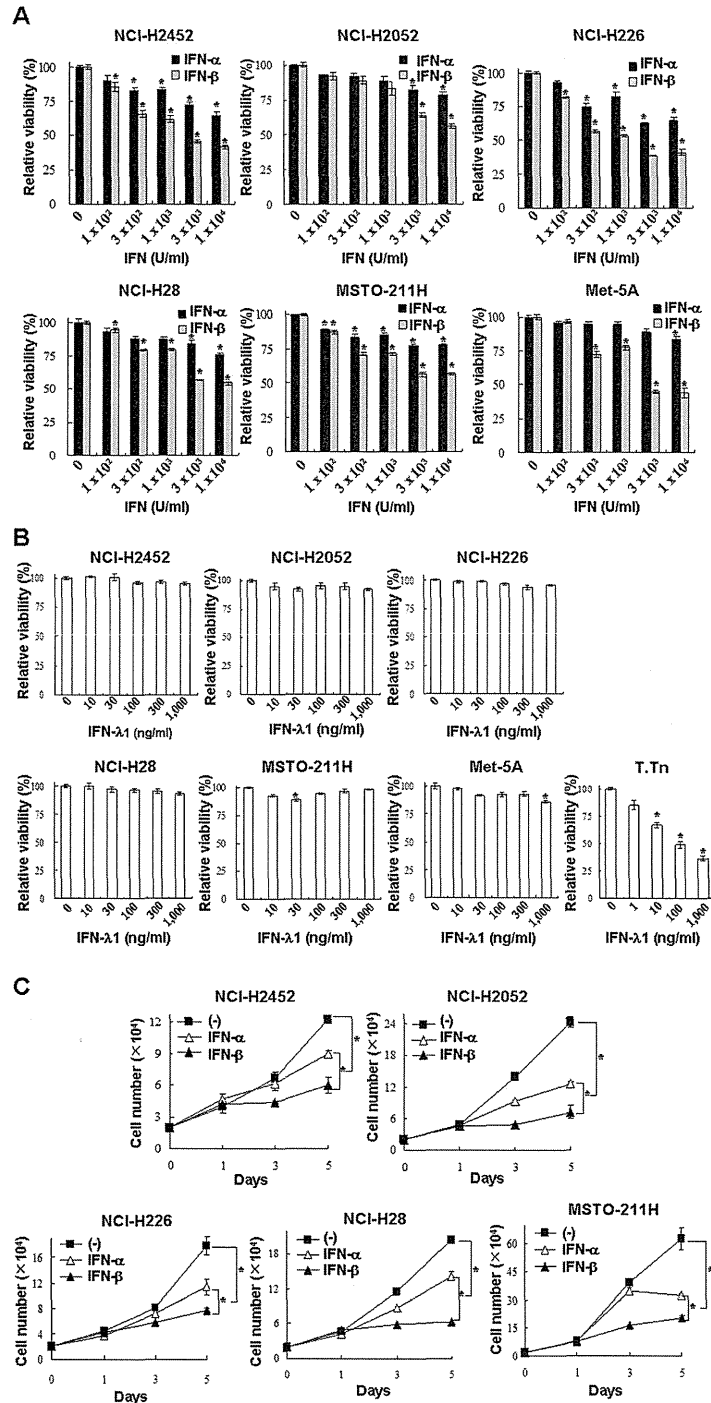
Figure 2. Growth inhibition by Type I and III IFNs. Cells were treated with various dose of IFN-α or -β (**A**), or IFN-λ1 (**B**) for 5 days, and the cell viabilities were measured with the WST assay. The relative viabilities were calculated based on the absorbance without any treatments. Means of triplicate samples and SE bars are shown (n=3). T.Tn cells, sensitive for IFN-λ1-mediated growth inhibition, were used as a positive control. *$P < 0.01$, comparing IFN-treated and untreated cells. (**C**) Cells were also cultured with or without IFN-α or -β (3,000 U/ml), and live cells numbers were determined with a dye exclusion test. Means and SE bars are shown (n=3), *$P < 0.01$.

Table 1. Cell cycle distributions after type I IFNs treatments.

Cell	Treatment	Time (hr)	Cell cycle distribution (%)			
			sub-G1	G0/G1	S	G2/M
NCI-H2452	(-)	48	1.5 ± 0.1	70.9 ± 0.3	10.5 ± 0.3	16.9 ± 0.5
	IFN-α	48	1.9 ± 0.1	61.3 ± 0.3	14.0 ± 0.2*	22.7 ± 0.3*
	IFN-β	48	3.2 ± 0.1*	45.8 ± 0.1	28.8 ± 0.6**	22.4 ± 0.5*
	(-)	72	2.2 ± 0.1	66.1 ± 0.2	12.4 ± 0.1	19.5 ± 0.2
	IFN-α	72	3.1 ± 0.2	55.3 ± 0.3	18.0 ± 0.2*	23.7 ± 0.2*
	IFN-β	72	6.3 ± 0.8*	40.1 ± 0.4	28.8 ± 0.6**	24.9 ± 0.5*
NCI-H2052	(-)	48	0.5 ± 0.1	77.9 ± 0.3	7.6 ± 0.2	14.1 ± 0.2
	IFN-α	48	0.7 ± 0.1	71.9 ± 0.6	10.9 ± 0.4*	16.8 ± 0.2
	IFN-β	48	1.1 ± 0.1	33.6 ± 1.5	36.8 ± 1.4**	29.3 ± 2.8**
	(-)	72	0.9 ± 0.1	86.8 ± 0.1	4.2 ± 0.2	8.3 ± 0.1
	IFN-α	72	0.7 ± 0.1	75.8 ± 0.3	8.7 ± 0.2*	15.1 ± 0.4*
	IFN-β	72	3.2 ± 0.1**	33.6 ± 0.6	37.8 ± 0.6**	26.2 ± 1.3**
NCI-H226	(-)	72	1.9 ± 0.2	73.2 ± 0.2	8.6 ± 0.3	16.1 ± 0.2
	IFN-α	72	5.2 ± 0.2**	66.3 ± 0.3	12.1 ± 0.3**	15.9 ± 0.1
	IFN-β	72	7.8 ± 1.2**	52.4 ± 1.2	20.5 ± 0.5**	18.5 ± 1.3
MSTO-211H	(-)	48	3.9 ± 0.1	63.1 ± 0.2	12.5 ± 0.3	21.1 ± 0.2
	IFN-α	48	5.9 ± 0.3	60.4 ± 0.5	15.1 ± 0.1*	19.4 ± 0.2
	IFN-β	48	9.1 ± 0.3*	51.7 ± 0.7	16.6 ± 0.3*	23.1 ± 0.9
	(-)	72	1.9 ± 0.1	83.7 ± 0.3	5.6 ± 0.2	8.9 ± 0.2
	IFN-α	72	4.6 ± 0.2*	69.5 ± 0.3	9.8 ± 0.2*	16.8 ± 0.4**
	IFN-β	72	16.7 ± 0.1**	44.8 ± 0.3	16.2 ± 0.1*	22.9 ± 0.3**
NCI-H28	(-)	48	1.3 ± 0.1	65.4 ± 0.3	13.6 ± 0.2	19.6 ± 0.3

Table 1 (continued).

Cell	Treatment	Time (hr)	Cell cycle distribution (%)			
			sub-G1	G0/G1	S	G2/M
	IFN-α	48	1.2 ± 0.1	70.5 ± 0.3	11.7 ± 0.2	16.6 ± 0.2
	IFN-β	48	7.2 ± 0.1*	47.4 ± 0.6	26.5 ± 0.6*	18.9 ± 0.3
	(-)	72	0.6 ± 0.1	73.2 ± 0.3	8.3 ± 0.3	17.9 ± 0.1
	IFN-α	72	2.3 ± 0.1*	72.4 ± 0.3	9.8 ± 0.2	15.6 ± 0.5
	IFN-β	72	12.4 ± 1.4*	50.8 ± 0.9	20.1 ± 0.8*	16.6 ± 1.4
Met-5A	(-)	48	3.9 ± 0.9	68.1 ± 0.7	11.2 ± 0.6	16.5 ± 0.9
	IFN-α	48	4.1 ± 0.7	61.9 ± 0.6	15.9 ± 0.2*	17.2 ± 0.5
	IFN-β	48	5.0 ± 0.8	53.2 ± 1.9	22.9 ± 1.0*	17.7 ± 1.6
	(-)	72	4.9 ± 0.7	72.5 ± 0.4	9.1 ± 0.4	13.1 ± 0.1
	IFN-α	72	4.8 ± 0.3	61.0 ± 0.4	14.9 ± 0.1*	18.5 ± 0.6*
	IFN-β	72	5.2 ± 0.8	51.9 ± 0.8	19.4 ± 0.3*	23.2 ± 0.8*

Cells were treated with or without IFN-α or - β (3,000 U/ml) for the indicated periods. Cell cycle profiles were analyzed with flow cytometry, and the percent mean and SE of each fraction are shown (n=3).
* $P < 0.05$, comparing IFN-α- or IFN-β-treated and untreated cells.
** $P < 0.01$, comparing IFN-α- or IFN-β-treated and untreated cells.

IFN-β and CDDP or PEM in NCI-H2052 and NCI-H28 cells (Table 2). The sub-G1-phase populations were greater in cells treated with IFN-β and CDDP or PEM than those treated with IFN-β or the agents alone. In particular, the sub-G1-phase fractions in NCI-H2052 cells treated with IFN-β and CDDP or PEM were significantly great compared with those in I IFN-β-, CDDP- or PEN-treated cells. CDDP or PEM treatments increased S-phase fractions in NCI-H2052 and NCI-H28 cells, but the influence on G2/M-phase populations was variable.

Discussion

In this study, we investigated cytotoxic effects of type I and type III IFNs and compared the biological activities between IFN-α and IFN-β. Moreover, we showed firstly to our knowledge combinatory cytotoxicity of IFN-β with the first-line anti-cancer agents for mesothelioma.

Type III IFNs was recently shown to produce anti-proliferative actions in a number of cancer cells [7,17], but the present study demonstrated that IFN-λ1 did not inhibit growth of mesothelioma cells due to the lack of IL-28Rα expression. Previous studies showed that expressions of type I IFN receptor complexes and IL-10Rβ were ubiquitous but that of IL-28Rα could be restricted in a tissue-specific manner [7]. Interestingly, Met-5A cells of mesothelium origin were positive for IL-28Rα in contrast to mesothelioma cells, suggesting that
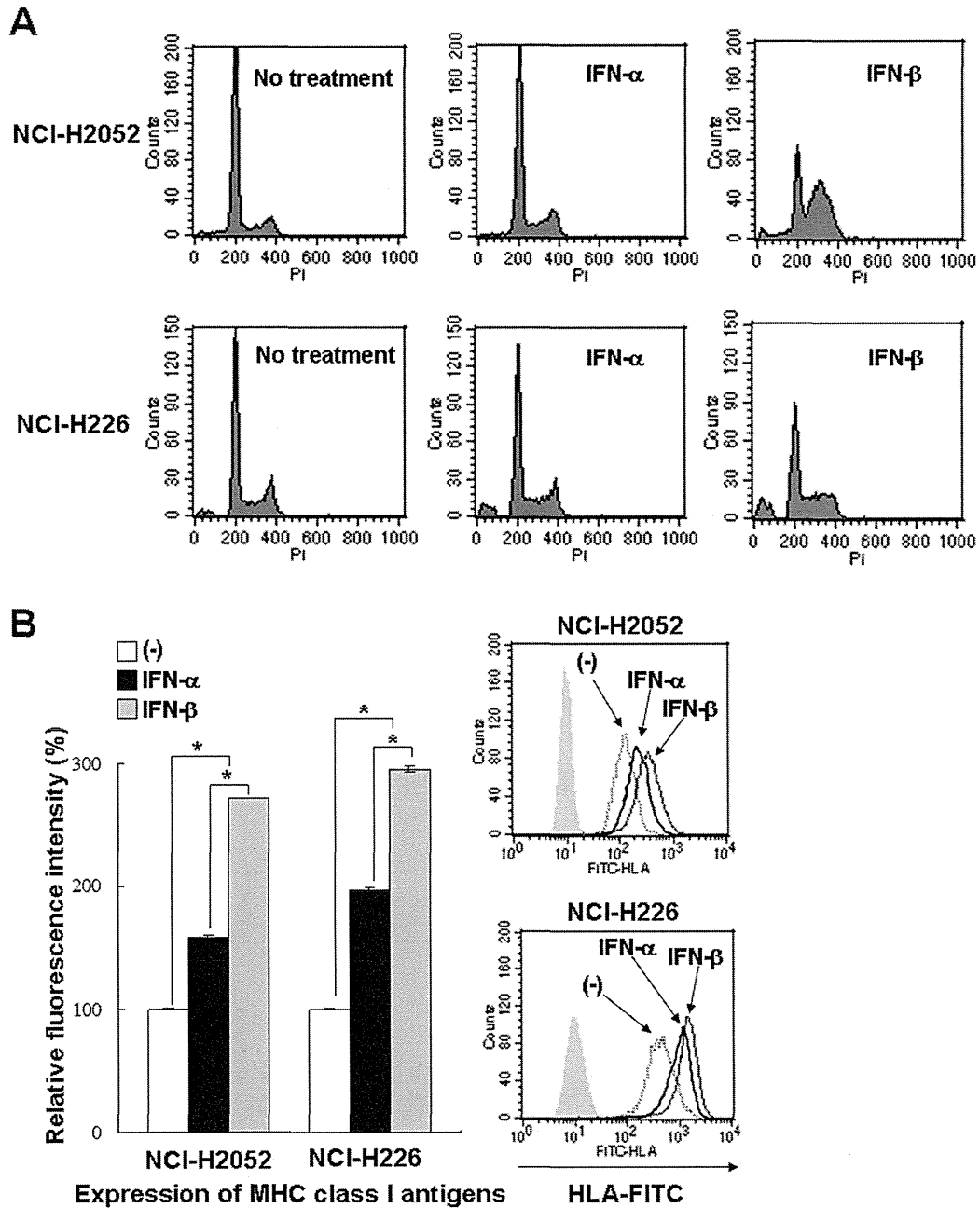
Figure 3. Compared biological activity between IFN-α and IFN-β. (A) NCI-H2052 and NCI-H226 cells were treated with or without IFNs (3,000 U/ml) and cultured for 72 hours. Cells were stained with PI and the cycle cycles were analyzed with flow cytometry. Representative data of 3 independent experiments are shown. (B) Cells were treated with or without type I IFNs (NCI-H2052; 300 U/ml, NCI-H226; 100 U/ml) and cultured for 24 hours. Mean fluorescence intensity of the MHC class I antigens analyzed with flow cytometry and the SE bars are also shown (n=3). *P < 0.01 Representative flow cytometrical data are also shown.

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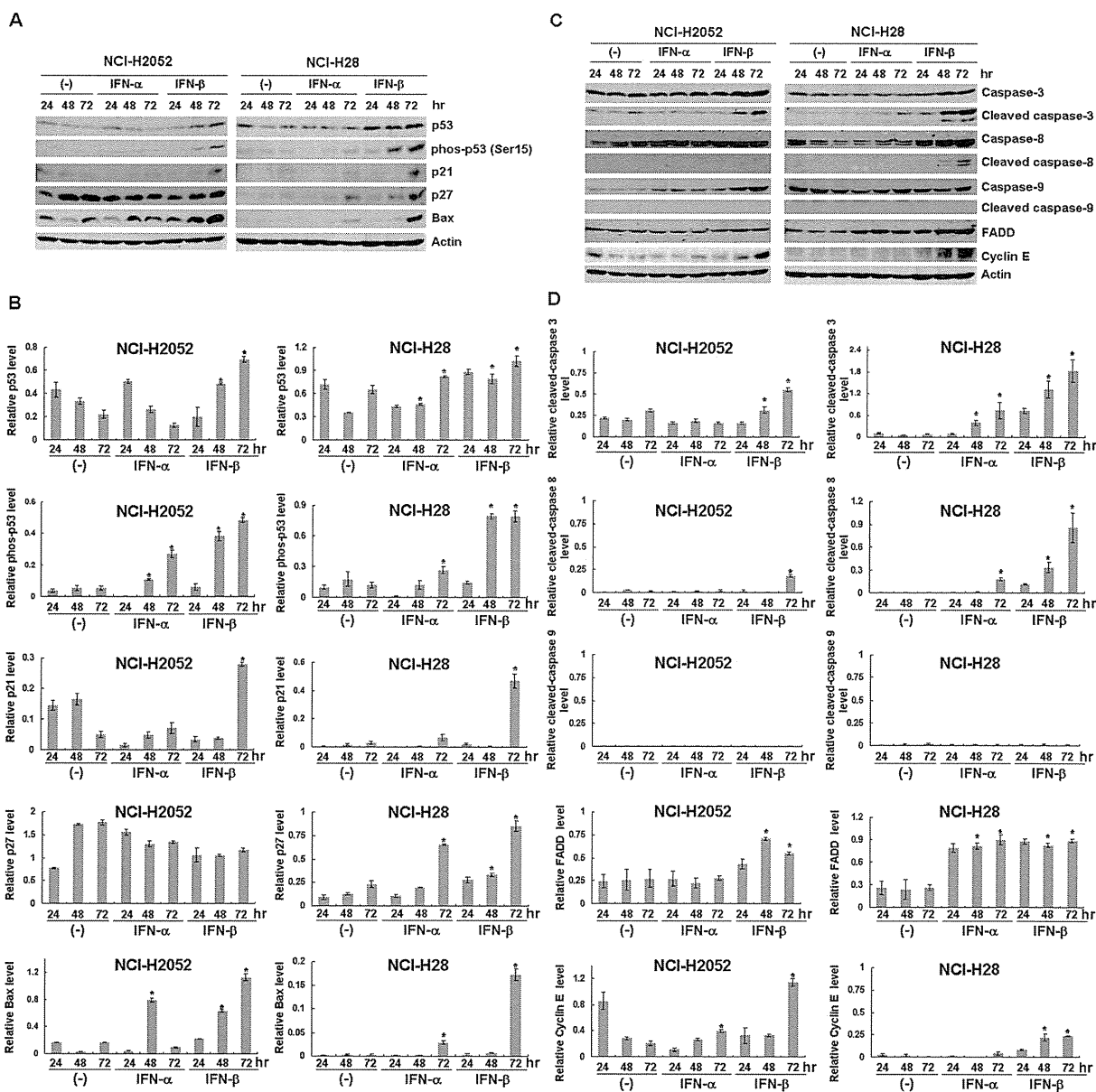
Figure 4. Expression of molecules involved in the p53 pathways, cell cycle and apoptosis. NCI-H2052 and NCI-H28 cells were treated with or without IFNs (3,000 U/ml) and cultured for the indicated time. Expression levels of (**A**) p53-, (**C**) cell cycle- and apoptosis-linked proteins were analyzed with Western blot analyses. Representative data of 3 independent experiments are shown and actin is used as a loading control. Relative expression levels of (**B**) p53 pathways-linked, (**D**) cell cycle-linked and apoptosis-linked molecules were quantified with the ImageJ software (http://rsb.info.nih.gov/ij/features.html). The relative intensity of each molecule was calculated based on (**B**) the corresponding actin, and (**D**) the uncleaved caspase (caspase-3, -8 and -9) or actin (FADD, cyclin E) expression level. (**B**) Relative phosphorylated p53 expression levels were determined based on the corresponding p53 expression level. Average intensities with SEs are shown (n=3). The statistical analysis was performed with ANOVA. *$P < 0.05$, comparing IFNs (3,000U/ml)-treated and untreated cells at 48 or 72 hr.

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IL-28Rα expression was activated by a process of immortalization due to expressed SV40 T antigen or was lost during tumorigenesis of mesothelial cells. Met-5A cells were relatively insensitive to type III IFNs despite being positive for

A

B
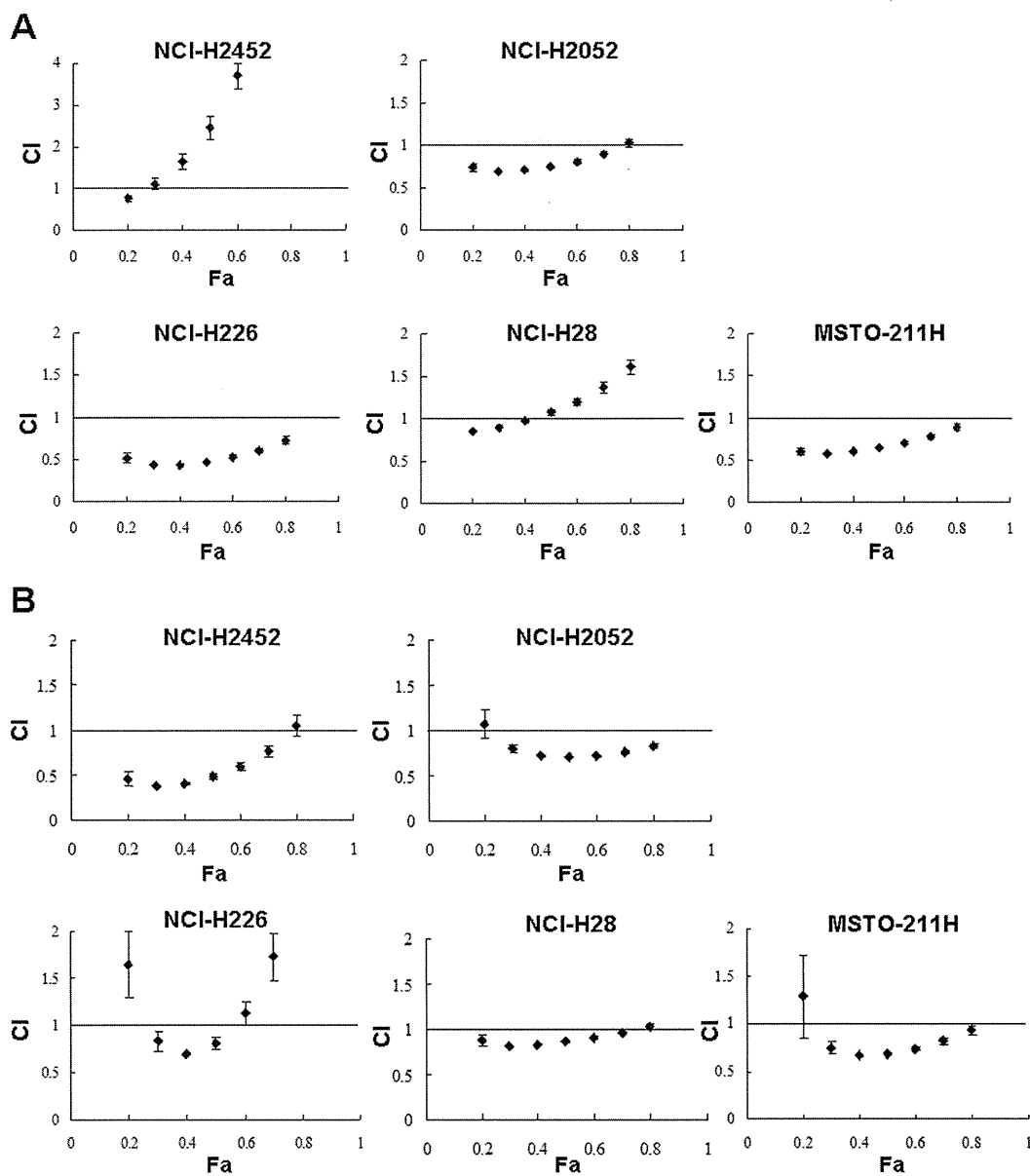
Figure 5. Combinatory effects of IFN-β and anti-cancer agents. Cells were treated either with (**A**) IFN-β (1,000U/ml) and CDDP (0, 0.3, 1, 3, 10 or 30 μM) or with (**B**) IFN-β (3,000 U/ml) and PEM (0, 3, 6, 12, 24 or 48 ng/ml), and were cultured for 4 days. CI values at respective Fa points are shown with SE bars (n=3).

doi: 10.1371/journal.pone.0072709.g005

both of the receptor molecules. The sensitivity to type III IFNs was therefore not completely attributable to the receptor expression. In fact our previous study showed that 9 esophageal carcinoma cell lines expressed both the IL-28α and IL-10β receptors, but the IFN-λ1-mediated growth inhibition was observed only in some of the cell lines [17,20]. Type III IFNs-mediated growth suppression is thus dependent on cell types as well as IL-28Rα expression. Biomarkers to detect the

growth inhibition are thereby required in the case of a possible clinical application with type III IFNs in future.

We also showed that IFN-β produced greater biological activities than IFN-α, which was evidenced by growth inhibitory actions, up-regulated expression levels of the MHC class I molecules and cell cycle changes. The mechanism underlying the greater biological functions of IFN-β than that of IFN-α is not well understood, but the differential binding affinity of both

Table 2. Cell cycle distributions by IFN-β and CDDP or PEM treatments.

Cell	IFN-β	Agent	Cell cycle distribution (%)			
			sub-G1	G0/G1	S	G2/M
NCI-H2052	(-)	(-)	0.3 ± 0.1	85.8 ± 1.1	4.3 ± 0.2	9.7 ± 0.3
	(+)	(-)	2.3 ± 0.1	53.4 ± 1.0	18.4 ± 0.9	26.5 ± 0.3
	(-)	CDDP	1.5 ± 0.1	14.0 ± 0.6	19.7 ± 0.3	65.4 ± 0.3
	(+)	CDDP	10.2 ± 0.1**	22.9 ± 0.4	53.9 ± 0.4	13.6 ± 0.2
	(-)	PEM	9.1 ± 0.4	43.9 ± 0.3	38.6 ± 0.5	9.6 ± 0.4
	(+)	PEM	22.2 ± 0.3*	49.8 ± 0.4	22.3 ± 0.6	6.6 ± 0.3
NCI-H28	(-)	(-)	0.2 ± 0.1	69.8 ± 0.3	11.4 ± 0.1	18.9 ± 0.3
	(+)	(-)	0.7 ± 0.1	61.6 ± 0.7	15.1 ± 0.1	23.4 ± 0.7
	(-)	CDDP	11.2 ± 0.2	56.6± 1.0	19.3 ± 1.0	13.5 ± 0.3
	(+)	CDDP	20.5 ± 0.4*	52.9 ± 0.6	15.6 ± 0.2	11.4 ± 0.2
	(-)	PEM	3.0 ± 0.1	31.3 ± 0.1	44.4 ± 0.2	22.6 ± 0.3
	(+)	PEM	8.1 ± 0.3*	37.5 ± 0.4	38.4 ± 0.5	17.0 ± 0.1

Cells were incubated with or without IFN-β (3,000 U/ml) and also treated with or without CDDP (NCI-H2052; 3 μM, NCI-H28; 50 μM) or PEM (30 ng/ml) for 96 hr (NCI-H2052) or 72 hr (NCI-H28). Cell cycle profiles were analyzed with flow cytometry, and the percent mean and SE of each fraction are shown (n=3).
* $P < 0.05$, comparing between IFN-β with CDDP- or PEM-treated cells and corresponding IFN-β-treated alone, or CDDP- or PEM-treated alone populations.
** $P < 0.01$, comparing between IFN-β with CDDP-treated cells and corresponding IFN-β-treated alone or CDDP-treated alone populations.

IFNs to type I IFN receptors and greater stability of IFN-β could be possible reasons [21].

Cell cycle analyses demonstrated that IFN-α and IFN-β treatments increased S- and G2/M-phase fractions, and then sub-G1-phase populations. Susceptibility of IFNs-mediated cell cycle changes was different among the mesothelioma cells tested. Interestingly, Met-5A cells were relatively resistant to increase of sub-G1-phase fractions although they showed increased S- and G2/M-phase populations. The lack of cell death in type I IFNs-treated Met-5A cells can be associated with the relative insensitivity to type III IFNs because Met-5A cells showed low proliferation activity compared with mesothelioma cells. IFNs up-regulated p53 and the phosphorylation levels, and subsequently induced p21 in mesothelioma cells, whereas IFNs did not induce G0/G1-phase arrests. IFN-β rather up-regulated cyclin E expressions which were linked with cell cycle shift to S-phase. Several lines of initial studies however showed that type I IFN-treated tumor cells became arrest at G0/G1-phase [22]. These data collective suggested that multiple factors were involved in the type I IFNs-induced cell cycle changes. Western blot analyses showed that IFN-β treatments increased caspase-3 cleavages in NCI-H28 cells. IFN-β also induced cleavage of caspase-8 and augmented FADD expression, but did not influence caspase-9 cleavage, indicating that the extrinsic death receptor-linked pathways play a role in the apoptosis. In contrast, previous studies showed that both the extrinsic and

the intrinsic pathways-mediated apoptosis were induced by type I IFNs [23], and therefore further investigations are required for understanding the mechanism underlying preferential activation of extrinsic pathways in mesothelioma.

The present study showed that type I IFNs activated the p53 pathways in mesothelioma cells bearing the wild-type p53 gene. The activated pathways were evidenced by phosphorylated p53 at Ser 15 residue and increased p53 levels together with augmented expression of p53 target molecules, p21, p27 and Bax. Takaoka et al. showed that IFN-β induced p53 protein expression through up-regulated p53 mRNA in fibroblasts, but did not induce the p53 phosphorylation [14]. In contrast, our present study demonstrated that IFN-β treatment itself induced p53 phosphorylation. Our previous study also showed that CDDP treatments increased endogenous p53 levels in mesothelioma cells, and the susceptibility to CDDP was augmented by forced p53 expression [5]. The synergistic combinatory effects between IFN-β and CDDP demonstrated in the present study can be due to augmented p53 activation. In contrast, a possible role of p53 in PEM-mediated cytotoxicity is controversial [24,25] although PEM induced DNA damages and apoptosis [26]. The present data of synergistic combinatory cytotoxicity between IFN-β and PEM rather suggested that PEM-mediated cytotoxicity could be enhanced by IFN-β-mediated p53 up-regulation. Several clinical trials of recombinant IFN-α with an anti-cancer agent have been conducted for mesothelioma [9–11], but possible combinatory effects with IFN-β and CDDP or PEM was not yet demonstrated in any of previous preclinical studies.

Sandoval et al. demonstrated that p14^{ARF} was required for type I IFNs-mediated apoptosis and for p53 up-regulation, and that IFN-β induced apoptosis even in a p53-defective state as long as p14 was intact [27]. The present study however demonstrated that IFN-β induced apoptosis and augmented p53 expression in NCI-H2052 and NCI-H28 cells which were defective of the p14^{ARF} gene (Figure S1). Moreover, Met-5A cells, with p14^{ARF} transcripts but loss of p53 functions, did not increase the sub-G1-phase populations with IFN-β treatments. It is currently unknown as to the p14 involvement in the type I IFNs-mediated apoptosis, but difference of genetic backgrounds, such as defective 16 expression and aberrant signal pathways often found in mesothelioma, can be responsible [28].

In summary, we demonstrated that IFN-β produced greater biological functions than IFN-α in mesothelioma, activated the p53 pathways and enhanced the anti-tumor effects of the first-line chemotherapeutic agents. Combinatory effects of IFN-β with the current first-line agents need to be re-evaluated since previous clinical studies for mesothelioma used IFN-α and non-current chemotherapeutic agents. The present study also suggests a possible clinical trial of intrapleural injections of adenoviruses expressing the IFN-β gene and systemic administrations of the first-line agents. Several clinical phase I studies with IFN-β-producing adenovirus vectors demonstrated feasibility of such gene therapy [12] but none of the clinical studies in combination with chemotherapy has been performed. Gene medicine with chemotherapeutic agents is one of the clinical trials to be examined in mesothelioma patients.

Supporting Information

Figure S1. Lack of the INK4A/ARF locus in mesothelioma.
(A) PCR to detect the p14^{ARF} gene consisting of exon 1β, 2 and 3, and the p16^{INK4A} gene consisting of exon 1α, 2 and 3. Both the p14^{ARF} and the p16^{INK4A} genes share the same exons 2 and 3. (B) RT-PCR to detect the p14^{ARF} and the p16^{INK4A} transcripts with primers designed between the exon 1β and the exon 2 for the p14^{ARF} and between the exon 1α to the exon 2 for the p16^{INK4A} gene. The data indicated that mesothelioma cells used in the present study did not express the p14^{ARF} or the p16^{INK4A} gene.
(TIF)

Table S1. All the experimental data of Figure 2A, 2B and 2C were analyzed with Bonferroni test at SPSS 13.0 version. The list shows only experiments with statistical significance ($P < 0.05$) and those without the significance (P above 0.05) were excluded. ^{a}IFN-α and IFN-β; U/ml, IFN-λ; ng/ml. ^{b}Live cell numbers cultured for 5 days were analyzed in the Figure 2C data.

References

1. Carbone M, Kratzke RA, Testa JR (2002) The pathogenesis of mesothelioma. Semin Oncol 29: 2-17. doi:10.1053/sonc.2002.37355. PubMed: 11836664.
2. Robinson BW, Musk AW, Lake RA (2005) Malignant mesothelioma. Lancet 366: 397-408. doi:10.1016/S0140-6736(05)67025-0. PubMed: 16054941.
3. Vogelzang NJ, Rusthoven JJ, Symanowski J, Denham C, Kaukel E et al. (2003) Phase III study of pemetrexed in combination with cisplatin versus cisplatin alone in patients with malignant pleural mesothelioma. J Clin Oncol 21: 2636-2644. doi:10.1200/JCO.2003.11.136. PubMed: 12860938.
4. Lee AY, Raz DJ, He B, Jablons DM (2007) Update on the molecular biology of malignant mesothelioma. Cancer 109: 1454-1461. doi:10.1002/cncr.22552. PubMed: 17348013.
5. Li Q, Kawamura K, Yamanaka M, Okamoto S, Yang S et al. (2012) Upregulated p53 expression activates apoptotic pathways in wild-type *p53*-bearing mesothelioma and enhances cytotoxicity of cisplatin and pemetrexed. Cancer Gene Ther 19: 218-228. doi:10.1038/cgt.2011.86. PubMed: 22223137.
6. Zhang SY, Boisson-Dupuis S, Chapgier A, Yang K, Bustamante J et al. (2008) Inborn errors of interferon (IFN)-mediated immunity in humans: insights into the respective roles of IFN-alpha/beta, IFN-gamma, and IFN-lambda in host defense. Immunol Rev 226: 29-40. doi:10.1111/j.1600-065X.2008.00698.x. PubMed: 19161414.
7. Li M, Liu X, Zhou Y, Su SB (2009) Interferon-λs: the modulators of antivirus, antitumor, and immune responses. J Leukoc Biol 86: 23-32. doi:10.1189/jlb.1208761. PubMed: 19304895.
8. George PM, Badiger R, Alazawi W, Foster GR, Mitchell JA (2012) Pharmacology and therapeutic potential of interferons. Pharmacol Ther 135: 44-53. doi:10.1016/j.pharmthera.2012.03.006. PubMed: 22484806.
9. Parra HS, Tixi L, Latteri F, Bretti S, Alloisio M et al. (2001) Combined regimen of cisplatin, doxorubicin, and alpha-2β interferon in the treatment of advanced malignant pleural mesothelioma: a phase II multicenter trial of the Italian Group on Rare Tumors (GITR) and the Italian Lung Cancer Task Force (FONICAP). Cancer 92: 650-656. doi:10.1002/1097-0142(20010801)92:3. PubMed: 11505411.
10. Halme M, Knuuttila A, Vehmas T, Tammilehto L, Mäntylä M et al. (1999) High-dose methotrexate in combination with interferons in the treatment of malignant pleural mesothelioma. Br J Cancer 80: 1781-1785. doi:10.1038/sj.bjc.6690597. PubMed: 10468296.
11. Metintas M, Ozdemir N, Uçgun I, Elbek O, Kolsuz M et al. (1999) Cisplatin, mitomycin, and interferon-alpha2a combination chemoimmunotherapy in the treatment of diffuse malignant pleural mesothelioma. Chest 116: 391-398. doi:10.1378/chest.116.2.391. PubMed: 10453867.
12. Sterman DH, Recio A, Carroll RG, Gillespie CT, Haas A et al. (2007) A phase I clinical trial of single-dose intrapleural IFN-β gene transfer for malignant pleural mesothelioma and metastatic pleural effusions: high rate of antitumor immune responses. Clin Cancer Res 13: 4456-4466. doi:10.1158/1078-0432.CCR-07-0403. PubMed: 17671130.
13. Sterman DH, Haas A, Moon E, Recio A, Schwed D et al. (2011) A trial of intrapleural adenoviral-mediated Interferon-{alpha}2b gene transfer for malignant pleural mesothelioma. Am J Respir Crit Care Med 184: 1395-1399. doi:10.1164/rccm.201103-0554CR. PubMed: 21642245.
14. Takaoka A, Hayakawa S, Yanai H, Stoiber D, Negishi H et al. (2003) Integration of interferon-α/β signalling to p53 responses in tumour suppression and antiviral defence. Nature 424: 516-523. doi:10.1038/nature01850. PubMed: 12872134.
15. Vannucchi S, Chiantore MV, Fiorucci G, Percario ZA, Leone S et al. (2005) TRAIL is a key target in S-phase slowing-dependent apoptosis induced by interferon-beta in cervical carcinoma cells. Oncogene 24: 2536-2546. doi:10.1038/sj.onc.1208403. PubMed: 15735750.
16. Ke Y, Reddel RR, Gerwin BI, Reddel HK, Somers AN et al. (1989) Establishment of a human in vitro mesothelial cell model system for investigating mechanisms of asbestos-induced mesothelioma. Am J Pathol 134: 979-991. PubMed: 2541616.
17. Li Q, Kawamura K, Ma G, Iwata F, Numasaki M et al. (2010) Interferon-λ induces G1 phase arrest or apoptosis in esophageal carcinoma cells and produces anti-tumor effects in combination with anti-cancer agents. Eur J Cancer 46: 180-190. doi:10.1016/j.ejca.2009.10.002. PubMed: 19879751.
18. Deshpande A, Sicinski P, Hinds PW (2005) Cyclins and cdks in development and cancer: a perspective. Oncogene 24: 2909-2915. doi:10.1038/sj.onc.1208618. PubMed: 15838524.
19. Satyanarayana A, Kaldis P (2009) Mammalian cell-cycle regulation: several Cdks, numerous cyclins and diverse compensatory mechanisms. Oncogene 28: 2925-2939. doi:10.1038/onc.2009.170. PubMed: 19561645.
20. Fujie H, Tanaka T, Tagawa M, Kaijun N, Watanabe M et al. (2011) Antitumor activity of type III interferon alone or in combination with type I interferon against human non-small cell lung cancer. Cancer Sci 102: 1977-1990. doi:10.1111/j.1349-7006.2011.02079.x. PubMed: 21883692.
21. de Weerd NA, Samarajiwa SA, Hertzog PJ (2007) Type I interferon receptors: biochemistry and biological functions. J Biol Chem 282: 20053-20057. doi:10.1074/jbc.R700006200. PubMed: 17502368.
22. Sangfelt O, Erickson S, Castro J, Heiden T, Gustafsson A et al. (1999) Molecular mechanisms underlying interferon-alpha-induced G0/G1 arrest: CKI-mediated regulation of G1 Cdk-complexes and activation of pocket proteins. Oncogene 18: 2798-2810. doi:10.1038/sj.onc.1202609. PubMed: 10362250.

(DOCX)

Table S2. NCI-H28 cells were untreated or treated with IFN-α or IFN-β (3,000 U/ml) for 5 days. The cells were then stained with PI and annexin V (Tali apoptosis kit, Life Technologies, Carlsbad, CA, USA) and were analyzed with Tali image-based cytometer (Life Technologies). The mean of stained cells percentage and the SE are shown (n = 3). The statistical analysis was performed with ANOVA. * $P < 0.05$, comparing IFN-α- or IFN-β-treated and untreated cells.
(DOCX)

Author Contributions

Conceived and designed the experiments: KT HS KH MT. Performed the experiments: QL KK SY SO. Analyzed the data: HK Y. Tada IS Y. Takiguchi MS. Contributed reagents/materials/analysis tools: HK Y. Tada IS Y. Takiguchi MS. Wrote the manuscript: QL MT.

23. Pokrovskaja K, Panaretakis T, Grandér D (2005) Alternative signaling pathways regulating type I interferon-induced apoptosis. J Interferon Cytokine Res 25: 799-810. doi:10.1089/jir.2005.25.799. PubMed: 16375608.

24. Lu X, Errington J, Curtin NJ, Lunec J, Newell DR (2001) The impact of p53 status on cellular sensitivity to antifolate drugs. Clin Cancer Res 7: 2114-2123. PubMed: 11448931.

25. Giovannetti E, Backus HH, Wouters D, Ferreira CG, van Houten VM et al. (2007) Changes in the status of p53 affect drug sensitivity to thymidylate synthase (TS) inhibitors by altering TS levels. Br J Cancer 96: 769-775. doi:10.1038/sj.bjc.6603639. PubMed: 17339891.

26. Yang TY, Chang GC, Chen KC, Hung HW, Hsu KH et al. (2013) Pemetrexed induces both intrinsic and extrinsic apoptosis through ataxia telangiectasia mutated/p53-dependent and -independent signaling pathways. Mol Carcinog 52: 183-194. doi:10.1002/mc.21842. PubMed: 22086658.

27. Sandoval R, Xue J, Pilkinton M, Salvi D, Kiyokawa H et al. (2004) Different requirements for the cytostatic and apoptotic effects of type I interferons. Induction of apoptosis requires ARF but not p53 in osteosarcoma cell lines. J Biol Chem 279: 32275-32280. doi:10.1074/jbc.M313830200. PubMed: 15169789.

28. Zucali PA, Ceresoli GL, De Vincenzo F, Simonelli M, Lorenzi E et al. (2011) Advances in the biology of malignant pleural mesothelioma. Cancer Treat Rev 37: 543-558. doi:10.1016/j.ctrv.2011.01.001. PubMed: 21288646.

A Microarray-Based Gene Expression Analysis to Identify Diagnostic Biomarkers for Unknown Primary Cancer

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Abstract

Background: The biological basis for cancer of unknown primary (CUP) at the molecular level remains largely unknown, with no evidence of whether a common biological entity exists. Here, we assessed the possibility of identifying a common diagnostic biomarker for CUP using a microarray gene expression analysis.

Methods: Tumor mRNA samples from 60 patients with CUP were analyzed using the Affymetrix U133A Plus 2.0 GeneChip and were normalized by asinh (hyperbolic arc sine) transformation to construct a mean gene-expression profile specific to CUP. A gene-expression profile specific to non-CUP group was constructed using publicly available raw microarray datasets. The t-tests were performed to compare the CUP with non-CUP groups and the top 59 CUP specific genes with the highest fold change were selected (*p*-value<0.001).

Results: Among the 44 genes that were up-regulated in the CUP group, 6 genes for ribosomal proteins were identified. Two of these genes (*RPS7* and *RPL11*) are known to be involved in the Mdm2–p53 pathway. We also identified several genes related to metastasis and apoptosis, suggesting a biological attribute of CUP.

Conclusions: The protein products of the up-regulated and down-regulated genes identified in this study may be clinically useful as unique biomarkers for CUP.

Introduction

Patients with cancer of unknown primary (CUP) present with metastatic disease for which the primary site cannot be found, despite extensive standard investigation. The prognosis of patients with CUP is usually poor for those receiving empiric treatments. The median survival period is 3–9 months even when newer combination treatment regimens are administered [1–5]. The survival of patients with CUP can be improved if the primary site can be identified and a site-specific therapy can be applied [6,7].

Clinically, CUPs exhibit common characteristics, such as rapid progression, early dissemination and a silent primary tumor, with signs and symptoms related to the metastatic site(s) [8]. The primary tumor may either have a slow growth pattern or may become involuted and undetectable. Existence of such common properties prompts us to hypothesize that there may be potential biological markers that elucidate CUP as a whole. Gene expression analysis is one of the means by which to identify genes characteristic to CUP.

Several studies using gene expression microarrays have demonstrated that the expression levels of thousands of genes can be used as a "molecular fingerprint" to classify a multitude of tumor types [9–15]. We are presently involved in a multicenter clinical study to predict the primary site of CUP based on the analysis of gene expression patterns. The analysis interprets the expression of ~22,000 genes in each specimen by applying normalization and classification algorithms to gene expression data from a microarray. The similarity of each tumor specimen's gene expression

pattern is then compared to the patterns for tumors from 24 known primary sites covered by the test. This study enabled the identification of genes that exhibited a unique expression pattern in CUP. Here, we present several genes encoding metastasis-and apoptosis-related proteins thus identified that may biologically characterize CUP.

Materials and Methods

Ethic Statement

All the patients provided written informed consent. Study approval was obtained from independent ethics committees of Kinki University, Shizuoka Cancer Center, Hyogo Cancer Center, Osaka City General Hospital, Chiba University, National Cancer Center Hospital East, Kobe University, Tochigi Cancer Center, Saitama Medical University, Tohoku University, and Cancer Institute Hospital. The study was undertaken in accordance with the Declaration of Helsinki.

Study Design

This study originated from currently ongoing multicenter, randomized, phase 2 prospective trial for the treatment of untreated CUP based on prediction of the primary site using data from a DNA chip. The patients had been diagnosed as having CUP between November 2008 and November 2010 at one of 13 centers of the West Japan Oncology Group (WJOG), a Japanese non-profit organization for conducting oncological clinical trials. The laboratory analyses were performed at 2 centers in Japan (Kinki University, Osaka-Sayama and Mitsubishi Chemical Medience Corporation, Tokyo).

Patients

All eligible patients had undergone a standard investigation for CUP. They were categorized into unfavorable subsets of CUP. Diagnoses of histologically or cytologically confirmed adenocarcinoma, poorly differentiated carcinoma, or squamous cell carcinoma were permitted. In each of the patients, a primary site had not been identified after a complete medical history, physical examination, chemistry profile, computed tomography (CT) scan of the chest, abdomen, and pelvis, mammography in women, measurements of the prostate-specific antigen (PSA) level in men, and a directed workup of any symptomatic areas. Patients in the following categories were excluded: women with adenocarcinoma involving only the axillary lymph nodes or the peritoneal cavity, patients with squamous cell carcinoma involving only cervical lymph nodes or inguinal lymph nodes, patients with poorly differentiated carcinoma consistent with a germ cell tumor (isolated midline structures, multiple pulmonary nodules, or elevated levels of β-human chorionic gonadotropin or α-human chorionic gonadotropin-fetoprotein), men with an elevated plasma PSA level or PSA-positive staining in a tumor, patients with a single, small, potentially resectable tumor, and patients with neuroendocrine carcinomas.

Sample Collection

Fresh frozen samples obtained from 60 patients with CUP were used for the analysis. All the samples were tested without knowledge of either the clinical characteristics or the subsequent response to treatment, except for the sex of the patient and the site of biopsy (mostly lymph nodes or ascites fluid).

Assay Procedure

RNA was extracted from the samples using an Isogene kit (Nippon Gene, Toyama, Japan). Spectrophotometry was used to assess whether an adequate total RNA concentration and purity was present. In general, the protocol for processing the RNA, amplifying and labeling fragments, hybridizing material on the microarray, and scanning was similar to the standard Affymetrix protocol for GeneChip® expression analysis. Affymetrix Gene-Chip® Human Genome U133 Plus 2.0 was used on an Affymetrix 3000 or 3000Dx GeneChip instrument (fluidics station and scanner) running Gene-Chip operating software to generate gene expression data (.CEL files).

Database Submission of Microarray Data

The microarray data were deposited in the Gene Expression Omnibus (GEO) database: http://www.ncbi.nlm.nih.gov/geo/. The GEO accession number for the platform is GSE42392, samples GSM1038716-GSM 1038775.

Data Analysis

All the microarray data were normalized using asinh (hyperbolic arc sine) transformation, which is a modified version of Huber's normalization with variance stabilization [16,17], and also a part of generalized log transformation (glog) [18]. Interinstitutional and array-to-array biases were corrected by subtracting their specific effects that were estimated by the mixed model [19]. The equation for asinh transformation is Igk/I.k, where I represents the expression value, g represents the gene, k represents the array, and the dot indicates the mean. The resulting asinh-transformed values, representing the relative expression of each gene, were used in further analyses.

The raw microarray datasets for 2,364 cancers of several primary types and 10 normal lymph nodes were obtained from the Gene Expression Omnibus (GEO) (Table 1). These datasets were normalized and used to construct gene-expression profiles specific to each type of cancer (n = 24) as well as an overall profile for cancer with known primary (CKP). The normal lymph node dataset was used as a reference. The data quality of CUP samples was monitored to ensure that data analysis of CUP samples was comparable to that of samples of CKP collected from GEO. Only the samples whose GAPDH, a housekeeping control gene, at 5′-terminal region (AFFX-HUMGAPDH/M33197_5_at) showed a minimum expression>500, and with the ratio of expression intensity (GAPDH at the 3′-region/5′-region)<3 were chosen.

The gene-expression profile specific to CUP was constructed using 30 CUP samples as training data and another 30 samples as test data (odd and even numbered cases, respectively). Of the 22,215 genes that were measured using both CUP samples (this work) and CKP samples (publicly accessed), a total of 5,645 genes with a present call for every sample were selected for further analysis. To identify CUP specific genes, the gene-expression profiles specific to CUP (training datasets) and normal lymph node were compared using t-tests. A histogram of the p-values is shown in Figure 1. The p-values for most of the genes were less than 0.001; when we selected the top 100 genes according to their p-values, the false discovery rate (FDR) was 4.56×10^{-12} [20]. To validate whether the genes identified using the CUP training datasets were significantly specific to CUP, the linear discriminant analysis (LDA) using these genes was performed for the CUP test datasets and the accuracy was estimated as described [21]. Heatmaps and a cluster dendrogram were then constructed using the Ward method [22].

Table 1. Number of cases for each cancer type and GEO series used for gene expression profiles.

Cancer type	n	GEO Series
Bladder	80	GSE2109, GSE3167, GSE7476
Brain	106	GSE2109, GSE3185, GSE4271
Breast (Basal)	25	GSE1456
Breast (ERBB2)	15	GSE1456
Breast (Inflammatory)	49	GSE1456
Breast (Luminal A)	39	GSE1456
Breast (Luminal B)	23	GSE1456
breast (No subtype)	20	GSE1456
Breast (Normal-like)	37	GSE1456
Cervical	89	GSE2109, GSE5787, GSE6791
Colon	365	GSE2109, GSE2509, GSE2742, GSE5486, MEXP101, MEXP170
Corpus_uteri	205	GSE2109
Gallbladder	2	GSE2109
Germ cell	101	GSE3218
Head (oral squamous cell)	42	GSE6791
Kidney	270	GSE2109, GSE6357, GSE781
Liver	13	GSE2109
Lung adenocarcinoma	61	GSE4127, MEXP231
Lymphoma	18	GSE2109, GSE4176
Ovarian	420	GSE2109, GSE3149
Panreas	56	GSE2109
Prostate	229	GSE2109, GSE3325, GSE7930, GSE8218
Stomach	42	GSE2109
Thyroid	57	GSE2109, MEXP97
Normal lymph node	10	GSE2665
CUP (This work)	60	GSE42392
Total	**2434**	

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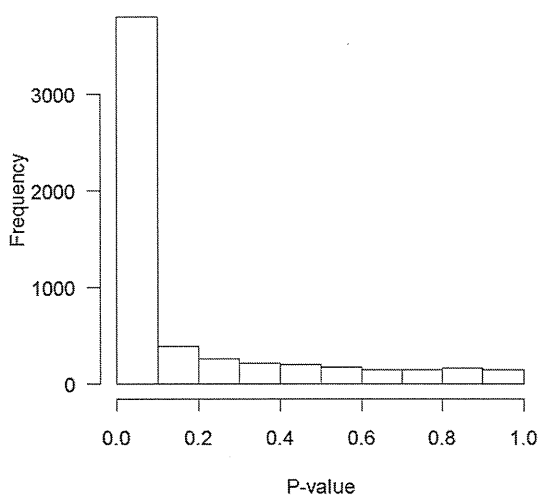
Figure 1. Frequency histogram of *p*-values.
doi:10.1371/journal.pone.0063249.g001

Results

Gene Expression Profile of CUP and Known Primary Cancers

A total of 237 genes were found to be either up-regulated or down-regulated by more than 2-fold between the normal lymph node and 30 CUP samples (training datasets). Of these, 59 genes with more than a 2.5-fold change (44 up-regulated and 15 down-regulated genes) are listed in Table 2. We designated the gene sets consisting of these CUP associated genes with >2 fold and >2.5 fold up-regulation or down-regulation as $M_{CUP}(2.0)$ and $M_{CUP}(2.5)$, respectively. Using these probe sets in $M_{CUP}(2.5)$, linear discriminant analysis (LDA) was performed for the CUP training datasets together with 2,364 cancers of various known types and 10 normal lymph nodes. As expected, all 2,404 samples were correctly discriminated. When the remaining 30 CUP samples (test datasets) were assessed using LDA that was modeled with the training datasets, 26 out of the 30 CUP samples were assigned correctly to "CUP", while only the 4 samples were predicted as "the other cancer". Thus, the accuracy of CUP was validated to be 86.7%, indicating that the 59 genes selected were of statistically significance as having biological attributes of CUP.

Figure 2 shows the supervised clustering of all 60 CUP samples performed together with 2,364 cancers of various known types and

Table 2. Genes identified as being up-regulatred or down-regulated in CUP.

Symbol	Gene description (Gene up-regulated in CUP)	Probe_set_ID	Log-fold change*	Fold change
RPL18A	Ribosomal protein L18A	200869_at	1.974	7.2
S100A4	S100 calcium binding protein A4	203186_s_at	1.587	4.9
PRG1	Proteoglycan 1, secretory granule	201858_s_at	1.539	4.7
SUB1	SUB1 homolog (S. cerevisiae)	214512_s_at	1.535	4.6
S100A6	S100 calcium binding protein A6	217728_at	1.523	4.6
RPS7	Ribosomal protein S7	200082_s_at	1.369	3.9
RPL11	Ribosomal protein L11	200010_at	1.245	3.5
PFN1	Profilin 1	200634_at	1.229	3.4
LOC23117	KIAA0220-like proteinKIAA0220	211996_s_at	1.212	3.4
TYROBP	TYRO protein tyrosine kinase binding protein	204122_at	1.196	3.3
TIMP1	TIMP metallopeptidase inhibitor 1	201666_at	1.178	3.2
SERF2	Small EDRK-rich factor 2	217756_x_at	1.173	3.2
YWHAZ	14-3-3 protein, zeta polypeptide	200641_s_at	1.169	3.2
LSM7	LSM7 homolog, U6 small nuclear RNA associated (S. cerevisiae)	204559_s_at	1.151	3.2
GSTP1	Glutathione S-transferase pi	200824_at	1.141	3.1
YWHAH	14-3-3 protein, eta polypeptide	201020_at	1.102	3.0
LAPTM5	Lysosomal associated multispanning membrane protein 5	201721_s_at	1.095	3.0
SNRPD2	Small nuclear ribonucleoprotein D2 polypeptide 16.5 kDa	200826_at	1.087	3.0
LOC392501	similar to 60 S ribosomal protein L26	222229_x_at	1.076	2.9
OAZ1	Ornithine decarboxylase antizyme 1	215952_s_at	1.073	2.9
POLR2J	Polymerase (RNA) II (DNA directed) polypeptide J, 13.3kDa	212782_x_at	1.062	2.9
EIF5A	Eukaryotic translation initiation factor 5A	201123_s_at	1.028	2.8
ATP5H	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit d	210149_s_at	1.023	2.8
APOC1	Apolipoprotein C-I	213553_x_at	1.018	2.8
LGALS1	Lectin, galactoside-binding, soluble, 1 (galectin 1)	201105_at	1.013	2.8
S100A11	S100 calcium binding protein A11	200660_at	1.010	2.7
SH3BGRL3	SH3 domain binding glutamic acid-rich protein like 3	221269_s_at	0.996	2.7
C1QB	complement component 1, q subcomponent, B chain	202953_s_at	0.984	2.7
RPS10	Ribosomal protein S10	216505_x_at	0.984	2.7
HSPA8	Heat shock 70 kDa protein 8	210338_s_at	0.972	2.6
NUTF2	Nuclear transport factor 2	202397_at	0.972	2.6
PRKDC	Protein kinase, DNA-activated, catalytic polypeptide	208694_at	0.967	2.6
NOLA3	Nucleolar protein family A, member 3 (H/ACA small nucleolar RNPs)	217962_at	0.957	2.6
TCEB2	Transcription elongation factor B (SIII), polypeptide 2 (18 kDa, elongin B)	200085_s_at	0.953	2.6
LOC442171	similar to ribosomal protein L10	217379_at	0.952	2.6
NEDD8	Neural precursor cell expressed, developmentally down-regulated 8	201840_at	0.944	2.6
LOC646417	similar to 60 S ribosomal protein L29 (P23)	216570_x_at	0.939	2.6
RPL36	Ribosomal protein L36	219762_s_at	0.937	2.6
VIM	Vimentin	201426_s_at	0.924	2.5
STK17A	Serine/threonine kinase 17a (apoptosis-inducing)	202693_s_at	0.922	2.5
NDUFS8	NADH dehydrogenase (ubiquinone) Fe-S protein 8, 23 kDa	203189_s_at	0.911	2.5
SELT	Selenoprotein T	217811_at	0.908	2.5
CST3	Cystatin C (amyloid angiopathy and cerebral hemorrhage)	201360_at	0.906	2.5
RPLP2	Ribosomal protein, large, P2	200909_s_at	0.901	2.5
Symbol	Gene description (Gene down-regulated in CUP)	Probe_set_ID	Log-fold change*	Fold change
ATP1B1 NGFRAP1	ATPase, Na+/K+ transporting, beta 1 polypeptide Nerve growth factor receptor (TNFRSF16) associated protein 1	201242_s_at 217963_s_at	−0.891 −0.968	0.4 0.4
FOXJ3	Forkhead box J3	206015_s_at	−0.978	0.4
GABARAPL1	GABA(A) receptor-associated protein like 1	211458_s_at	−0.984	0.4

Table 2. Cont.

Symbol	Gene description (Gene up-regulated in CUP)	Probe_set_ID	Log-fold change*	Fold change
CD24	CD24 molecule	216379_x_at	−0.995	0.4
IVNS1ABP	Influenza virus NS1A binding protein	206245_s_at	−1.000	0.4
SCAMP1	Secretory carrier membrane protein 1	212417_at	−1.037	0.4
SEC22B	SEC22 vesicle trafficking protein homolog B (S. cerevisiae)	214257_s_at	−1.047	0.4
ITM2B	Integral membrane protein 2B	217731_s_at	−1.071	0.3
PDIA3	Protein disulfide isomerase family A, member 3	208612_at	−1.071	0.3
PIN4	Protein (peptidylprolyl cis/trans isomerase) NIMA-interacting, 4 (parvulin)	214224_s_at	−1.087	0.3
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog	214352_s_at	−1.175	0.3
DICER1	Dicer1, Dcr-1 homolog (Drosophila)	213229_at	−1.264	0.3
SWAP70	SWAP-70 protein	209306_s_at	−1.342	0.3
VAPA	VAMP (vesicle-associated membrane protein)-associated protein A, 33 kDa	208780_x_at	−2.720	0.1

Each of the gene symbols, description, probe set in HG-U133 plus 2.0, log fold change and fold change are given in the table.
*Natural logarithm of fold change (CUP/normal lymph node).
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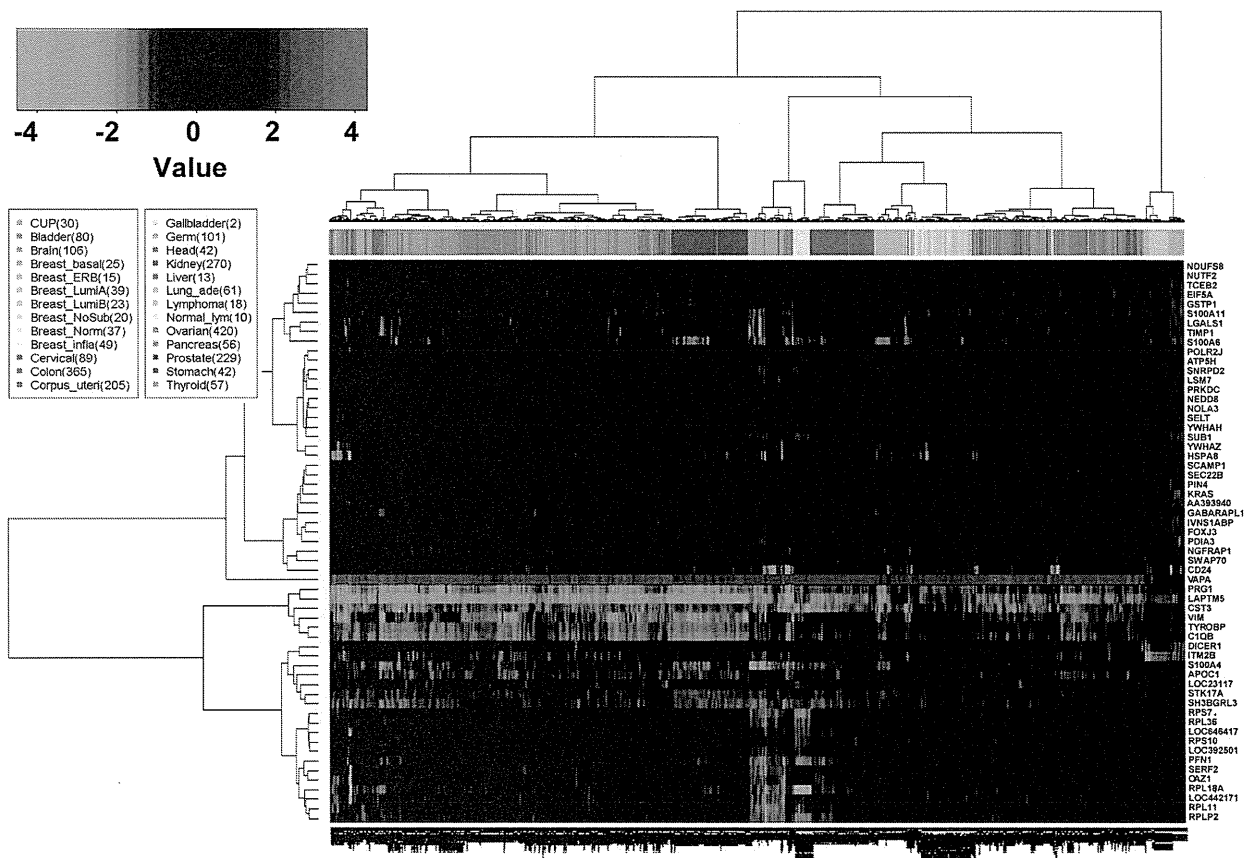
Figure 2. Heatmap representing the expressions of 59 genes with significant different expression in CUP compared with other cancer types or normal lymph nodes. Genes are indicated on the right. The colored bar above the heatmap represents the different cancer types, and the legend key is on the left. On the heatmap, red represents up-regulated genes and green represents down-regulated genes, relative to the expression levels in normal lymph nodes, with the scale shown in the upper left corner. The gene expression profiling datasets for normal lymph nodes and 24 known cancer types other than CUP were obtained from publicly available sources, as described in the Materials and Methods.
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10 normal lymph nodes using the 59 genes. The CUP samples were split into 2 groups with lung adenocarcinoma (LAC) clustered in between (right most part of the heat map). The larger group consisted of 42 samples, while the smaller consisted of 15 samples. Only 3 CUP samples were not included in any of these groups and instead were included in the clusters for normal lymphoma, brain tumors, and ovarian cancer, respectively. These were among the 4 samples that were predicted as "the other cancer" in the LDA. The *VAPA* gene, which was overexpressed in most of the cancer samples but not in CUP or LAC, revealed a striking contrast between CUP/LAC and other samples, which may have influenced the clustering analysis. When we re-analyzed

the data after excluding the *VAPA* gene, the grouping for CUP was unchanged, but the smaller group with 15 samples was no longer clustered with LAC (Figure S1). The mean gene expression profiles (GEPs) for CUP, normal lymphoma, and 24 known cancer types were compared to create a dendrogram representing the quantified relations among CUP and the known cancer types, which again showed the clustering of CUP together with LAC (Figure S2).

Selection of CUP Associated Genes

Although the functions were diverse or unknown for the 44 up-regulated genes in the $M_{CUP}(2.5)$ datasets (Table 2), we found that
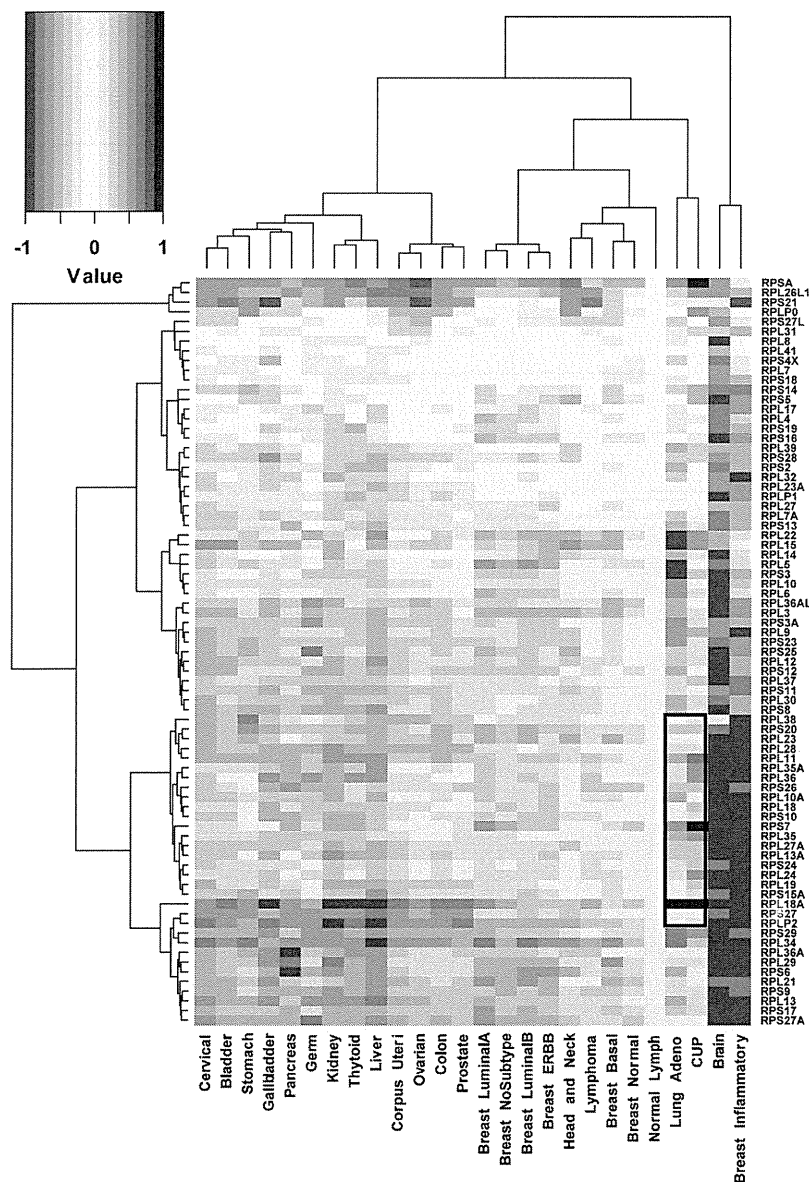
Figure 3. Heatmap representing the expression of 77 ribosomal protein genes in CUP, normal lymph nodes, and other cancer types. Ribosomal protein genes are indicated on the right. On the heatmap, purple represents up-regulated genes and green represents down-regulated genes, relative to the expression levels in normal lymph nodes, with the scale shown in the upper left. The genes that were exclusively overexpressed in CUP and lung adenocarcinoma are highlighted.
doi:10.1371/journal.pone.0063249.g003
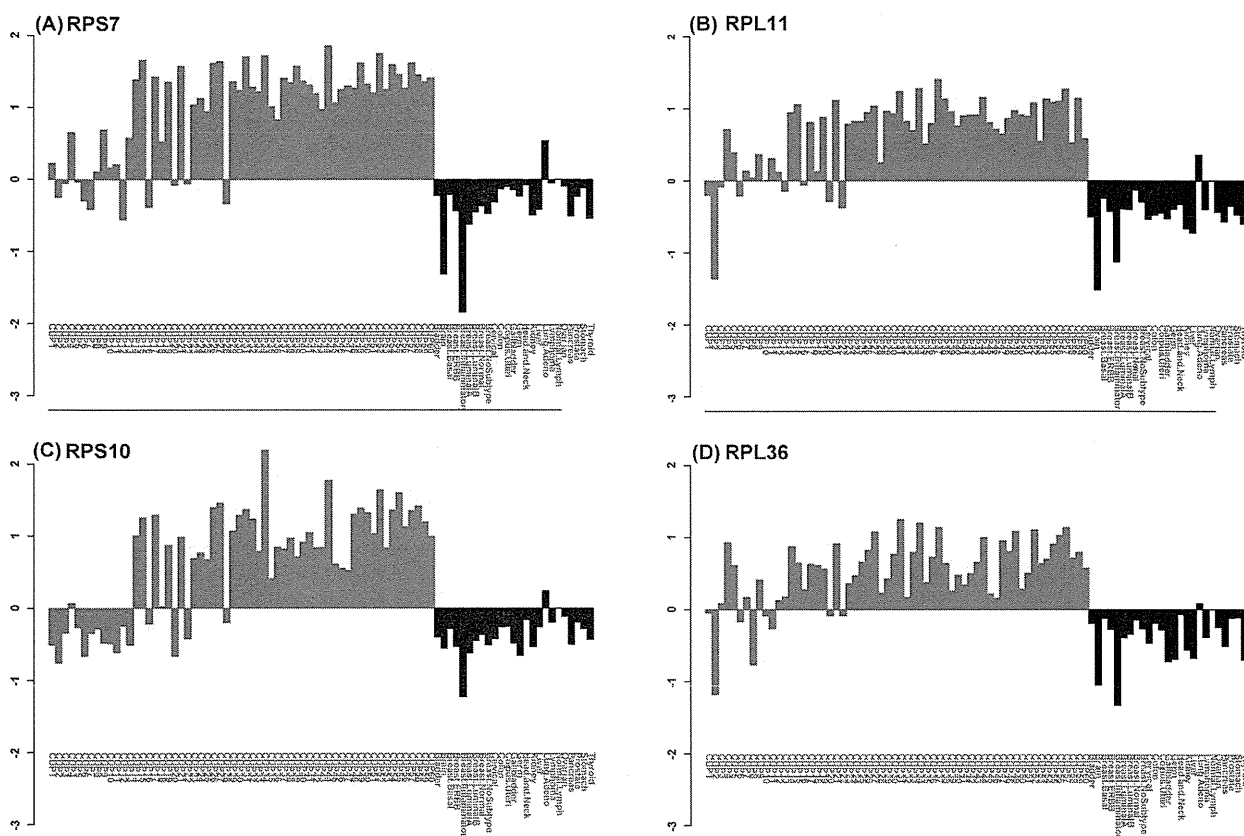
Figure 4. Relative expression levels for 4 ribosomal proteins. The relative expression levels of (A)RPS7, (B)RPL11, (C)RPS10, and (D)RPL36 were compared using individual CUP samples (n = 60), the mean expression levels of known cancer types, and a normal lymph node samples (n = 25). The asinh-transformed values for each gene were used for the calculations.

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14 genes (*S100A4, PRG1, S100A6, GSTP1, EIF5A, LGALS1, S100A11, PRKDC, VIM, CST3, TIMP1, YWHAZ, NEDD8, STK17A*) could be characterized after a search using the keywords "metastasis" and "apoptosis". Some of these genes were associated with the epithelial-to-mesenchymal transition (EMT), a function that has been increasingly recognized as a key step in cancer metastasis [23].

In the $M_{CUP}(2.5)$ dataset, 15 genes were down-regulated. Of these genes, we focused on *CD24, KRAS* and *DICER1*. The known functions of the above-mentioned up-regulated and down-regulated genes will be discussed in detail below.

Relative Expression of Up-Regulated Ribosomal Proteins

In the $M_{CUP}(2.5)$ dataset, we also identified 6 ribosomal proteins (*RPL18A, RPS7, RPL11, RPS10, RPL36, and RPLP2*). We found 11 more genes for ribosomal proteins (*RPL24, RPL35, RPL35A, RPS20, RPL13A, RPL28, RPS26, RPS14, RPL27A, RPL19, and RPL29*) in the $M_{CUP}(2.0)$ dataset. Ribosomal proteins are assembled into small and large ribosomal subunits. The small 40 S and large 60 S ribosomal subunits contain approximately 32 and 47 ribosomal proteins (known as RPS and RPL proteins), respectively [24]. The increased expression of ribosomal proteins has been associated with increased proliferation and growth; in some cases, however, increased expression has also been shown to suppress tumorigenesis [25,26].

To examine whether ribosomal protein genes can be used as biomarkers to discriminate CUP from other cancer types, the mean GEPs for a total of 77 ribosomal protein genes were compared using clustering for CUP, normal lymphoma, and 24 known cancer types (Figure 3). The ribosomal protein genes that were up-regulated in CUP were also up-regulated in LAC.

The relative mRNA expression levels of 4 ribosomal protein genes that were up-regulated in CUP (*RPS7, RPL11, RPS10, and RPL36*) were compared with the levels in normal lymphoma and 24 known cancer types (Figure 4). The 42 CUP samples that consistently contained large amounts of these mRNAs belonged to the larger CUP cluster, while the remaining 15 sample that showed relatively smaller amounts of these mRNAs belonged to the smaller cluster, as shown in Figure 2. As expected, the increased expressions of these mRNAs were also observed in LAC, but not in the other cancer types (Figure 4).

Discussion

Accumulating data sets from gene-expression microarray analyzed for various types of tumors have enabled the establishment of organ- and tumor-specific expression profiles that improve precise prediction of primary site of CUP [9,10,14,15]. Our official phase 2 study to corroborate the feasibility of CUP prediction using our algorithm is currently ongoing and will provide genes that exhibit unique expression pattern in CUP. A

compelling theory to explain CUP is that the primary cancer is microscopic and may disappear because of marked apoptosis after seeding metastases that are able to proliferate into more significant tumors in different tissues [27]. As a high metastasis potential and vulnerability to apoptosis would explain the properties of CUP well, we first searched for genes related to metastasis and apoptosis among all the genes that were up-regulated by more than 2.5-fold in the CUP samples ($M_{CUP}(2.5)$ dataset).

Of the 14 up-regulated genes that were found (*S100A4, PRG1, S100A6, GSTP1, EIF5A, LGALS1, S100A11, PRKDC, VIM, CST3, TIMP1, YWHAZ, NEDD8, STK17A*), three (*S100A4, S100A6, S100A11*) belong to a group of S100 proteins involved in the Ca^{2+} signaling network and regulate a variety of intracellular activities including cell growth and motility [28]. The expressions of these genes are observed in several epithelial tumors and have been linked to metastasis [29,30]. *S100A4*, together with *VIM*, has also been used as an EMT marker [31]. The overexpression of *EIF5A* induces the EMT, thereby promoting the tumor metastasis of colorectal and hepatocellular carcinoma [32]. Serglycin, a gene product of *PRG1*, is a proteoglycan that has been functionally identified as a significant regulator of metastasis in nasopharyngeal carcinoma (NPC) [33]. The elevated expression of Serglycin in NPC cells can mediate the level of vimentin (*VIM*) expression, which is not only a marker of the EMT, but also has an important role in the regulation of cellular migration [31,34]. Lewis lung carcinoma cells in mice show metastasis to the lung when the cells express Galectin-1 (Gal-1), a large carbohydrate-binding protein encoded by *LGALS1*, suggesting novel targeting strategies for Gal-1 in cancer [35].

Both metastatic cells and drug-resistant cells have similar gene expression patterns of survival-related molecules, suggesting that metastatic cancer may be difficult to treat because of resistance to anticancer drugs. DNA-dependent protein kinase (DNA-PK), a gene product of *PRKDC*, is one of the proteins up-regulated in several metastatic and drug-resistant cancer cells [36]. Because the up-regulation of DNA-PK was observed in the CUP patients in our cohort, who had never been treated with chemotherapy, DNA-PK may indicate essential resistance, rather than acquired resistance, to chemotherapy. GSTP1 has also been postulated in several cancer types to enhance the metastatic potential and the development of resistance to drugs that induce reactive oxygen species (ROS), such as paclitaxel and cisplatin [37,38]. Other genes up-regulated in CUP also reveal a significant role in chemoresistance and may be linked to the metastatic potential. Breast cancer cells overexpressing TIMP-1, a well-known inhibitor of matrix metalloproteinase, exhibit a reduced sensitivity to the chemotherapeutic drugs paclitaxel and epirubicin through the activation of transcription factor NF-κB [39]. The knocked-down expression of 14-3-3 ζ, a gene product of *YWHAZ*, sensitizes head and neck cancer cells to chemotherapy [40]. A small molecule inhibitor of NEDD8 activating enzyme (NAE) may be active against tumors that are resistant to other chemotherapeutic agents [41].

Unlike the hitherto described genes, cystatin C (*CST-3*) and STK17A function as direct pro-apoptotic factors by antagonizing TGF-β signaling and by modulating ROS, respectively. Cystatin C has been shown to interact with the TGF-β type II receptor, thereby preventing TGF-β binding and subsequent EMT induction [42]. TGF-β has been accepted as a main initiator of EMT; however, NF-κB was recently found to promote EMT in some cells that are unresponsive to TGF-β because they lack functional SMAD4, representing an alternative pathway leading to EMT that can replace TGF-β signaling [43]. NF-κB signaling may predominately induce EMT in CUP. Both TIMP-1, which can

activate NF-κB, and vimentin, which is activated by NF-κB, were among the genes (proteins) that were up-regulated in CUP as described above, making this hypothesis more likely [39,43]. STK17A is up-regulated in response to oxidative stress in a p53-dependent manner [44]. Since STK17A is known as a positive regulator of the apoptotic pathway and its expression level in colorectal carcinomas is enhanced in lesions with lymph node metastasis, the apoptotic process could be involved in the node metastasis of carcinomas, including CUP [45].

Of the 15 down-regulated genes in the $M_{CUP}(2.5)$ dataset, *CD24, KRAS* and *DICER1* are of particular interest. CD24 is the most widely used marker, together with CD44, for identifying tumor-initiating cells in breast carcinomas. $CD44^{+}/CD24^{-/low}$ breast cancer cells have the ability to metastasize, since the enrichment of these stem-like cells is significantly observed in patients with positive lymph nodes [46]. A subset of kras mutant cancer cells exhibit "kras addiction" and have a differentiated epithelial phenotype. The induction of EMT has been shown to convert kras-dependent cancer cells to kras-independent cells, which do not require the continued expression of kras [47]. *Dicer1* functions as a haploinsufficient tumor suppressor gene [48]. Frequent loss of one allele of *Dicer1* has been observed in several different tumor types causing a global reduction of steady-state micro RNA levels that could be functionally suppressive to the oncogenesis and metastasis of CUP.

The increased expression of several ribosomal proteins was found in CUP. Whether these changes in expression are causally related to the generation of CUP is unknown. In some cases, the overexpression of ribosomal proteins, including RPL5, RPL11, RPL23 and RPS7 has been shown to suppress tumorigenesis [49,50]. These proteins activate p53 by binding to MDM2 and inhibiting MDM2-mediated p53 ubiquitination and degradation in response to nucleolar stress (also called ribosomal stress). RPL11 and RPS7 were recently shown to be required for p53 activation induced by DNA-damaging agents [51], suggesting that these ribosomal proteins may play a crucial role in p53 activation in response to diverse stressors. Furthermore, neddylation, the process by which the ubiquitin-like protein NEDD8 is conjugated to its target, is essential for RPL11's role in the mediation of p53 signaling [49]. Interestingly, these two ribosomal proteins and NEDD8 were included in our $M_{CUP}(2.5)$ dataset. The tumor suppressor function performed by these proteins may be related to the vulnerability to apoptosis that CUP (at the primary site) exhibits as one of its properties.

For functional analyses of the identified genes, overexpression or knockdown experiments using appropriate cell lines would be plausible to pursue if the gene of interest confers change in growth or in metastatic ability to the cells. The metastatic process can be evaluated *in vitro* by monitoring cell invasion through Matrigel and adhesion of cells to plates, etc. Synthetic inhibitors specific to Gal-1, DNA-PK and 14-3-3 ζ have been developed [52–54]. Thus, it will be intriguing to investigate the effect of these inhibitors on the cells overexpressing the respective gene *in vitro* or *in vivo*, which may lead to targeted therapy for CUP.

To our surprise, the gene expression profile (GEF) of CUP closely resembled that of lung adenocarcinoma (LAC), which may simply reflect the relatively high metastatic potential of LAC. In a study using ^{18}F-fluoro-2-deoxyglucose positron emission tomography (FDG-PET), the most commonly detected location of the primary tumor in patients with CUP was the lung [55]. In CUP, the primary cancer and its metastasis (-ses) behave very differently in respect to proliferation, leading to the assumption that the molecular profiles of CUP specimens from the two sites would differ. We are unable to compare these differences because the

primary cancer is unidentifiable. A differential gene expression analysis using primary and metastatic tumor tissues from advanced lung cancer patients may provide some clues to this question.

In conclusion, we identified several genes that were up-regulated in CUP and that may contribute to the acquisition of a metastatic phenotype as well as resistance to anticancer drugs in many cases. Proapoptotic factors were also identified. The combinational effects of the multiple functions of genes that are highly expressed in CUP could be involved in regulating CUP behaviors, such as apoptosis and metastasis. Immunohistochemical-based or PCR-based validation of the candidate genes is needed to refine the molecular classification of CUP.

Supporting Information

Figure S1 Heatmap constructed as in Figure 1 but excluding the *VAPA* gene.
(TIF)

References

1. Greco FA, Burris HA, Litchy S, Barton JH, Bradof JE, et al. (2002) Gemcitabine, carboplatin, and paclitaxel for patients with carcinoma of unknown primary site: a Minnie Pearl Cancer Research Network study. J Clin Oncol 20: 1651–6.
2. Pavlidis N, Briasoulis E, Hainsworth J, Greco FA (2003) Diagnostic and therapeutic management of cancer of an unknown primary. Eur J Cancer 39: 1990–2005.
3. Hainsworth JD, Spigel DR, Farley C, Thompson DS, Shipley DL, et al. (2007) Phase II trial of bevacizumab and erlotinib in carcinomas of unknown primary site: the Minnie Pearl Cancer Research Network J Clin Oncol 25: 1747–52.
4. Schneider BJ, El-Rayes B, Muler JH, Philip PA, Kalemkerian GP, et al. (2007) Phase II trial of carboplatin, gemcitabine, and capecitabine in patients with carcinoma of unknown primary site. Cancer 110: 770–5.
5. Pimiento JM, Teso D, Malkan A, Dudrick SJ, Palesty JA (2007) Cancer of unknown primary origin: a decade of experience in a community-based hospital. Am J Surg 194: 833–7(Disc 7–8).
6. Abbruzzese JL, Abbruzzese MC, Lenzi R, Hess KR, Raber MN (1995) Analysis of a diagnostic strategy for patients with suspected tumors of unknown origin. J Clin Oncol 13: 2094–103.
7. Varadhachary GR, Raber MN, Matamoros A, Abbruzzese JL (2008) Carcinoma of unknown primary with a colon-cancer profile-changing paradigm and emerging definitions. Lancet Onc 9: 596–9.
8. Abbruzzese JL, Abbruzzese MC, Hess KR, Hess KR, Raber MN, et al. (1994) Unknown primary carcinoma: natural history and prognostic factors in 657 consecutive patients. J Clin Oncol 12: 1272–80.
9. Dennis JL, Hvidsten TR, Wit EC, Komorowski J, Bell AK, et al. (2005) Markers of adenocarcinoma characteristic of the site of origin: development of a diagnostic algorithm. Clin Cancer Res 11: 3766–72.
10. Bloom G, Yang IV, Boulware D, Kwong KY, Coppola D, et al. (2004) Multi-platform, multi-site, microarray-based human tumor classification. Am J pathol 164: 9–16.
11. Giordano TJ, Shedden KA, Schwartz DR, Kuick R, Taylor JM, et al. (2001) Organ-specific molecular classification of primary thyroid, lung, colon, and ovarian adenocarcinomas using gene expression profiles. Am J Pathol 159: 1231–8.
12. Ramaswamy S, Tamayo P, Rifkin R, Mukherjee S, Yeang CH, et al. (2001) Multiclass cancer diagnosis using tumor gene expression signatures. Proc Natl Acad Sci USA 98: 15149–54.
13. Shedden KA, Taylor JM, Giordano TJ, Kuick R, Misek DE, et al. (2003) Accurate molecular classification of human cancers based on gene expression using a simple classifier with a pathological tree-based framework. Am J Pathol 163: 1985–95.
14. Su AI, Welsh JB, Sapinoso LM, Kern SG, Dimitrov P, et al. (2001) Molecular classification of human carcinomas by use of gene expression signatures. Cancer Res 61: 7388–93.
15. Tothill RW, Kowalczyk A, Rischin D, Bousioutas A, Haviv I, et al. (2005) An expression-based site of origin diagnostic method designed for clinical application to cancer of unknown origin. Cancer Res 65: 4031–40.
16. Huber W, von Heydebreck A, Sultmann H, Poustka A, Vingron M, et al. (2002) Variance stabilization applied to microarray data calibration and to the quantification of differential expression. Bioinformatics 18: S96–S104 (Suppl 1).
17. Kurahashi I, Ito Y, Matsuyama Y, Ohashi Y, Nishio K (2007) Evaluation of normalization methods for cDNA macroarray data (in Japanese). J Japan Statist Soc 36: 147–63.
18. Durbin B, Rocke DM (2003) Estimation of transformation parameters for microarray data. Bioinformatics 19: 1360–7.
19. Laird NM, Ware JH (1982) Random-Effects Models for Longitudinal Data. Biometrics 38: 963–74.

20. Benjamini Y, Hochberg Y (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing. J Roy Statist Soc 57: 289–300.
21. Hastie T, Tibshirani R, Friedman J (2009) The Element of Statistical Learning: Data mining, Inference, and Prediction (ed 2).New York, NY: Springer.
22. Anderberg MR (1973) Cluster analysis for applications. New York, NY: Academic Press.
23. Mani SA, Guo W, Liao MJ, Eaton EN, Ayyanan A, et al. (2008) The epithelial-mesenchymal transition generates cells with properties of stem cells. Cell 133: 704–15.
24. Rodnina MV, Wintermeyer W (2009) Recent mechanistic insights into eukaryotic ribosomes. Curr Opin Cell Biol 21: 435–43.
25. Ruggero D, Pandolfi PP (2003) Does the ribosome translate cancer? Nat Rev Cancer 3: 179–92.
26. Dai MS, Lu H (2008) Crosstalk between c-Myc and ribosome in ribosomal biogenesis and cancer. J Cell Biochem 105: 670–7.
27. Park JS, Yim JJ, Kang WJ, Chung JK, Yoo CG, et al. (2011) Detection of primary sites in unknown primary tumors using FDG-PET or FDG-PET/CT. BMC Res Notes 4: 56.
28. Schafer BW, Heizmann CW (1996) The S100 family of EF-hand calcium-binding proteins: functions and pathology. Trends Biochem Sci 21: 134–40.
29. Maelandsmo GM, Florenes VA, Mellingsaeter T, Hovig E, Kerbel RS, et al. (1997) Differential expression patterns of S100A2, S100A4 and S100A6 during progression of human malignant melanoma. Int J cancer 74: 464–9.
30. Melle C, Ernst G, Schimmel B, Bleul A, von Eggeling F (2008) Colon-derived liver metastasis, colorectal carcinoma, and hepatocellular carcinoma can be discriminated by the Ca(2+)-binding proteins S100A6 and S100A11. PloS one 3: e3767.
31. Strutz F, Okada H, Lo CW, Danoff T, Carone RL, et al. (1995) Identification and characterization of a fibroblast marker: FSP1. J Cell Biol 130: 393–405.
32. Tang DJ, Dong SS, Ma NF, Xie D, Chen L, et al. (2010) Overexpression of eukaryotic initiation factor 5A2 enhances cell motility and promotes tumor metastasis in hepatocellular carcinoma. Hepatology 51: 1255–63.
33. Li XJ, Ong CK, Cao Y, Xiang YQ, Shao JY, et al. (2011) Serglycin is a theranostic target in nasopharyngeal carcinoma that promotes metastasis. Cancer Res 71: 3162–72.
34. Ivaska J, Pallari HM, Nevo J, Eriksson JE (2007) Novel functions of vimentin in cell adhesion, migration, and signaling. Exp Cell Res 313: 2050–62.
35. Banh A, Zhang J, Cao H, Bouley DM, Kwok S, et al. (2011) Tumor galectin-1 mediates tumor growth and metastasis through regulation of T-cell apoptosis. Cancer Res 71: 4423–31.
36. Um JH, Kwon JK, Kang CD, Kim MJ, Ju DS, et al. (2004) Relationship between antiapoptotic molecules and metastatic potency and the involvement of DNA-dependent protein kinase in the chemosensitization of metastatic human cancer cells by epidermal growth factor receptor blockade. J Pharmcol Exp Ther 11: 1062–70.
37. Raj L, Ide T, Gurkar AU, Foley M, Schenone M, et al. (2011) Selective killing of cancer cells by a small molecule targeting the stress response to ROS. Nature 475: 231–4.
38. Jiffar T, Yilmaz T, Lee J, Hanna E, El-Naggar A, et al. (2011) KiSS1 mediates platinum sensitivity and metastasis suppression in head and neck squamous cell carcinoma. Oncogene 30: 3163–73.
39. Fu ZY, Lv JH, Ma CY, Yang DP, Wang T (2011) Tissue inhibitor of metalloproteinase-1 decreased chemosensitivity of MDA-435 breast cancer cells to chemotherapeutic drugs through the PI3K/AKT/NF-κB pathway. Biomed Pharmacother 65: 163–7.

Author Contributions

Conceived and designed the experiments: IK TK YK K. Nakagawa K. Nishio. Performed the experiments: YF YK KS. Analyzed the data: IK YF TA. Contributed reagents/materials/analysis tools: TK K. Matsumoto MT KT YT NY AT NM H. Mukai H. Minami NC YY K. Miwa Shin Takahashi Shunji Takahashi. Wrote the paper: IK YF K. Nishio.

40. Macha MA, Matta A, Chauhan S, Siu KM, Ralhan R (2010) 14-3-3 zeta is a molecular target in guggulsterone induced apoptosis in head and neck cancer cells. BMC cancer 10: 655.

41. Soucy TA, Dick LR, Smith PG, Milhollen MA, Brownell JE (2010) The NEDD8 conjugation pathway and its relevance in cancer biology and therapy. Genes & Cancer 1: 708–16.

42. Sokol JP, Neil JR, Schiemann BJ, Schiemann WP (2005) The use of cystatin C to inhibit epithelial-mesenchymal transition and morphological transformation stimulated by transforming growth factor-beta. Breast Cancer Res 7: R844–853.

43. Maier HJ, Schmidt-Strassburger U, Huber MA, Wiedemann EM, Beug H, et al. (2010) NF-kappaB promotes epithelial-mesenchymal transition, migration and invasion of pancreatic carcinoma cells. Cancer lett 295: 214–28.

44. Mao P, Hever MP, Niemaszyk LM, Haghkerdar JM, Yanco EG, et al. (2011) Serine/threonine kinase 17A is a novel p53 target gene and modulator of cisplatin toxicity and reactive oxygen species in testicular cancer cells. J Biol Chem 286: 19381–91.

45. Bandres E, Catalan V, Sola I, Honorato B, Cubedo E, et al. (2004) Dysregulation of apoptosis is a major mechanism in the lymph node involvement in colorectal carcinoma. Oncol Rep 12: 287–92.

46. Al-Hajj M, Wicha MS, Benito-Hernandez A, Morrison SJ, Clarke MF (2003) Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983–8.

47. Singh A, Greninger P, Rhodes D, Koopman L, Violette S, et al. (2009) A gene expression signature associated with "K-Ras addiction" reveals regulators of EMT and tumor cell survival. Cancer Cell 15: 489–500.

48. Kumar MS, Pester RE, Chen CY, Lane K, Chin C, et al.(2009) Dicer1 functions as a haploinsufficient tumor suppressor. Genes Dev 23: 2700–4.

49. Sun XX, Wang YG, Xirodimas DP, Dai MS (2010) Perturbation of 60 S ribosomal biogenesis results in ribosomal protein L5- and L11-dependent p53 activation. J Biol Chem 285: 25812–21.

50. de Marval PM, Lutfeali S, Jin JY, Leshin B, Selim MA, et al. (2011) CYLD inhibits tumorigenesis and metastasis by blocking JNK/AP1 signaling at multiple levels. Cancer Prev Res 4: 851–9.

51. Zhu Y, Poyurovsky MV, Li Y, Biderman L, Stahl J, et al. (2009) Ribosomal protein S7 is both a regulator and a substrate of MDM2. Molecular Cell 35: 316–26.

52. Iurisci I, Cumashi A, Sherman AA, Tsvetkov YE, Tinari N, et al. (2009) Synthetic inhibitors of Galectin-1 and -3 selectively modulate homotypic cell aggregation and tumor cell apoptosis. Anticancer Res 29: 403–10.

53. Zhao Y, Thomas HD, Batey MA, Cowell IG, Richardson CJ, et al. (2006) Preclinical evaluation of a potent novel DNA-dependent protein kinase inhibitor NU7441. Cancer Res 66: 5354–62.

54. Matta A, Siu KM, Ralhan R (2012) 14-3-3 zeta as novel molecular target for cancer therapy. Expert Opin Ther Targets 16: 515–23.

55. Kwee TC, Kwee RM (2009) Combined FDG-PET/CT for the detection of unknown primary tumors: systematic review and meta-analysis. Eur Radiol 19: 731–44.