Title	Temporal proteome analysis of cisplatin-resistant cancer cells for discovering novel regulation of cancer drug resistance
Sub Title	
Author	山久保, 純(Yamakubo, Jun)
Publisher	慶應義塾大学湘南藤沢学会
Publication year	2015
Jtitle	生命と情報 No.22 (2015.) ,p.40- 46
JaLC DOI	
Abstract	Many cases of cancer recurrence occur because some cancer cells acquire drug resistance and proliferate. This represents one of the major problems for cancer treatment. Acquisition of drug resistance in cancer cells has been investigated in many previous studies, but drug resistance has not yet been overcome, because resistant cells express a variety of proteins that contribute to drug resistance. Proteome analysis allows the comprehensive measurement of the expression of many proteins and identifies proteins that contribute to drug resistance. Additionally, phosphoproteome analysis can reveal signaling pathways involved in drug resistance. However, previous studies have focused on only one time point. The dynamic changes that occur after drug treatment in drug resistant cancer cells remain unknown. Here we compared the differential cellular responses to cisplatin in ovarian cancer cell lines that were drug sensitive (A2780) and resistant (A2780cis) to cisplatin, using temporal proteome and phosphoproteome analysis. By analyzing temporal data, the differential DNA damage and cell cycle responses to cisplatin in A2780 and A2780cis were highlighted, and A2780cis exhibited various drug resistance mechanisms to escape the effects of cisplatin. Additionally, we detected differences in the expression and phosphorylation of proteins that were not previously known to be involved in cisplatin resistance. From these proteins, we confirmed mRNA expression of FABP5, NOLC1, PHGDH, SDHA, and 14-3-3 sigma. In this study, we estimated the contribution of these proteins to drug resistance for applying cancer treatment.
Notes	慶應義塾大学湘南藤沢キャンパス先端生命科学研究会 2015年度学生論文集 修士論文ダイジェスト
Genre	Technical Report
URL	https://koara.lib.keio.ac.jp/xoonips/modules/xoonips/detail.php?koara_id=KO92001004-00000022-0040

Temporal proteome analysis of cisplatin-resistant cancer cells for discovering novel regulation of cancer drug resistance

Jun Yamakubo

Abstract

Many cases of cancer recurrence occur because some cancer cells acquire drug resistance and proliferate. This represents one of the major problems for cancer treatment. Acquisition of drug resistance in cancer cells has been investigated in many previous studies, but drug resistance has not yet been overcome, because resistant cells express a variety of proteins that contribute to drug resistance. Proteome analysis allows the comprehensive measurement of the expression of many proteins and identifies proteins that contribute to drug resistance. Additionally, phosphoproteome analysis can reveal signaling pathways involved in drug resistance. However, previous studies have focused on only one time point. The dynamic changes that occur after drug treatment in drug resistant cancer cells remain unknown. Here we compared the differential cellular responses to cisplatin in ovarian cancer cell lines that were drug sensitive (A2780) and resistant (A2780cis) to cisplatin, using temporal proteome and phosphoproteome analysis. By analyzing temporal data, the differential DNA damage and cell cycle responses to cisplatin in A2780 and A2780cis were highlighted, and A2780cis exhibited various drug resistance mechanisms to escape the effects of cisplatin. Additionally, we detected differences in the expression and phosphorylation of proteins that were not previously known to be involved in cisplatin resistance. From these proteins, we confirmed mRNA expression of FABP5, NOLC1, PHGDH, SDHA, and 14-3-3 sigma. In this study, we estimated the contribution of these proteins to drug resistance for applying cancer treatment.

Key Words

1. Cancer, 2. Drug resistance, 3. Cisplatin, 4. Proteomics, 5. Phosphoproteomics

1.Introduction and our study

Chemotherapy is frequently used because anti-cancer drugs can have adverse effects on many ovarian cancer patients. Cisplatin, one of the common anti-cancer drugs, has long been used as a conventional treatment[1]. This drug binds to the N7 atoms of guanine and adenine and induces cell death by interfering with DNA replication. However, many cases have been reported where cancers have relapsed after the shrinkage of a tumor following the use of chemotherapy; these relapsed cancers acquire drug resistance against the primary drug used and are often not affected by other drugs. Hence, cancer drug resistance has been a growing problem and complicates the treatment[2]. As an example of drug resistance mechanisms, transporters that are efflux pomp are confirmed to be overexpress in various resistant cells[3]. In addition to this mechanism, there are various other mechanisms of drug resistance such as DNA repair that is activated against a DNA targeted drug and conjugation that combines drugs for detoxicating[4,5]. Cancer cells acquire drug resistance by expressing a simultaneous plural drug resistance mechanism. Furthermore, protein phosphorylation is also known to contribute to drug resistance by interfering with apoptotic signaling and inducing cell survival. Since many proteins and phosphorylated proteins contribute to drug resistance, a comprehensive understanding of the problem is necessary to overcome such drug resistance. However, resistance mechanisms comprise complex networks and these systems are still unknown. Many researchers have used proteome analysis as an effective approach for comprehensive analysis such as targeting various proteins[6]. Proteome analysis can simultaneously identify and qualify various proteins. Furthermore, this analysis can also identify protein modification. Phosphoproteome analysis can search phosphorylation sites that reveal an overview of cell signaling[7]. These analyses have been successful for identifying many proteins that contribute to drug resistance and are applied to chemotherapy[8]. Almost previous studies investigated differentially expressed proteins between drug sensitive (parental) and resistant cancer cells using proteome

analysis and examined whether the proteins contribute to drug resistance[9–12]. However, these studies only focused on a single time point of protein expression and were not investigated dynamic change. In particular, cell signaling is important in the response to drugs, and the differential response between drug sensitive and resistant cells may be closely involved in drug resistance. Here we performed proteome and phosphoproteome analysis of cisplatin resistant ovarian cancer cells at several time points after cisplatin treatment; we aimed to understand the dynamic change in drug resistant cells and to identify novel proteins that critically contributed to drug resistance (Fig. 1). In this study, we predicted a novel protein candidate contributing to drug resistance by searching differential expression or response, and some candidate proteins were confirmed by mRNA expression.

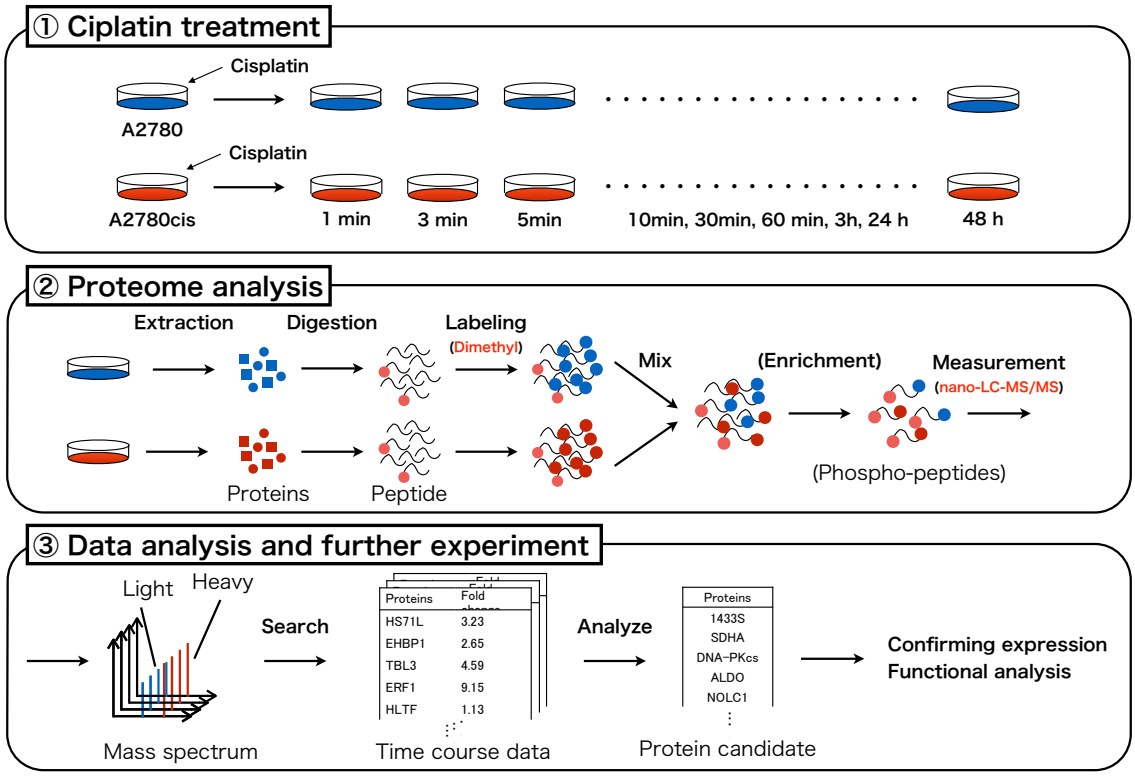


Figure 1. Overview of this study
An overview of this study is shown.

2. Materials and methods.
Materials and methods are omitted. Please check master thesis.

3. Results and discussions
3.1 Searching for novel drug resistance-related protein
We investigated novel candidate genes implicated in cisplatin resistance by the proteome analysis. We selected proteins up or down-regulated by over 1.5 fold at all time points in three duplicate experiments (Table 1, 2). The expressions of these proteins significantly differed between A2780 and A2780cis, as assessed using a t-test. Up-regulation of Galectin-1 has previously been reported in cisplatin-resistant ovarian cancer, consistent with our proteome data[13]. Adenosylhomocysteinase (ALDO), succinate dehydrogenase (ubiquinone) flavoprotein subunit, mitochondrial (SDHA), and D-3-phosphoglycerate dehydrogenase (PHGDH) are metabolic enzymes and their differential expression was consistent with the metabolome analysis performed by Guo Jing's study[14]. Additionally, down regulation of ALDO was previously reported in a study that performed proteome analysis of A2780cis[15]. As a result of this proteome analysis, we suggested that fatty acid-binding protein, epidermal (FABP5), nucleolar and coiled-body phosphoprotein 1 (NOLC1), SDHA, PHGDH,

nestin, and pericentriolar material 1 protein may contribute to cisplatin resistance. These proteins have not previously been reported to be involved in cisplatin resistance.

Table 1 Differentially expressed proteins at all time points

Protein name	Fold change (A2780cis/A2780)	p-value
Fatty acid-binding protein, epidermal	9.09	7.90E-32
Galectin-1	3.02	7.82E-20
Nucleolar and coiled-body phosphoprotein 1	12.88	1.29E-15
Adenosylhomocysteinase	3.96	2.20E-29
Succinate dehydrogenase [ubiquinone] flavoprotein subunit, mitochondrial	3.87	1.35E-17
14-3-3 protein theta	0.41	2.37E-16
Fructose-bisphosphate aldolase A	0.36	1.89E-21
Mesoderm-specific transcript homolog protein	0.16	1.20E-14
D-3-phosphoglycerate dehydrogenase	0.05	2.17E-29
Vinculin	0.39	2.73E-22
Fermitin family homolog 2	0.19	2.54E-19

This protein table shows differentially expressed proteins (more than 1.5 up- or down-regulated) between A2780 and A2780cis at all time point (N = 3). p-value was calculated by t-test.

Table 2 Differentially expressed phosphorylated proteins in all time points

Protein name	Fold change (A2780cis/A2780)	p-value
Nestin	0.18	1.24E-16
Pericentriolar material 1 protein	0.24	6.72E-17

This table shows proteins expression or phosphorylation which differs by more than 1.5 fold between A2780 and A2780cis cells at all studied time points (N = 3). p-value was calculated by t-test.

3.2 mRNA expressions of candidates by RT-PCR

We investigated mRNA levels of FABP5, NOLC1, SDHA, and PHGDH as novel candidates of cisplatin resistance using RT-PCR to confirm expression in A2780 and A2780cis from predicting by the proteome analysis (Table 1). Cellular levels of FABP5 mRNA were higher in A2780cis than in A2780 and consistent with our proteome analysis (Fig.2A).

Up-regulation of FABP5 and cellular retinoic acid binding protein-1 (CRABP1) are reported to induce cell survival and inhibition of retinoic acid used in cancer treatment[16]. By contrast, when FABP5 and CRABP1 expression is low and expression of CRABP2 is high, proliferation is suppressed. In this experiment, cellular levels of CRABP1 and CRABP2 mRNA were lower in A2780cis than in A2780 (Fig.2B). Although CRABP1 is highly expressed in retinoic acid-resistant cancer, its expression in cisplatin-resistant cells was low. However, high expression of FABP5 and low expression of CRABP2 in A2780cis is same pattern to retinoic acid resistance. Therefore, we suggested that A2780cis up-regulates FABP5 for promoting cell survival by increasing nuclear translocation of FABP5 and down-regulates CARBP2 for suppression of inhibiting cell proliferation by decreasing nuclear translocation of CARBP2.

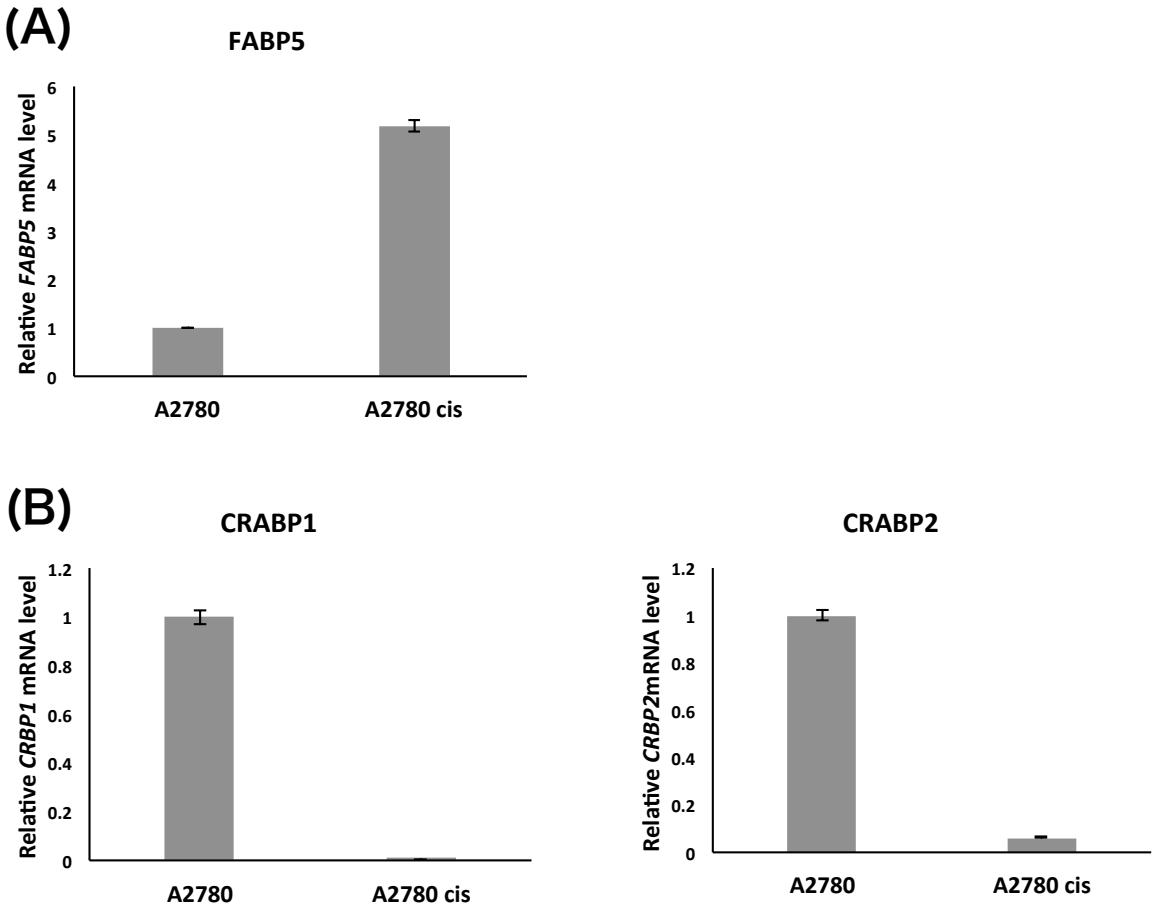
Expression levels of NOLC1 are higher in A2780cis than in A2780, as indicated by proteome analysis, but the difference of mRNA expression is very small (Fig.2C). NOLC1 is a transcription factor and may play a roll in proliferation and apoptosis[17]. Thus, it may be involved in cisplatin resistance.

Levels of PHGDH mRNAs were low in A2780cis and consistent with results of proteome and metabolome analysis (Fig.2D). PHGDH, belonging to upper stream of serine synthesis pathway, is known to activate DNA synthesis and support abnormal proliferation of cancer cells[18]. However, A2780cis down-regulate PHGDH, and it might suppress cisplatin-mediated DNA damage due to low expression levels of PHGDH.

Inconsistent with the proteome analysis, levels of SDHA mRNA were low in A2780cis (Fig.2E). But levels of fumaric acid, a metabolite of SDHA, were increased in A2780cis. Thus, results of proteome metabolome analysis are consistent with matabolome data, and a significant difference

between expression levels of SDHA and its metabolite in A2780 and A2780cis were observed. Therefore, some regulation in translation from mRNA to protein may reverse differences in expression between A2780 and A2780cis. SDHA is known to play a roll in complex II of mitochondrial electron transport, apoptosis, and proliferation[19]. Thus, up-regulation of SDHA may contribute inhibiting apoptosis in A2780cis.

We also confirmed dynamic changes in 14-3-3 sigma. RT-PCR revealed that 14-3-3 sigma in A2780 was largely up-regulated at 24 and 48 h after cisplatin treatment (Fig.2F). On the other hand, 14-3-3 sigma was not up regulated in A2780cis, and it consistent with proteome analysis. Therefore, this result reveals that expression of 14-3-3 sigma in A2780 was increased after cisplatin exposure than that in A2780cis. 14-3-3 sigma is implicated in DNA damage responses and has been reported to contribute to cisplatin resistance[20–22]. However, there are no reports about ovarian cancer with cisplatin resistance, and the reports of other types of cancer report inconsistent results about cisplatin resistant cells.



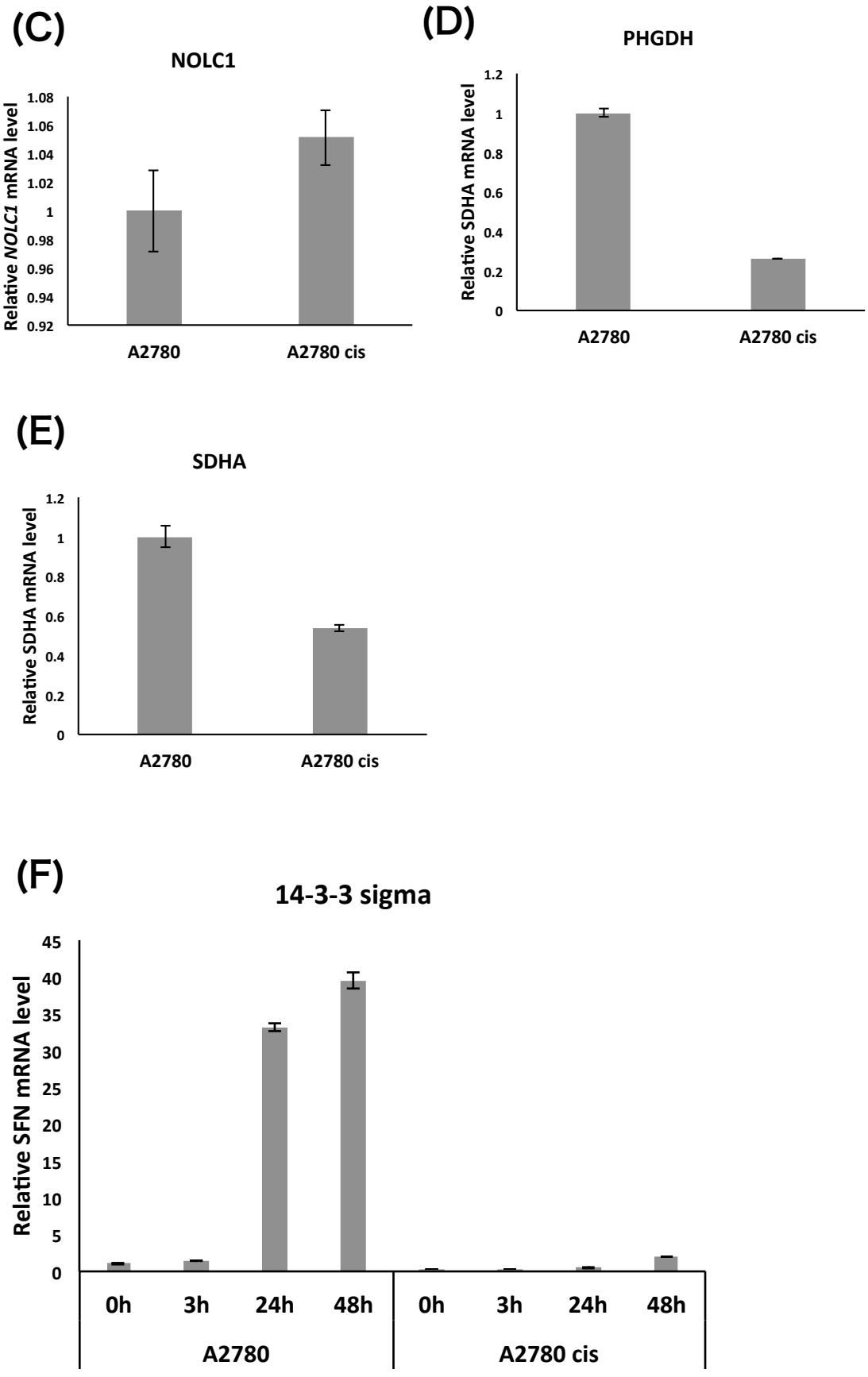

(C)
NOLC1
Relative NOLC1 mRNA level
1.08
1.06
1.04
1.02
1
0.98
0.96
0.94
0.92
A2780
A2780 cis
(D)
PHGDH
Relative SDHA mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
A2780
A2780 cis
(E)
SDHA
Relative SDHA mRNA level
1.2
1
0.8
0.6
0.4
0.2
0
A2780
A2780 cis
(F)
14-3-3 sigma
Relative SFN mRNA level
45
40
35
30
25
20
15
10
5
0
0h
3h
24h
48h
0h
3h
24h
48h
A2780
A2780 cis

Figure 2. mRNA expression of novel candidate related to drug resistance

mRNA expression of novel candidate in A2780 and A2780cis (normalized by expression of actin). (A–E) are relative mRNA levels in A2780 and A2780cis before cisplatin treatment. (F) is relative mRNA levels in A2780 and A2780cis after each cisplatin exposure time (0, 3, 24, and 48 h).

4. Conclusions

We predicted that proteins and phosphorylated proteins were expected to be involved in cisplatin resistance. Furthermore, we confirmed mRNA expression, and the results of FABP5, NOLC1, PHGDH, and 14-3-3sigma were consistent with proteome analysis. The expression of PHGDH and SDHA from proteome analysis is also consistent with the results of metabolome analysis. It was reported for the first time that the differential expression of these proteins (FABP5, NOLC1, PHGDH, and SDHA) might be due to acquired cisplatin resistance. Because FABP5 was up regulated and CRABP2 was downregulated in A2780cis, it suggests that cell survival as well as the retinoic acid resistance mechanism was induced. NOLC1 was also expected to contribute to cisplatin resistance because NOLC1 has some interactions of cancer factors and is involved in apoptosis. PHGDH expression level was recently reported to be increased and closely related to cancer in previous study; therefore, PHGDH was suggested to be a novel target for cancer treatment[23]. However, inhibiting PHGDH will not affect cisplatin-resistant cancer because PHGDH was down regulated in cisplatin-resistant cell in our study. 14-3-3 sigma expression was newly predicted to be differently dynamic change after cisplatin treatment in A2780 and A2780cis from proteome analysis, and a similar differentially dynamic change was observed for its mRNA expression. This protein belongs to the DNA damage response pathway, and it is suggested that A2780cis acquires cisplatin resistance due to unresponsiveness against cisplatin compared with the excessive response of A2780. From these candidates, we investigated the contribution of SDHA to cisplatin resistance using SDHA inhibitors. However, sensitizing cisplatin of A2780cis by SDHA inhibitors was not observed. In this study, we attempted to investigate only SDHA, but other candidates may contribute to cisplatin resistance and are expected to use as a novel cancer treatment for overcoming cisplatin resistance.

Acknowledgement

I would like to thank my adviser Dr. Masaru Mori who taught me about proteome analysis and gave much advice. Additionally, I would like to thank the Proteome group's member. Ms. Guo Jing helped me with my experiments and Mr. Tamaki taught me statistical and informatics analysis. Ms. Midori Saito and Ms. Sumiko Ohnuma also supported my experiment. Prof. Naoyuki Sugiyama provided guidance concerning analysis of proteomic data.

I would like to thank other groups. Dr. Kentaro Hayashi and Dr. Vincent Piras advised me with regards to analysis and cancer. Prof. Kumar Selvarajoo also provided support and advice throughout my studies.

Dr. Sho Tabata taught me how to perform the experiments, after proteome analysis, and Ms. Fumie Nakasuka performed RT-PCR experiments. I would like to thank these kind people.

Finally, I would like to express special gratitude to Prof. Masaru Tomita for providing me with the opportunity and environment to conduct my research so far.

Reference

1. Siddik, Z. H. Cisplatin: mode of cytotoxic action and molecular basis of resistance. *Oncogene* **22,** 7265–79 (2003).
2. Gottesman, M. M. Mechanisms of Cancer Drug Resistance. *Annu. Rev. Med.* **Vol. 53,** (2002).
3. Mccarthy, N. & Kirkpatrick, P. ABCs of drug resistance. *Nat. Rev. Cancer* **4,** 2004 (2004).
4. Holohan, C., Van Schaeybroeck, S., Longley, D. B. & Johnston, P. G. Cancer drug resistance: an evolving paradigm. *Nat. Rev. Cancer* **13,** 714–726 (2013).
5. Galluzzi, L. *et al.* Molecular mechanisms of cisplatin resistance. *Oncogene* **31,** 1869–83 (2012).
6. Larance, M. & Lamond, A. I. Multidimensional proteomics for cell biology. *Nat. Rev. Mol. Cell Biol.* **16,** 269–280 (2015).
7. Roux, P. P. & Thibault, P. The coming of age of phosphoproteomics--from large data sets to

inference of protein functions. *Mol. Cell. Proteomics* **12,** 3453–64 (2013).
8. Li, X. H., Li, C. & Xiao, Z. Q. Proteomics for identifying mechanisms and biomarkers of drug resistance in cancer. *J. Proteomics* **74,** 2642–2649 (2011).
9. Oyama, M. *et al.* Integrated quantitative analysis of the phosphoproteome and transcriptome in tamoxifen-resistant breast cancer. *J. Biol. Chem.* **286,** 818–29 (2011).
10. Buchi, F. *et al.* Acetylome and phosphoproteome modifications in imatinib resistant chronic myeloid leukaemia cells treated with valproic acid. *Leuk. Res.* **35,** 921–931 (2011).
11. Hekmat, O. *et al.* TIMP-1 Increases Expression and Phosphorylation of Proteins Associated with Drug Resistance in Breast Cancer Cells. *J Proteome Res* **12 ,** 4136–4151 (2013).
12. Liu, Y., Liu, H., Han, B. & Zhang, J.-T. Identification of 14-3-3 as a Contributor to Drug Resistance in Human Breast Cancer Cells Using Functional Proteomic Analysis. *Cancer Res.* **66,** 3248–3255 (2006).
13. Albrethsen, J., Angeletti, R. H., Horwitz, S. B. & Yang, C.-P. H. Proteomics of cancer cell lines resistant to microtubule-stabilizing agents. *Mol. Cancer Ther.* **13,** 260–9 (2014).
14. Jing, G. The role for glutamine as a key factor of cisplatin resistance in ovarian cancer cell line. *Master Thesis* 1–42 (2014).
15. Jin, L. *et al.* Down-regulation of Ras-related protein Rab 5C-dependent endocytosis and glycolysis in cisplatin-resistant ovarian cancer cell lines. *Mol. Cell. Proteomics* **13,** 3138–51 (2014).
16. Liu, R.-Z. *et al.* CRABP1 is associated with a poor prognosis in breast cancer: adding to the complexity of breast cancer cell response to retinoic acid. *Mol. Cancer* **14,** 129 (2015).
17. Hwang, Y.-C. *et al.* NOLC1, an enhancer of nasopharyngeal carcinoma progression, is essential for TP53 to regulate MDM2 expression. *Am. J. Pathol.* **175,** 342–54 (2009).
18. Gromova, I. *et al.* High level PHGDH expression in breast is predominantly associated with keratin 5-positive cell lineage independently of malignancy. *Mol. Oncol.* **9,** 1636–1654 (2015).
19. Kluckova, K. *et al.* Ubiquinone-binding site mutagenesis reveals the role of mitochondrial complex II in cell death initiation. *Cell Death Dis.* **6,** e1749 (2015).
20. Kim, I. *et al.* 14-3-3σ attenuates RhoGDI2-induced cisplatin resistance through activation of Erk and p38 in gastric cancer cells ABSTRACT: **4,** (2013).
21. Zheng, G. *et al.* 14-3-3σ regulation by p53 mediates a chemotherapy response to 5-fluorouracil in MCF-7 breast cancer cells via Akt inactivation. *FEBS Lett.* **586,** 163–8 (2012).
22. Peng, C. *et al.* The 14-3-3σ / GSK3β / β-catenin / ZEB1 regulatory loop modulates chemo-sensitivity in human tongue cancer. **6,** (2015).
23. Luo, J. Cancer's sweet tooth for serine. *Breast Cancer Res.* **13,** 317 (2011).