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Proteomic Analysis toward Understanding a Retinoid-Hypersensitive Embryonal Carcinoma Cell Mutant

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OVERVIEW

One of the great strengths of proteomics is its potential to aid in the generation of novel hypotheses and/or to lead to new insights into biological systems. RA functions on a cellular level to block proliferation, promote differentiation, and induce apoptosis, which make it an ideal candidate as a new type of anti-cancer drug. However, all-trans retinoic acid syndrome remains the major side effect of ATRA treatment. The aim of this study was to determine how RA works molecularly to identify those element most crucial for mediating and amplifying retinoid response in the process of tumor initiation and progression through comparison of a tumorigenic vs. a mutant non-tumorigenic embryonal carcinoma (EC) cell line. This application described how the use of iTRAQ isobaric tagging combined with off-line multidimensional liquid chromatography have enabled the identification and differential profiling of low level proteins from cancer cell lines.

INTRODUCTION

Retinoic acid (RA) functions as a crucial signaling molecule that promotes differentiation in the developing limb, CNS, heart, vertebrae and neural crest cells. RA also functions on a cellular level to block proliferation, promote differentiation, and induce apoptosis. Treatment with physiological levels of RA promote exit from the cell cycle and exposure to excess levels of RA can also result in programmed cell death, [i.e., apoptosis]. These activities of RA – i.e., promoting cell cycle arrest, differentiation, apoptosis – make it an ideal candidate as a cancer therapeutic agent. One approach to maximizing the benefits and minimizing the drawbacks of retinoid anti-cancer therapy is to determine exactly how retinoids work molecularly to identify those elements most crucial for mediating and amplifying retinoid response in tumor cells. In order to identify genes/proteins that might regulate differentiative and/or oncogenic potentials, we used a mutagenesis scheme to create an EC mutant (NR1-6), containing only a single retroviral insert, which is morphologically aberrant, no longer tumorigenic in syn-geneic mice, and demonstrates an extreme hypersensitivity to RA. In order to identify the cause of the RA hypersensitivity, quantitative proteomic analysis has been employed to identify the potential RA-targets in the EC cell line.

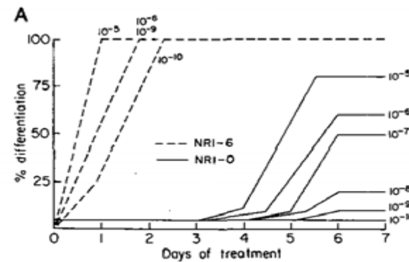


Figure 1: Response of NR1-0 and NR1-6 cells to varying RA concentration. Cells were grown in monolayer culture in DMEM with 10% FCS and RA at indicated molar concentrations. Differentiation was examined microscopically and determined by alteration in cell morphology.

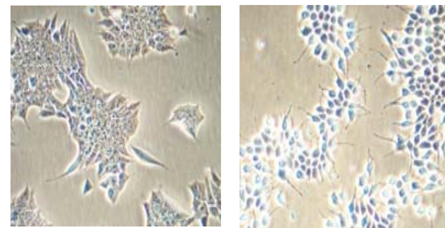
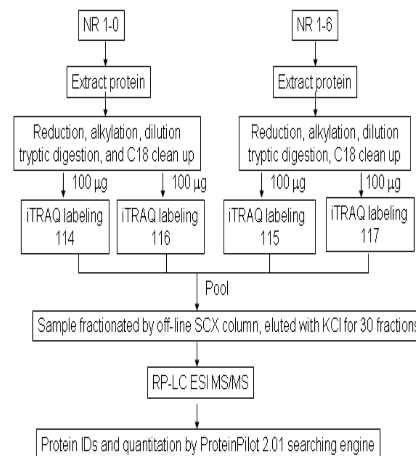


Figure 2. Morphology of NR1-0 and NR1-6

EXPERIMENTAL



A scheme of the work flows of four-channel iTRAQ analysis

Protein preparation and iTRAQ isobaric labeling. EC Cells (6×10^6) were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. The cells were washed with cold PBS 3 times, lysated in 400 µl sample buffer (100 mM Tris-HCl, 3%SDS, 10 mM DTT, pH 7.5), and followed by ultracentrifugation. Acetone precipitation was used to pellet the protein mixture in the supernatant. The protein mixture mixtures were re-dissolved in iTRAQ buffer followed by reduction and alkylation. Protein concentration was measured by MicroBCA. iTRAQ labeling was followed according to the manufactory.

Off-line strong cation exchange chromatography. The labeled samples were then pooled and acidified to a total volume of 2.0 ml followed by injection into a Agilent 1100 system with a Zorbax 300-SCX column (4.6 ×250 mm). Fractions of 25-30 were collected and dried by a speed-vac prior to LC-MS/MS analysis.

On-line Nano-LC ESI QqTOF MS Analysis. The nanobore LC system was from Dionex and interfaced to a QSTAR XL QqTOF mass spectrometer with a NanoSpray ion source). The Picofrit column (Magic C18, 100 A pore 75 µm ID ×150 mm) was packed in house. Peptides mixture (reconstituted in 125 µl of 5% formic acid) was injected and eluted from the column with a 110 min mobile phase B gradient. Identification and quantitation was performed using ProteinPilot 2.01 software.

RESULTS

Table 1 – Example of differentially expressed proteins as determined by iTRAQ

N	Unused	Total	% Cov	Accession #	Name	115:114	116:114	117:114
1	81.63	81.63	91.3	gi|123681	RecName: Full=Heat shock protein HSP 90-beta; AltNa...	0.9643	0.9862	0.9634
2	54.96	54.96	90.1	gi|114152161	RecName: Full=Serine/arginine repetitive matrix protein 2	0.9884	1.0030	0.9903
3	49.36	49.36	79.6	gi|128843	RecName: Full=Nucleolin; AltName: Full=Protein C23	1.0422	0.9974	1.0762
4	46.13	46.13	77.2	gi|81869784	RecName: Full=A-kinase anchor protein 12; AltName: F...	1.0047	1.0113	0.9777
5	39.99	39.99	84.8	gi|13959325	RecName: Full=Nucleolar RNA helicase 2; AltName: Fu...	0.9377	1.0285	0.9078
6	36.24	36.24	93.9	gi|46397854	RecName: Full=DNA replication licensing factor MCM2;...	1.0647	1.0091	1.0353
7	36.07	36.07	76.7	gi|3183181	RecName: Full=Transcription intermediary factor 1-beta...	0.8850	1.1035	0.8062
8	34.08	34.08	90.6	gi|6753036	mitochondrial aldehyde dehydrogenase 2 [Mus musculus]	0.9310	1.0098	0.9376
9	29.05	29.05	85.4	gi|81914516	RecName: Full=Eukaryotic translation initiation factor 3...	1.0115	1.0275	0.9792
10	27.75	27.75	84.0	gi|267150	RecName: Full=DNA topoisomerase 2-alpha; AltName:...	1.0461	1.0101	1.0321
11	26.73	26.73	77.3	gi|29293809	ATP citrate lyase [Mus musculus]	0.8900	1.0159	0.9367
12	25.73	25.73	96.9	gi|1526545	14-3-3 epsilon [Mus musculus]	0.8764	0.9910	0.9112

❖iTRAQ isobaric labeling coupled with off-line multidimensional chromatography has been developed and applied for analysis protein changes in a cancer cell line

❖ Total of 25-30 SCX fractions were used for the LC-MS/MS analysis resulted in 10498 MS/MS spectra. Total of 3638 spectra were identified with a confidence of 99%

❖ Over 500 proteins including integral membrane proteins, hydrophobic proteins and proteins with extreme pIs have been identified in an EC mutant cell line.

❖Up-regulated proteins by retinoic acid in hyposensitive cell line include: ribosomal protein, thymosin, splicing factor, and dihydropyrimidinase-like 3 isoform1.

❖Down-regulated proteins include: KRAB-A-interacting protein, ATP citrate lyase,14-3-3 epsilon, Calnexin; Flags: Precursor, promyelocytic leukemia isoform 2, Facilitates chromatin transcription complex subunit SSRP1, Hemidesmosomal plaque protein, Cullin-3, Mammalian branch point-binding protein mBBP, 60S ribosomal protein L22-like 1, Ankyrin repeat and coiled-coil structure-containing protein, Zinc finger homeobox protein 4, Palladin, Activated RNA polymerase II transcriptional coactivator p15, Alcohol dehydrogenase class 4 mu/sigma chain.

ACKNOWLEDGEMENTS

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