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Human Fibroblasts for Large-Scale “Omics” Investigations of ATM Gene Function

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Abstract

ATM (gene mutated in ataxia-telangiectasia) is a critical central component of the pleiotropic responses of cells to ionizing radiation-induced stress. To gain insight into molecular mechanisms and to enhance our understanding of ATM functions, we have advanced a human model cell system, derived from genetically defined immortal fibroblasts, and we have applied high-throughput genomic, proteomic and metabolomic technologies for a systems level analysis. The cellular characterizations reported here provide the background for application of a systems analysis to integrate transcription, post-translational modifications and metabolic activity induced by exposure of cells to ionizing radiation. We present here a summary of the derivation and characterization of cells comprising this model cell system and review applications of this model to systems analysis of ATM functions.

15.1 Introduction

Investigations of the human genetic disease, ataxia-telangiectasia (AT) have led to the discovery of ATM, the gene mutated in AT, as the central factor leading to a complex clinical syndrome marked by progressive neurological degeneration, as well as immunological deficiency and extreme sensitivity to ionizing radiation [1–3]. Established primary cells, lymphoid and fibroblasts derived from AT patients are available; however, growing such cells has proven difficult and most mechanistic studies utilize virus immortalized cell lines. We have used one such cell line, AT5BIVA to develop a model human fibroblast system for investigating the role of ATM in regulating gene expression, protein expression and post-translational modification, as well as metabolite generation. Here we characterize the cells and demonstrate feasibility for high-throughput analysis to globally define ATM mediated cellular responses in the genetically defined model cell system. Bioinformatic integration of the genomic, proteomic and metabolomic analyses using commercially available software permits a systems view of cellular responses to radiation stress.

Although the clinical syndrome of AT is multi-faceted, the disease is attributed to mutation in the single gene, ATM [4]. ATM spans more than 150 kb, consisting of 66 exons and transcribing a 13-kb transcript. The 3,056 amino acid gene product belong to the PI-3 kinase family of proteins and functions by phosphorylating and activating key molecules involved in cell cycle regulation, DNA repair, immune response, transcriptional regulation and genomic stability [4–6].

The activation of ATM in response to DNA damage results in phosphorylation of proteins involved in critical cellular processes, including cell cycle regulation and DNA repair. The phosphorylation cascade ultimately leads to transcriptional activation, and siRNA silencing of ATM has shown a significant impact on the transcriptional profile in the cell [7]. To our knowledge, there has been no comprehensive analysis of global gene expression changes in human cells in which ATM function has been restored. Therefore, our initial aim was to establish model cells suitable for investigating ATM-dependent and ATM-independent response to ionizing radiation exposure.

15.1.1 Establishment of the (ATM $\pm$) Model Cell System

To establish a model cell system for gene expression analysis we selected AT human fibroblasts (AT5BIVA) with a known mutation in ATM, which leads to a truncated gene product. Introduction of the full-length *ATM* in a pcDNA3 expression vector resulted in a clonal cell line (ATCL8) with corrected radiation phenotype. A second important cell line was established following gene transfer and radiation selection experiments [8]. Cell line ATCL11 was found to have normal radiation response parameters in a background of mutant ATM. These cells have been previously reported and represent ATM-independent enhancement of cellular responses to radiation exposure attributed to the introduction of a mutated I κ B- α , modifying cellular NF- κ B regulation [8]. Figure 15.1 provides an overall schema of cell line derivation.

15.1.2 Characterization of Fibroblast Cell Lines

The radiation responses shown in Fig. 15.2 illustrate the survival of AT5BIVA cells to graded doses of γ -radiation exposure. Parameters derived from the single hit, multitarget model of cellular radiation survival, D_0 determinations provide a convenient assessment of radiation sensitivity as illustrated in Table 15.1 [9]. The smaller the value of D_0 , the more sensitive are the cells to radiation killing. In our laboratories, cells with normal responses to ionizing radiation demonstrate D_0 values ranging from 1.2 to 1.7 Gy. Together, these several cell lines provide a unique, genetically defined model of the extremely sensitive fibroblasts of AT, AT5BIVA and the pcDNA transfected cells, as well as the radiation sensitivity complemented ATCL8 derived by the introduction of the “wild type” ATM. The cell line, ATCL11, contains a truncated I κ B- α , a dominant negative factor which complements the radiation responses of AT5BIVA cells in an ATM-independent manner [8]. The MRC5CV1 cells are immortal human fibroblasts with “normal” radiation sensitivity ($D_0 \cong 1.4$ Gy) but in a different genetic background, serving as additional controls in radiation studies.

15.1.3 Gene Expression Profiling in Human AT Fibroblasts

ATM has been implicated as a primary DNA damage sensing molecule in the cell [9]. To assess the effect of ATM on transcriptional regulation, we investigated gene expression patterns of the several AT5BIVA derived cells. A line graph of microarray analyses in Fig. 15.3 compares basal gene expression levels of cells in exponential growth showing the impact of ATM gene product, resulting in enhanced and suppressed gene “outliers.” To assure reproducibility and quality of the data, experiments were performed in triplicate and samples were split prior to cRNA library preparation. This resulted in the analysis of six microarray chips per experimental point. Multidimensional scaling and gene-tree analysis of these samples from the genetically defined cell lines confirmed distinct separation by cell line, as reported elsewhere [10].

The expression differences demonstrated by microarray data were validated by quantitative Real-Time PCR (qRT-PCR) assays (Table 15.2). All samples were normalized to GAPDH controls. Overall, expression trends were remarkably consistent with data obtained by array analyses, albeit the more sensitive qRT-PCR generally showed higher expression levels.

15.1.4 Proteomic Studies of AT Cells

The product of the ATM gene triggers signaling cascades that regulate DNA damage and repair, cell cycle and apoptosis and stress response. These signaling pathways are governed by translational and post-translational events, leading to the need for analysis of differential protein levels. Since the primary focus has been on the radiation stress response, global changes in the proteome were determined by comparing 2-D gel patterns with and without radiation exposure [11].

A total of 435 and 630 differentially expressed proteins were identified for AT5BIVA and ATCL8 cell lines, respectively, across the time course study. We selected proteins with a high confidence score (>95%) for gene ontology analysis which showed a predominance of proteins involved in signaling, transcription, cell cycle and cytoskeletal structure and regulation. As an example, Table 15.3 summarizes selected differential protein levels as a function of time after exposure to ionizing radiation. Since 2-D gel analysis reflects extent of post-translational modifications, as well as changes in expression levels, the interpretation of upregulated and downregulated events are less relevant than the observations of changes in levels of a given protein or modified status.

Previously, we reported the effects of ATM on differential protein levels and/or post-translational modifications under basal conditions, by resolving whole cell protein lysates on 2-D gel electrophoresis [12]. A predominant representation of proteins was involved in pathways regulating cancer progression, cell growth and proliferation, cell death, small molecule biochemistry and amino acid metabolism. The pathways for purine, pyrimidine and amino acid metabolism were significantly altered in ATCL8 as compared to AT5BIVA [12]. Moreover, the integrated analysis identified significant ATM impact on RRM2, a potential constriction point in purine metabolism [12].

15.1.5 Metabolomic Studies of AT Cells

To determine effects of ATM restoration on levels of metabolic products, we performed ultra-performance liquid chromatography coupled with time of flight (UPLC TOF) mass spectrometry. The resultant features were analyzed by multivariate data analysis to yield ion rankings for putative identification of biomarkers showing relative changes in metabolomic profiles. These ions comprised the dataset for mass based chemical identification using the Madison Metabolomic Consortium Database (MMCD). KEGG (Kyoto Encyclopedia of Genes and Genomes) IDs associated with metabolites showing significant fold changes and *p*-values for functional pathway analysis using “Ingenuity” software (ingenuity pathway analysis, IPA). Pathways showing significant perturbations included purine, pyrimidine and aminosugars metabolism (Fig. 15.4) [11].

15.1.6 Integration of Databases

To integrate the several functional pathways, the proteomics and microarray datasets were scored for differentially expressed proteins or genes known to participate in the common metabolic pathways using the IPA tool. As an example, Table 15.4 illustrates pathway integration across all three levels of expression for purine metabolism. The selected ions representing small molecule metabolites were validated by mass spectrometry and empirical formulae by comparing MS/MS fragmentation patterns and retention times of the target ion and the standard metabolite [11].

The work summarized here utilized the iProX-press proteomic analysis system, the commercial software, Ingenuity (IPA) and the KEGG (<http://www.genome.ad.jp/kegg/>) pathway, one of the most widely used databases with curated pathway maps for metabolism and other cellular processes.

15.2 Discussion

We have described a genetically defined model cell system, established by transferring genes into the mutant AT5BIVA fibroblasts and selecting for clones exhibiting correction of the radiation sensitive phenotype. A full-length ATM expressing vector was used to derive ATCL8 and Western analysis has demonstrated characteristic ATM phosphorylation at serine 1981 1 h following exposure of cells to ionizing radiation in ATCL8, but not in AT5BIVA, confirming the presence of an activated ATM in ATCL8. Further characterization of the ATM competent and ATM deficient cells has included transcriptomic, proteomic and metabolomic studies, followed by an integrated analysis.

Collectively, our data show that ATM expression results in profound changes in the radiation phenotype and in gene expression. Comparisons of gene expression patterns of AT5BIVA to cells expressing a functional ATM following radiation exposure, demonstrate an attenuated, less robust response in AT5BIVA. Differences were observed in 15 functional categories, including those expected; such as apoptosis regulation, cell cycle and growth regulation. Therefore, these cells should permit detailed evaluation of ATM-dependent and ATM-independent radiation responses of cells.

The integrated systems approach to the global studies of cellular processes identifies common links at the mRNA, protein and small molecule metabolite levels. Purine metabolism was found to be a major ATM mediated pathway at all three levels of analysis.

Finally, this report shows the feasibility of performing integrated “omics” analysis for studying global changes that result from complex perturbations in a single gene. The technical aspects of the approach extending from genes to metabolites by integration within metabolic pathways have been demonstrated. The data highlight potential biomarker discovery and hypothesis generation in studies of complex phenotypes within the ATM model fibroblast system.

15.3 Materials and Methods

15.3.1 Cell Cultures

AT5BIVA and MRC5CV1 cells were obtained from the National Institute of General Medical Sciences (NIGMS). Cells were maintained in modified Eagle's medium with 20% fetal bovine serum, 100 U/penicillin, and 100 pg/ml streptomycin. Cells were grown to 80% confluence and serum starved for 24 h for experiments. The ATCL8 cell line was established by transfecting AT5BIVA cells with the wild type, full-length “ATM” in a pCDNA expression vector and selected by screening for correction of radiation sensitivity.

15.3.2 Affymetrix Microarray Analysis

Total RNA was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA). RNA labeling and hybridization were performed according to the Affymetrix protocol for one-cycle target labeling. For each experiment, fragmented cRNA was hybridized in triplicates to Affymetrix GeneChip HG-U95 arrays (Affymetrix, Santa Clara, CA). Affymetrix data analysis included pre-processing of the probe-level Affymetrix data (CEL files). We applied RMA for background adjustment, quantile method for normalization and the “median polish” for summarization. The triplicate arrays representing the same subject were averaged. Probe sets were considered statistically significant if their *p*-values were less than 0.001. Pathway analysis was performed with Database for Annotation, Visualization and Integrated Discovery (DAVID).

15.3.3 Quantitative RT-PCR

Total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). qRT-PCR was performed in triplicate using TaqMan Gene Expression Assays (Applied Biosystems) on the Applied Biosystems 7900HT Fast Real-time PCR System. Amplification of 18 S rRNA provided endogenous control to standardize the amount of sample added to the reaction. The comparative cycle threshold (CT) method was used to analyze the data by generating relative values of the amount of target cDNA (Applied Biosystems). The statistical analyses of these data were performed with a two-sided *t* test since the expression data showed normal distribution.

15.3.4 Two-Dimensional Protein Gel Electrophoresis

Five hundred microgram of each whole cell lysate were focused on the Protean IEF Cell from Bio-Rad. Proteins were resolved on a second dimension on 12% SDS-PAGE gels, fixed and stained with coomassie blue G250. Protein patterns were compared using Dimension imaging software (version 1.5, Syngene).

15.3.5 In-Gel Tryptic Digestion And Protein Identification by Mass Spectrometry

The protein spots of interest were manually excised from 2-D-gels and processed as previously described [13]. Tryptic peptides were extracted in acetonitrile and mass spectra were recorded with a matrix assisted laser desorption/ionization-time of flight, time of flight (MALDI-TOF-TOF) spectrometer (4700 Proteomics Analyzer, ABI, USA).

Peptide masses were compared with the theoretical masses derived from the sequences contained in Swiss-Prot databases using MASCOT. A subset of predicted proteins was validated using western blotting and the trend with respect to fold change was observed to be consistent with the experimental findings. For a time course comparisons of molecular networks, 30 min and 3 h samples were selected as representative of early and late responses following radiation exposure. Functional pathways were determined using the ingenuity pathway analysis (IPA).

15.3.6 Mass Spectrometry for Metabolomic Analyses

Samples were prepared and subjected to ultra-performance liquid chromatography-time of flight mass spectrometry (UPLC TOF MS). All reference chemicals were purchased from Sigma, St. Louis, MO. All solvents were LCMS grade (Fisher Scientific, USA– Optima grade).

15.3.7 Western Analyses

Protein extracts of AT5BIVA and ATCL8 cells were resolved by 1-D gel electrophoresis and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore Corporation, Bedford MA). Antibodies were purchased from Santa Cruz Biotechnology (USA). Antiphosphorylated serine 1981 ATM antibody was procured from Rockland Chemicals, USA.

15.3.8 Functional Pathway Analyses

IPA software was used to generate the pathways that are related to transcripts, proteins and metabolites. Network analysis generated functional association networks based on curated literature information of protein interaction, co-expression, and genetic regulation.

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**Model Cell System is Based on AT5BIVA
Human Fibroblasts**

Fig. 15.1.
Schematic diagram of cell model system

Fig. 15.2.

Radiation clonogenic survival of AT cells. Logarithmically growing cells were exposed to graded doses of γ -radiation. Clonogenic survivors were measured and fit to the single-hit, multitarget and linear quadratic models as shown in a semi-logarithmic plot of the data. Points and bars represent means of SEMs from triplicate flasks

Fig. 15.3.
Line graph of differential gene expression comparing AT5BIVA, vector control, ATCL8 and ATCL11 cells

Fig. 15.4.

Canonical pathways associated to the metabolites that showed significant changes in expression in response to radiation exposure of ATCL8 (**a**) and AT5BIVA (**b**) cells, respectively

Table 15.1

Radiobiological parameters of model human fibroblasts

Cell line	Radiation category	(Gy)	$\tilde{N}$
AT derived cells and controls			
AT5BIVA	ERS	0.8	2.7
AT5BIVA/pcDNA	ERS	0.8	3.5
ATCL8	RS	1.6	1.2
ATCL11	RS	1.6	1.0
MRC5CV1	RS	1.4	5.2

ERS extreme radiation sensitivity; *RS* normal level of radiation sensitivity

Table 15.2

qRT-PCR validation of microarray determined expression differences comparing ATCL8 to AT5BIVA

Mean fold change (SEM)			
Accession number	Description	PCR	Microarray
MCL1	Myeloid cell leukemia sequence	−2.0 (0.183)	−2.1 (0.381)
RELA	Reticuloendotheliosis viral oncogene	1.52 (0.123)	1.88 (0.166)
GAS6	Growth arrest specific 6	−6.96 (1.64)	−10.24 (5.29)
CASP3	Caspase 3	−1.62 (0.258)	−1.55 (0.273)
CDH11	Cadherin 11	16.0 (2.39)	17.28 (1.71)
EPHB1	Ephrin B1	21.11 (5.87)	21.07 (3.29)
ATM	Ataxia telangiectasia mutated	34.30 (4.76)	20.17 (10.73)
SLC16A4	Solute carrier family 16	−13.93 (9.87)	−21.72 (16.83)
HDAC1	Histone deacetylase 1	2.30 (0.496)	1.47 (0.097)

Table 15.3

Summary of selected proteins, expressed differentially in ATCL8 cells determined at indicated intervals following experiments 5 Gy γ -radiation

Protein name (transcription factors)	30 min	1 h	3 h	24 h
(Q99497) DJ-1 protein	ND	↓	↓	ND
(P67809) Nuclease sensitive element binding protein	ND	↑	↑	ND
(O60675) Transcription factor MafK	ND	↑	ND	ND
(Q86WZ6) Zinc finger protein 227	ND		ND	ND
(Q99676) Zinc finger protein 184	ND		ND	ND
(Q9Y265) RuvB-like 1	ND	ND		ND

Protein name (stress response)	30 min	1 h	3 h	24 h
(P07900) Heat shock protein – 90 alpha	↑	↑	↑	↑
(P08107) Heat shock protein 70	↓	↓	↓	↓
(P11021) 78 kDa glucose regulated protein	ND	↓	↑	↑

Protein name (DNA/RNA/protein processing)	30 min	1 h	3 h	24 h
(P24534) Elongation factor 1	↓	↓	ND	ND
(P31942) Heterogeneous nuclear riboprotein	↓	ND	ND	ND
(P17987) T-complex protein 1	↑	ND	ND	↓
(P49411) Elongation factor Tu	ND	↑	↓	↓
(P31948) Stress-induced phosphoprotein	ND	↓	↓	↓
(P19338) Nucleolin	ND	↓	ND	ND
(Q01105) SET protein	ND	ND	↓	ND
(P35232) Prohibitin	ND	ND	↓	ND

Protein name (cell cycle/proliferation)	30 min	1 h	3 h	24 h
(Q05682) Caldesmon	ND	ND	↑	ND
(O60861) Growth arrest specific protein	ND	↓	ND	ND
(P12004) Proliferating cell nuclear antigen	ND	↓	ND	ND
(P11802) Cell division protein kinase 4	ND	ND	ND	↓
(P82979) Nuclear protein Hcc-1	ND	ND	ND	↑
(Q9HCT0) Fibroblast growth factor-22	ND	ND	ND	↑

Table 15.4

Functional pathway analysis for network integration of “3-omics” data

Pathway	Genes			Proteins			Metabolites		
	Symbol	Description	ATCL8/AT5B1A	Symbol	Description	Acc ID	ATCL8/AT5B1A	Name	KEGG ID
Purine metabolism	ABCC1	ATP-binding cassette	↓	ACTC1	Actin	P68032	↓	Adenine	C00147
	ADSL	Adenylosuccinate lyase	↓	ATP5B	ATP synthase	P06576	↓	Adenosine	C00212
	ATP1B1	ATPase	↓	BAT1	HLA-B	Q13838	↑	AMP	C00020
	ENPP2	Phosphodiesterase 2	↓	DDX39	DEAD	O00148	↑	GMP	C00144
	POLA2	DNA polymerase	↑	MYH1	Myosin	P12882	↓	Guanine	C00242
	POLR3C	RNA polymerase III	↓	POLRMT	RNA polymerase	O00411	↓	Xanthine	C00385
	SMARCA5	SWI/SNF related	↓	PSMC4	Proteasome	P43686	↓		
	TRAP1	TNF receptor-associated	↓	RUVBL2	RuvB-like 2	Q9Y230	↑		