論文内容の要旨

論文題目

Identification of EPSIN3 and CYSTATIN C as novel p53-inducible targets and their functional analysis

（*p53* 新規下流標的としてのエプシン 3, シスタチン C の同定および機能解析）

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Background

p53 is one of the most intensively studied tumor suppressor genes. Recent cancer genomic analyses using next-generation sequencing technique revealed that mutations of the *p53* gene are still the most common alteration observed in the majority of human cancers. Alterations in the *p53* gene are clustered in its DNA-binding domain, implying that transactivation of target genes is pivotal mechanism of tumor suppressive function of p53, and therefore identification and functional analysis of p53-targets are of importance in cancer research. A number of p53 target genes have been previously isolated; however, the full picture of the p53 downstream pathway still remains to be elucidated. To identify novel p53 targets, we here performed transcriptome and proteome analysis, and we further analyzed the function of the newly identified genes.

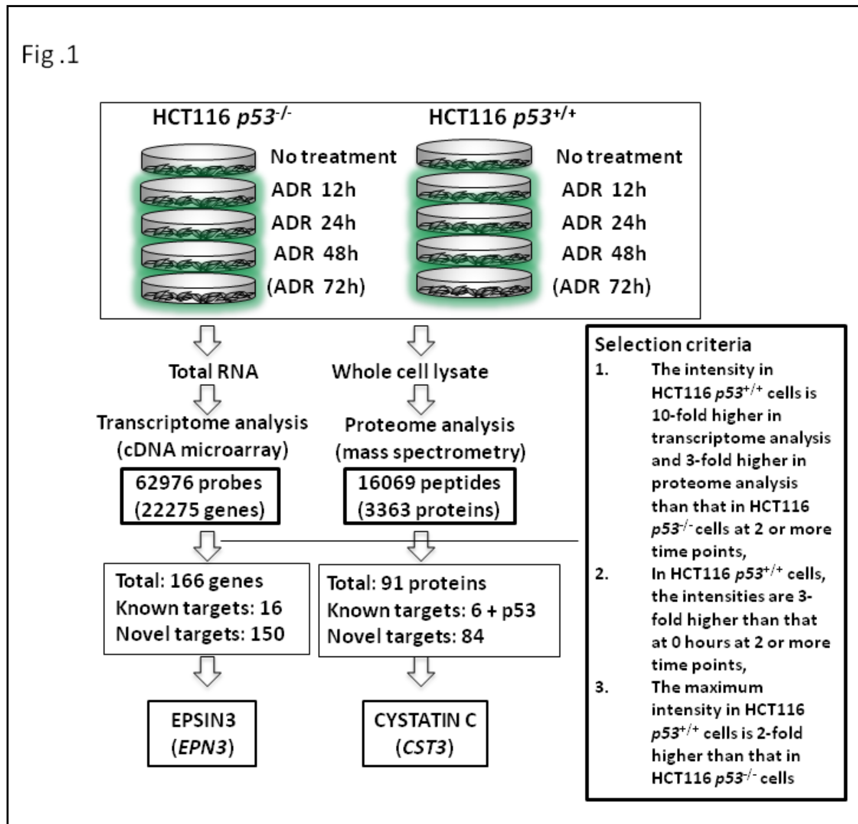
Materials and Methods

To identify genes and proteins which are induced by DNA damage in a p53-dependent manner, cDNA microarray and mass spectrometry analysis were performed using human colon adencarcinoma cell lines HCT116 *p53*$^{-/-}$ and HCT116 *p53*$^{+/+}$ treated with adriamycin (ADR). Among potential candidates, we selected EPSIN3 and CYSTATIN C from transcriptome and proteome analysis for further functional analysis, respectively. Quantitative real-time PCR (qPCR) and western blotting analysis were performed to verify the results of screening step. To investigate whether p53 transactivates these candidates and directly binds to the genomic regions, gene reporter assay and chromatin immunoprecipitation assay were conducted, respectively. To elucidate the role of EPSIN3 and CYSTATIN C in proliferation and apoptosis of cancer cells, cell proliferation analysis using ATP measurement assay and western blotting analysis using anti-caspase3 specific antibody were conducted using ADR treated cells after gene knockdown by small interference RNAs (siRNAs).

Results

Screening of p53-targets and verification of the results

To identify novel p53 targets, we conducted cDNA microarray analysis and mass spectrometry analysis using mRNAs and whole cell lysates, respectively, isolated from HCT116*p53*$^{+/+}$ and HCT116 *p53*$^{-/-}$ cells that were treated with 2 μg/ml of ADR. RNA and protein were collected at 0, 12, 24, 48, and 72 h after ADR treatment. We obtained expression data of totaling 22,275 genes from the transcriptome analysis and 3,363 proteins from the proteome analysis. To screen candidates that were induced by ADR damage in a p53-dependent manner, we applied the criteria as shown in Fig.1. After this screening, we identified 166 genes and 91 proteins including 16 and 6 known p53-downstream targets, respectively.

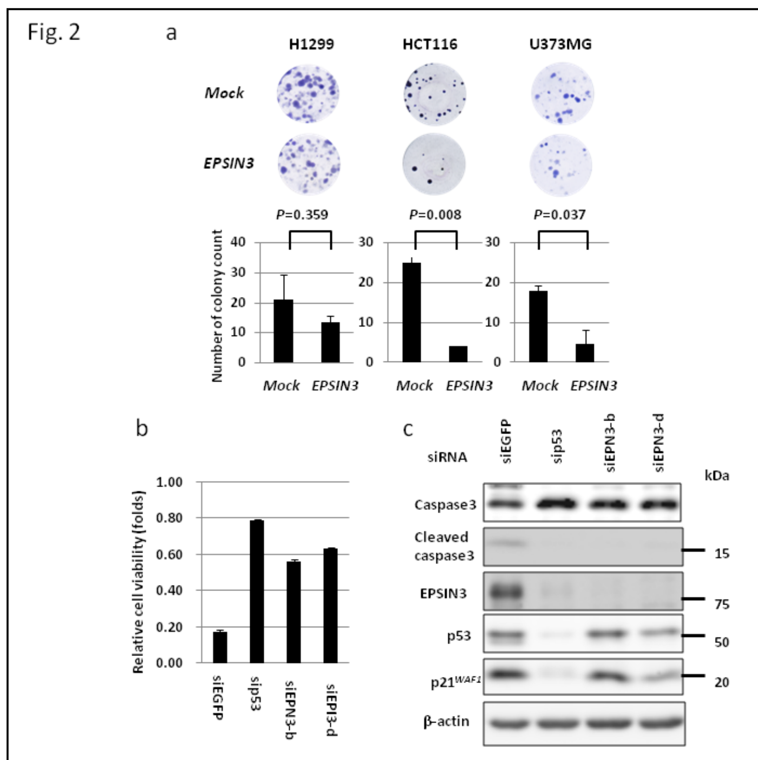


Among the novel candidates, we selected *EPSIN 3* from transcriptome analysis and CYSTATIN C from proteome analysis. To confirm the result of cDNA microarray and mass spectrometry analysis, we performed qPCR analysis and western blotting analysis. We found that EPSIN 3 and CYSTATIN C were remarkably induced by ADR in a p53-dependent manner. Next, we surveyed for p53-binding sequence (p53BS) within their genetic loci and identified two potential binding sequence (p53BS1 and p53BS2) in the promoter region of the *EPSIN 3* gene and one p53BS in first intron in the *CYSTATIN C* gene. The DNA fragment containing p53BS1 and p53BS2 (p53BS1+2) of *EPSIN 3* and p53BS of *CYSTATIN C* gene were amplified and subcloned upstream of the minimal promoter in pGL4.24 vector (pGL4.24/p53BS). The result of reporter assay revealed that U373MG cells transfected with pGL4.24/p53BS1+2 or pGL4.24/p53BS showed increased luciferase activity only in the presence of plasmid expressing wild-type p53. To verify whether p53 could directly bind to p53BSs, we performed ChIP assay using U373MG cells that were infected with either

Ad-p53 or Ad-LacZ. After precipitation by an anti-p53 antibody, DNA fragments harboring BSs were quantified by qPCR. As a result, p53 specifically bound to p53BS2 (*EPSIN 3*) and p53BS (*CYSTATIN C*) in cells infected with Ad-p53.

functional analysis of EPSIN 3

Epsins are accessory proteins implicated in clathrin-mediated endocytosis by binding ubiquitin moieties on the cytoplasmic part of membrane proteins. To date, three family members (EPSIN 1-3) have been identified in vertebrates. EPSIN 1 and EPSIN 2 are ubiquitously expressed in most type of tissues and were shown to be involved in the internalization of membrane proteins. However, the roles of EPSIN 3 in both normal and cancer cells are largely unknown. To explore the role of EPSIN 3 in the growth of cancer cells, we performed colony formation assay using three cancer cell lines: H1299, HCT116, and U373MG. As a results, colony formation was significantly impaired in HCT116 and U373MG cells which had been transfected with EPSIN 3 expressing plasmid compared with mock (Fig. 2a). We subsequently analyzed cell viability by ATP measurement assay and found that

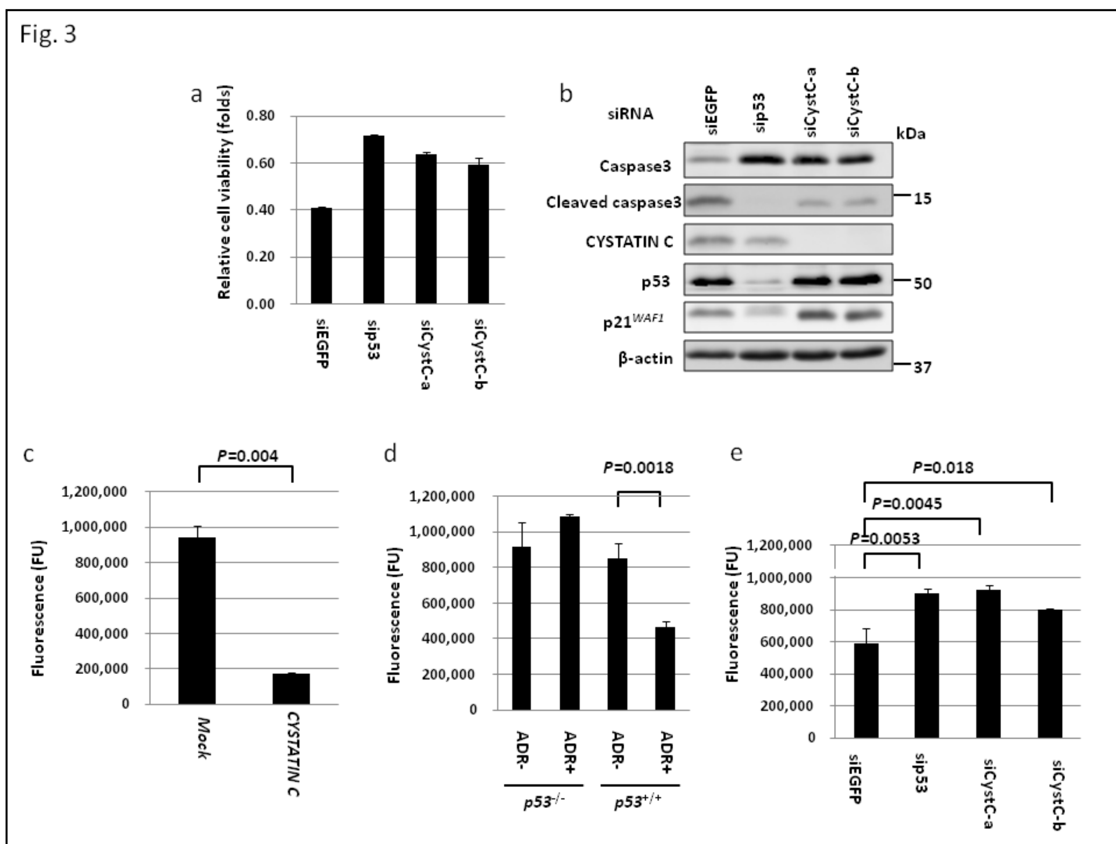


EPSIN 3 knockdown inhibited the ADR-induced growth suppression to the same degree as cells treated with sip53 (Fig. 2b). We further examined the impact of EPSIN 3 on ADR-induced apoptosis. Interestingly, knockdown of EPSIN 3 in ADR-treated HCT116 cells increased pro-caspase 3 and reduced cleaved caspase 3, indicating the regulation of apoptosis by EPSIN 3 (Fig. 2c).

Functional analysis of CYSTATIN C

Cystatins are reversible, tight binding inhibitors against C1 cysteine proteases, which exert various physiological functions. Among the family, CYSTATIN C has been the most intensively studied and shown to inhibits cathepsin L, a lysosomal cysteine protease, that is

highly expressed in various cancer cells and is involved in cancer development and progression. First, we analyzed cell viability by ATP measure assay and found that CYSTATIN C knockdown inhibited the ADR-induced growth suppression to the same degree as cells treated with sip53 (Fig. 3a). Next, we found that knockdown of CYSTATIN C in ADR-treated HCT116 cells increased pro-caspase 3 and reduced cleaved caspase 3, indicating the regulation of apoptosis by CYSTATIN C (Fig. 3b). When HEK293T cells were transfected with plasmid expressing CYSTATIN C, cathepsin L activity was markedly decreased compared with mock-transfected cells (Fig. 3c). Then we measured cathepsin L activity in HCT116 *p53*$^{-/-}$, *p53*$^{+/+}$ or CYSTATIN C-silenced *p53*$^{+/+}$ cells that were treated with ADR. As a result, cathepsin L activity was significantly reduced in HCT116 *p53*$^{+/+}$ cells after ADR treatment, while ADR treatment increased cathepsin L activity in *p53*$^{-/-}$ cells or CYSTATIN C-silenced *p53*$^{+/+}$ cells (Fig. 3d,e). These results indicated that p53-CYSTATIN C pathway regulates cathepsin L activity.



Conclusion

We identified EPSIN 3 and CYSTATIN C as novel p53 targets using transcriptome and proteome analysis, respectively. Both genes promote apoptosis of cancer cells in regulation of p53.