Biochemical and Biophysical Research Communications 463 (2015) 389–394

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Ablation of Dicer leads to widespread perturbation of signaling pathways

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ARTICLE INFO

Article history:
Received 23 April 2015
Accepted 15 May 2015
Available online 30 May 2015

Keywords:
Dicer
Phosphotyrosine
Pathways
Receptor tyrosine kinase
SILAC

ABSTRACT

Dicer is an essential ribonuclease involved in the biogenesis of miRNAs. Previous studies have reported downregulation of Dicer in multiple cancers including hepatocellular carcinoma. To identify signaling pathways that are altered upon Dicer depletion, we carried out quantitative phosphotyrosine profiling of liver tissue from Dicer knockout mice. We employed antibody-based enrichment of phosphotyrosine containing peptides coupled with SILAC spike-in approach for quantitation. High resolution mass spectrometry-based analysis identified 349 phosphotyrosine peptides corresponding to 306 unique phosphosites of which 75 were hyperphosphorylated and 78 were hypophosphorylated. Several receptor tyrosine kinases including MET, PDGF receptor alpha, Insulin-like growth factor 1 and Insulin receptor as well as non-receptor tyrosine kinases such as Src family kinases were found to be hyperphosphorylated upon depletion of Dicer. In addition, signaling molecules such as IRS-2 and STAT3 were hyperphosphorylated. Activation of these signaling pathways has been implicated previously in various types of cancers. Interestingly, we observed hypophosphorylation of molecules including focal adhesion kinase and paxillin. Our study profiles the perturbed signaling pathways in response to dysregulated miRNAs resulting from depletion of Dicer. Our findings warrant further studies to investigate oncogenic effects of downregulation of Dicer in cancers.

1. Introduction

Dicer is a ribonuclease involved in the biogenesis of miRNAs and RNA silencing. It is required for growth and development and its deficiency causes embryonic lethality [1,2]. As miRNAs are key mediators of post-transcriptional gene regulation, lack of Dicer leads to dysregulation of Dicer-dependent miRNAs, which subsequently impacts the downstream transcriptome and proteome. It has been demonstrated that Dicer regulates various cellular processes including proliferation, differentiation and regulation of metabolism [3–5]. Interestingly, downregulation of Dicer is shown to associate with tumorigenesis and metastasis, and lower expression of Dicer is observed in various types of cancers such as lung, ovarian and liver cancers [6–8] Han et al. have demonstrated activation of Akt upon ablation of Dicer [9]. Hypoxia-mediated downregulation of Dicer was observed to lead expression of several hypoxia associated factors including HIF-α, a process known to be associated with cancer development [10]. Taken together, these studies suggest that there might be a more global dysregulation of signaling pathways upon ablation of Dicer.

One of the key protein post-translational modifications in signal transduction is the phosphorylation of tyrosine residues.

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http://dx.doi.org/10.1016/j.bbrc.2015.05.077
0006-291X/

Phosphotyrosine signaling controls various cellular processes, cell–cell and cell–environment interactions. With an established association of downregulation of Dicer with various types of cancer, we sought to investigate the phosphotyrosine proteome downstream of Dicer. Owing to its low abundance, anti-phosphotyrosine antibody-based enrichment methods have been developed to increase the detection of tyrosine phosphoproteome [11]. To quantify the enriched phosphotyrosine-containing peptides, a variety of methods have been established. As *in vitro* labeling such as iTRAQ or label-free methods are more prone to introducing experimental errors, the SILAC approach for the metabolic labeling now is widely adopted [12]. Recently, SILAC spike-in approach was developed, where SILAC labeled cell lysates are spiked at a fixed ratio for relative quantitation [13]. In principle, the spiked-in peptides serve as the internal reference standards to compare the relative abundance of phosphopeptides.

To understand the *in vivo* alterations of the phosphotyrosine-mediated signaling associated with Dicer depletion, we employed SILAC spike-in approach. We carried out replicate analysis of unlabeled liver lysates of control and Dicer knockout mice spiked with SILAC labeled Hepa 1–6 cell lysates. High resolution mass spectrometry-based analysis identified 306 phosphotyrosine sites, of which 75 were hyperphosphorylated. We further carried out bioinformatics revealed enrichment of pathways such as MET and IGF1R which are associated with oncogenesis. Our findings shed light on possible association of downregulation of Dicer and activation of oncogenic signaling pathways.

2. Material and methods

2.1. Generation of Dicer knockout mice and SILAC labeled Hepa 1–6 cells

We selected Cre-loxP system to generate inducible knockout mice, as described previously [5]. In brief, ROSA26-CreERT2 mice and mice with floxed Dicer exons 21 and 22 were crossed. Administration of tamoxifen resulted in deletion of floxed *Dicer* exon 21 and 22. On the day 8 post-induction, mice were starved for 3 h prior to euthanasia and necropsy to harvest liver from 5 Dicer knockout mice. Similarly, 5 uninduced control mice were also starved prior to euthanasia and necropsy. Mouse hepatoma Hepa 1–6 cells were (kind gift from Kensler Lab, School of Public Health, Johns Hopkins University) adapted to $^{13}C_6$-Lysine; $^{13}C_6$-Arginine containing custom DMEM media to label proteins *in vivo*.

2.2. In-solution trypsin digestion

Lysates were prepared in urea lysis buffer containing 20 mM HEPES pH 8.0, 9 M urea, 1 mM sodium orthovanadate, 2.5 mM sodium pyrophosphate and 1 mM β-glycerophosphate supplemented with Complete protease inhibitor cocktail (Roche Diagnostic Systems). The lysates were sonicated and cleared by centrifugation at 3000 × g at 4 °C for 10 min. Protein estimation was carried out using BCA protein assay. Equal amount of protein was pooled from each mouse to obtain pooled control and pooled Dicer knockout liver lysates. ~30 mg protein from each pooled sample (spiked with SILAC labeled Hepa 1–6 lysate at 5:1 w:w ratio) was reduced with 5 mM dithiothreitol and alkylated with 10 mM iodoacetamide. *In-solution* digestion was carried out using TPCK-treated trypsin on an orbital shaker at 25 °C overnight. The protein digest was desalted using SepPak C18 cartridge (Waters Incorporation, USA) and eluted peptides were lyophilized for phosphotyrosine peptide enrichment.

2.3. Anti-phosphotyrosine Western blot and immunoaffinity enrichment of phosphotyrosine peptides

Phosphotyrosine protein profile of liver from control and Dicer knockout mice was analyzed by Western blot using 4G10 anti-phosphotyrosine antibody essentially as described before [14].

For immunoaffinity enrichment, the lyophilized peptides were reconstituted in 1.4 ml of immunoaffinity purification (IAP) buffer

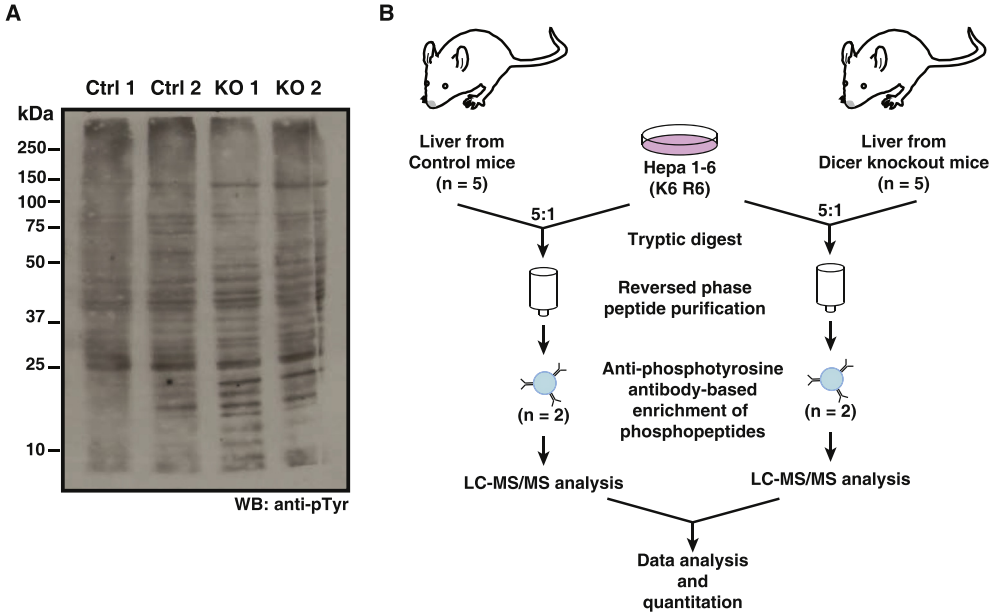

Fig. 1. Investigation of phosphotyrosine profile upon ablation of Dicer A) Phosphotyrosine profile of control and Dicer knockout mice. Ctrl1 and Ctrl2 represent liver lysates from two control mice and KO 1 and KO 2 represent liver lysates from 2 Dicer knockout mice. The lysates were immunoblotted using anti-phosphotyrosine antibody. B) Schematic representation of workflow followed to identify differentially phosphorylated tyrosine sites upon ablation of Dicer. Pooled liver lysates from 5 control and 5 Dicer knockout mice were spiked with $^{13}C_6$-lysine and $^{13}C_6$-ariginine labeled Hepa 1–6 cell lysates and replicate analysis was carried out. Phosphotyrosine containing peptides were enriched using pY100 anti-phosphotyrosine antibody. LC-MS/MS analysis was carried out using LTQ-Orbitrap Velos followed by data analysis.