Mutation Research 707 (2011) 15–23

Contents lists available at ScienceDirect

Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Differential expression of the *klf6* tumor suppressor gene upon cell damaging treatments in cancer cells

Ricardo C. Gehrau, Diego S. D'Astolfo, Verónica Andreoli, José L. Bocco*, Nicolás P. Koritschoner

Centro de Investigaciones en Bioquímica Clínica e Inmunología (CIBICI-CONICET), Departamento de Bioquímica Clínica, Facultad de Ciencias Químicas, Universidad Nacional de Córdoba, Haya de la Torre y Medina Allende, Ciudad Universitaria, 5000 Córdoba, Argentina

ARTICLE INFO

Article history:
Received 22 June 2010
Received in revised form
27 November 2010
Accepted 6 December 2010
Available online 13 December 2010

Keywords:
KLF6
Kruppel
Cancer
Cellular damaging
Chemotherapy

ABSTRACT

The mammalian Krüppel-like factor 6 (KLF6) is involved in critical roles such as growth-related signal transduction, cell proliferation and differentiation, development, apoptosis and angiogenesis. Also, KLF6 appears to be an emerging key factor during cancer development and progression. Its expression is thoroughly regulated by several cell-damaging stimuli. DNA damaging agents at lethal concentrations induce a p53-independent down-regulation of the *klf6* gene.

To investigate the impact of external stimuli on human *klf6* gene expression, its mRNA level was analyzed using a cancer cell line profiling array system, consisting in an assortment of immobilized cDNAs from multiple cell lines treated with several cell-damaging agents at growth inhibitory concentrations (IC_{50}).

Cell-damaging agents affected the *klf6* expression in 62% of the cDNA samples, though the expression pattern was not dependent on the cell origin type. Interestingly, significant differences ($p < 0.0001$) in KLF6 mRNA levels were observed depending on the cellular p53 status upon cell damage. KLF6 expression was significantly increased in 63% of p53-deficient cells (122/195). Conversely, KLF6 mRNA level decreased nearly 4 fold in more than 70% of p53+/+ cells. In addition, *klf6* gene promoter activity was down-regulated by DNA damaging agents in cells expressing the functional p53 protein whereas it was moderately increased in the absence of functional p53. Consistent results were obtained for the endogenous KLF6 protein level.

Results indicate that human *klf6* gene expression is responsive to external cell damage mediated by IC_{50} concentrations of physical and chemical stimuli in a p53-dependent manner. Most of these agents are frequently used in cancer therapy. Induction of *klf6* expression in the absence of functional p53 directly correlates with cell death triggered by these compounds, whereas it is down-regulated in p53+/+ cells. Hence, *klf6* expression level could represent a valuable marker for the efficiency of cell death upon cancer treatment.

© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Krüppel-like factor 6 (KLF6) belongs to the Sp1/KLF family of transcription factors, which are frequently involved in critical roles such as cell proliferation and differentiation, development, apoptosis and angiogenesis, depending on cell stimulation [1,2]. The KLF6 transcription factor is an evolutionarily conserved and ubiquitously expressed protein that was identified as an activator of pregnancy-specific genes [3–6]. KLF6 has been postulated as a tumor suppressor since its gene is frequently inactivated by loss of heterozygozity (LOH), somatic mutations and/or decreased expression in human cancers [7]. Transactivation of the cell cycle inhibitor p21 in a p53-independent manner, reduction of cyclin D1/cdk4 complexes and inhibition of c-Jun proto-oncoprotein activities are some of the mechanisms proposed by which KLF6 mediates growth suppression [8–10]. Nevertheless, other studies on human neoplasms have shown that genetic alterations of *klf6* gene are rare events [11–17] and that a KLF6 overexpression was found in certain hepatocellular carcinoma cells (HCC) [13].

Several independent reports described apoptotic or anti-apoptotic functions for KLF6. Apoptosis mediated by KLF6 involved: (i) overexpression of KLF6 in non-small cell lung cancer cells, (ii) up-regulation of ATF-3 gene expression by KLF6 in prostate cancer cells and (iii) transactivation of DAPK2 gene through KLF6/E2F1 in lung cancer cell lines [18–20]. However, siRNA-mediated KLF6 knock down in hepatocarcinoma cell lines lead to a reduction in cell cycle progression and cells became more susceptible to DNA-damage induced apoptosis, indicating that KLF6 function is required for cell proliferation and survival in these cells [21,22].

* Corresponding author. Tel.: +54 351 434 4973/4974/4975/4976x110; fax: +54 351 433 3048.
E-mail address: jbocco@fcq.unc.edu.ar (J.L. Bocco).

0027-5107/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.mrfmmm.2010.12.002

KLF6 expression has been shown to be responsive to external cues triggered by several physical and chemical stimuli. In this sense, KLF6 expression was increased upon carotid balloon injury and thereby regulates key genes involved in vascular remodeling and angiogenesis [23–25]. A similar expression pattern was found in hemorrhagic and hypoxic processes [26,27], chemical intoxication (CCl_4) or chronic drug injuries [28,29]. Tumor promoter and proliferating cell signaling generated by phorbol esters (PMA) also produced a transient increase in the KLF6 expression level, which triggers an enhanced c-Jun degradation by a proteasome-dependent pathway, leading to a reduced cell proliferation rate [8,30]. Also, PMA-induced growth arrest of non-small cell lung cancer cells (NSCLC) was mediated by the induction of KLF6 expression, following PKC activation [31].

In opposite to stimuli that induce up-regulation of KLF6, many reports demonstrated that DNA damage mediated by UV radiation [8] and chemotherapeutic drugs (cisplatin and adriamycin) induced a p53-independent down-regulation of KLF6 protein level at lethal and apoptotic doses [7,8,21,32,33]. Additionally, recent studies from our laboratory have demonstrated a down-regulation of KLF6 gene promoter activity and transcript level in hepatocellular carcinoma cell lines treated with apoptotic concentration of cisplatin [21].

To gain insights into the KLF6 response to cell treatments aimed to induce death of tumor cells; its mRNA level was analyzed in multiple cell lines systematically exposed to cell growth inhibitory (IC_{50}) concentrations of chemotherapeutic agents, oxidative stress and irradiation conditions. Results are consistent with a differential *klf6* gene expression associated to the functional p53 status of the cell. In this regard, the *klf6* transcript level was increased in p53−/− cell background but, conversely, it was decreased in p53+/+ cells treated with the same stimuli. This differential response of mRNA expression is in line with the endogenous KLF6 protein level and involves the regulation of the *klf6* gene promoter activity.

2. Material and methods

2.1. Cancer cell line profiling array

KLF6 transcript level was determined in a cancer cell line profiling array (BD Biosciences Clontech, CA, USA). The array system consists of a nylon membrane spotted with total cDNA of 26 cell lines representing twelve tissue organs treated with 25 cell damage stimuli including chemotherapeutic agents, oxidative stress and serum deprivation conditions, and gamma and UV irradiations. As indicated by the manufacturer, all cell lines were incubated in 10-cm tissue culture plates, in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and 50 μg/ml of penicillin and streptomycin, and in an atmosphere containing 5% CO_2 at 37 °C. Each cell line was exposed to the described cell damage stimuli at concentrations to induce a 50% growth inhibition (IC_{50}) after 48 h of treatment. Cell viability was determined using an MTT assay. For chemical agents, cells were seeded into 96-well microtiter plates at a density of 10,000 cells per well an exposed to an optimized concentration. For UV-irradiation, cells were exposed to a 365 nm light from a distance of 15 cm for an optimized time interval. Cells were exposed to Gamma irradiation using a ^{60}Co source to receive an overall dose of 2 Gy. Heat shock was induced by incubating the cell suspension at an elevated temperature (44 °C) in a water bath for an optimized length of time. Treatment conditions are described for each cell line in Table S1A. Additionally, a treatment procedure with 0.5% FCS was included for each cell line. Cells cultured in 10% FCS supplemented DMEM medium were considered as the non-treated control for each cell line.

2.2. Probe generation

The cDNA spotted array was hybridized with a specific probe covering the 3′-untranslated region (3′ UTR) of the KLF6 cDNA (nucleotides 1134–1616 from KLF6 cDNA – GI:236460447). It was obtained by digesting the KLF6 cDNA cloned into the p513HA plasmid with BamHI and PstI restriction endonucleases. The 584 bp restriction fragment obtained included 482 bp corresponding to the 3′ UTR region of the human KLF6 gene (Fig. 1A) [8]. The 482 bp DNA sequence of the probe is conserved in all the splice variants of KLF6 transcripts and its specificity was tested by blastn and megablast BLAST algorithms (http://blast.ncbi.nlm.nih.gov/Blast.cgi) to verify that it does not share homology with regions of other genes, especially with other Krüppel-like family members (data not shown). The generated fragment was removed and purified from a 1% TAE–agarose gel using Qiaquick Gel Extraction Test kit (Qiagen, CA, USA) and the concentration was estimated by spectrophotometer readings at A260 nm. To generate the probe, 20 ng of DNA fragment were radio-labeled for 1 h at 37 °C in a final volume of 50 μl containing 1× random primer buffer (RPB, Boehringer Mannheim GmbH, Germany), 0.075 mM dNTPs (Invitrogen, CA, USA) minus dATP, 5 U of Klenow fragment of DNA polymerase (Promega, Madison, WI, USA) and 1 μCi/μl [α-P^{32}-dATP] (Amersham Pharmacia Biotech). The radio-labeled probe was adjusted to 100 μl of solution and precipitated for 30 min at −20 °C with 10 μl 3 M sodium acetate and 2.5 volumes of 100% ethanol, including 50 μg of sheared DNA of salmon sperm. The DNA pellet was washed once with 70% ethanol and solubilized in 200 μl of sterile deionized water.

2.3. Nylon membrane hybridization and autoradiography

The total cDNA spotted nylon membrane was pre-hybridized for 30 min at 68 °C in 10 ml of pre-warm BD ExpressHyb hybridization solution treated with 1.5 mg heat denatured sterile sheared DNA of salmon sperm at 95–100 °C in an AutoBlot Micro-Hybridization Oven (Bellco Glass Inc., Vineland, NJ, USA). The radio-labeled probe was denaturalized by adding 50 μl of 2 M NaOH and incubated for 10 min at room temperature. After the addition of 150 μg sheared DNA of salmon sperm, the probe was added to the 10 ml of pre-hybridization solution and hybridized over-night with continuous rotation at 68 °C. Wash procedures were performed as indicated by the manufacturer in the Disease Profiling Arrays User Manual. Autoradiography of the hybridized membrane was obtained by exposing to X-ray films for different times at −70 °C with an intensifying screen (Fig. S1). Following, the nylon membrane was stripped and checked for complete stripping as described in the Disease Profiling Arrays User Manual. A similar procedure was performed to generate a radio-labeled probe for the ubiquitin gene used as an internal standard control provided by the manufacturer. The radioactive signal pattern of the ubiquitin probe developed in an X-ray film exposure (Fig. S2).

2.4. Data analysis and statistical calculation

KLF6 and ubiquitin probes radioactive intensities were quantified using radiation detector Fuji Laboratory Analyzer FLA3000 and by the Image Gauge software (Fuji Corporation, Japan). Low and high exposed films were employed to enhance the sensitivity in those spots with under and over saturated specific KLF6 probe intensity, respectively (Fig. S1). For those particular cases, KLF6 probe intensity from non-treated control spots were also re-quantified from the same X-ray film exposure time and following used for fold changes calculation as following describe. X-ray film background was subtracted from each positive spot for both labeled specific probes.

KLF6/ubiquitin probes intensities ratios were calculated for normalization. Normalized KLF6 probe signal was expressed as fold changes with respect to the non-treated control for each cell line. Assuming normal sample data distribution, fold change cut-off value was set as mean plus one standard deviation (mean + SD) for positive and negative fold change mean. These data represents fold changes included into the 68% confidence interval (CI) and were excluded from the analysis. Data outside of the set CI, representing 32% of values, were considered for analysis. In this regard, ±1.5 fold change value was considered to be statistically different to identify up (+1.5 fold change) and down-regulated (−1.5 fold change) *klf6* gene expression respect to non-treated cells controls. Following, fold changes considered for *klf6* gene expression analysis were grouped as increased (≥1.5-fold), decreased (≤−1.5 fold), and non-modified (1.4 to −1.4 fold) (Table 2 and Table S1B).

Chi-square (χ^2) test (contingency tables analysis) was performed to statistically analyze treated cells grouped as non-modified, increased and decreased depending on the *klf6* gene expression level related to the cellular p53 background. Significant differences were considered as $p < 0.05$ (two-tails).

2.5. DNA damaging agents

The DNA damaging and synthesis inhibitors hydroxyurea (H8627), 5-fluorouracil (F6627), cisplatin (*cis*-diammineplatimun (II) dichloride, crystalline; P4394) and amethopterin (A6770; methotrexate hydrate) were purchased from Sigma–Aldrich (St. Louis, MO, USA).

2.6. Transfection and luciferase reporter assays

Human hepatocellular carcinoma-derived HepG2 and Hep3B cell lines were cultured in high glucose DMEM medium supplemented with 10% (v/v) FCS, 2 mM L-glutamine, 50 U/ml penicillin and 50 μg/ml streptomycin (Gibco-Invitrogen, Carlsbad, CA, USA) at 37 °C and 5% CO_2 atmosphere. Twenty-four hours before transfection cells were seeded in 96-well plate (18,000 cells per well) in culture medium without antibiotics. Both cell lines were transfected with 100 ng of PROM6 luciferase reporter vector containing the *klf6* gene promoter region, and 10 ng of pRL-TK (Renilla) used as an internal transfection control, both in the presence of 0.22 μl lipofectamine 2000 transfection lipid (Invitrogen, Carlsbad, CA, USA). The plasmid DNA/lipid mixture was diluted in Opti-MEM medium (Invitrogen, Carlsbad, CA, USA) without FCS. For promoter activity analyses, cells were cultured for 36 h post-transfection. For DNA damaging agent treatments, the complete medium volume was replaced by fresh medium containing hydroxyurea (2 mM), 5-fluorouracil (60 μM), cisplatin (5 μg/ml) or amethopterin (92 μg/ml). These DNA damaging

Download English Version:

https://daneshyari.com/en/article/2146670

Download Persian Version:

https://daneshyari.com/article/2146670

Daneshyari.com