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In vivo footprinting analysis of the Glypican 3 (GPC3) promoter region in neuroblastoma cells

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Abstract

Glypican 3 (GPC3) is an X-linked gene that has its peak expression during development and is down-regulated in all studied tissues after birth. We have shown that GPC3 was expressed in neuroblastoma and Wilms' tumor. To understand the mechanisms regulating the transcription of this gene in neuroblastoma cells, we have focused our study on the identification of putative transcription factors binding the promoter. In this report we performed *in vivo* dimethylsulfate, UV type C irradiation and DNaseI footprinting analyses coupled with ligation-mediated PCR on nearly 1000 bp of promoter in two neuroblastoma cell lines, SJNB-7 (expressing GPC3) and SK-N-FI (not expressing GPC3). Nucleosome signature footprints were observed in the most distal part of the studied region in both cell lines. We detected eight large differentially protected regions, suggesting the presence of binding proteins in both cell lines but more DNA–protein interactions in GPC3-expressing cells. Sp1 was previously shown to be able to bind some of these regions. Here by combining electromobility shift assays and chromatin immunoprecipitations we showed that the transcription factor NFY was part of the DNA–protein complex found in footprinted regions upstream of the described minimal promoter. These studies performed on chromatin *in situ* suggest that NFY and yet unknown cell type-specific factors may play an important role in the regulation of GPC3.

Keywords: GPC3; Neuroblastoma; In *vivo* footprinting; Transcription factor

1. Introduction

Glypican 3 (GPC3) has been shown to be expressed in embryonal tumors including neuroblastoma (NB), Wilms' tumor and hepatoblastoma [1,2]. This gene is expressed in a tissue-specific manner, has its peak expression during development and is down-regulated in adult tissues [3–5]. The product of this gene, located at chromosome Xq26 [6,7] is a heparan sulfate proteoglycan located on the cell surface and attached to the cellular membrane by a glycosyl-phosphatidyl inositol anchor [7]. The role of this protein has not yet been defined. Many studies suggested that GPC3 is a negative cellular growth regulator [7–12]. A germline mutation of the human GPC3 gene is associated with the Simpson–Golabi–Behmel overgrowth syndrome [7] and GPC3 knock-out mice partly recapitulate the syndrome [8]. On the other hand, GPC3 has been shown to be over-expressed in hepatocellular carcinomas [4,13,14] and to be associated with advanced stages as well as with invasive potential of this cancer [4]. Moreover, colorectal carcinoma-associated liver metastases express GPC3 significantly more than primary tumors [15]. These data suggest that, depending on the cellular context, GPC3 is regulating different growth and survival factors [16].

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The mechanisms regulating the transcription of GPC3 are of particular interest to understand the altered expression of GPC3 in cancer cells. We recently showed that female-derived NB cells presented a loss of methylation in the promoter region, suggesting that the demethylation of the inactive X-linked GPC3 allele may allow the expression of the gene at least in NB cells [17]. This hypothesis is supported by the fact that GPC3 is over-expressed preferentially in female as compared to male hepatocellular carcinomas [4]. However, methylation is not necessary for gene silencing in NB, indicating that permissive conditions for GPC3 expression must prevail at another regulatory level [17]. Transcription factors (TFs) are key players in the transcriptional regulation of genes. The factor Sp1 has been shown to be involved in the activation of the GPC3 transcription [18], but being expressed in virtually every cell, is not likely to regulate alone the tissue-specific expression of GPC3.

In this report, we carried out a comprehensive *in vivo* footprinting analysis using dimethylsulfate (DMS), 254-nm ultraviolet type C (UVC) light and deoxyribonuclease I (DNase I) treatments coupled with the ligation-mediated PCR approach to identify other regulatory elements in the GPC3 promoter in two NB cell lines, SJNB-7 and SK-N-FI, associated with opposite GPC3 expression pattern [1]. This method allows the probing of DNA–protein interactions in the native nuclear context, which is believed to have a great importance in the regulation of genes. We found several *in vivo* footprints specific to the GPC3 expressing cells. Two of these promoter regions have been further investigated *in vitro* by electrophoresis mobility shift assays and chromatin immunoprecipitations. Our results provide evidence that NFY binds the promoter region in its *in vivo* context but that other unknown cell-specific factors could be involved in the regulation of GPC3 expression.

2. Materials and methods

2.1. DMS, UVC and DNase I treatments of cells

SK-N-FI (obtained from ATCC, Manassas, Virginia) and SJNB-7 (obtained from T. Look), both male-derived NB cell lines, were grown in DMEM supplemented with 10% of FBS. These cell lines were treated *in vivo* with DMS (0.02%) or irradiated with 254-nm UVC light (1500 J/m^2) as previously described [19]. The *in vivo* DNase I treatment was performed carrying out the permeabilization (using 0.05% of Lysolecithin) and the enzymatic digestion (using 8.75 μg/mL of DNaseI, Worthington biochemical corporation, Lakewood, NJ, USA) simultaneously in 4 mL of digestion buffer (150 mM sucrose, 80 mM KCl, 35 mM HEPES pH 7.4, 5 mM $MgCl_2$, and 2 mM $CaCl_2$) for 20 min at room temperature. At the end of the DNase I reaction, the cells were pelleted, and the supernatant was removed. The reaction was stopped and the cells were lysed by the addition of 500 μL of lysis buffer (150 mM NaCl, 5 mM EDTA pH 7.8), 500 μL of buffer C (20 mM Tris–HCl pH 8, 20 mM NaCl, 20 mM EDTA, 1% sodium dodecyl sulfate (SDS)), and proteinase K to a final concentration of 300 μg/mL. This mixture was incubated at 37 °C for 3 h. RNA was removed by digestion with RNase A (100 μg/mL) at 37 °C for 1 h. Following phenol, phenol/chloroform and chloroform extractions, DNA was ethanol precipitated and dissolved in water. For footprinting controls with naked DNA, genomic DNA isolated from each cell line was treated in vitro with DMS, UVC or DNase I as previously described [19]. To generate a sequence marker (see Ligation-mediated PCR), genomic DNA from human peripheral blood leukocytes was chemically damaged according to Maxam and Gilbert's protocol [20]. Cyclobutane pyrimidine dimers (CPD) from UVC irradiation were converted into strand breaks by successive T4 endonuclease V and photolyase digestions [19]. Methylated guanines from DMS exposures and chemically damaged DNA were converted into strand breaks by hot piperidine treatment. Strand break frequencies were estimated on an alkaline agarose gel [21].

2.2. Ligation-mediated PCR (LMPCR)

LMPCR was carried out in duplicate as previously described [19] using the primer set shown in Table 1, allowing the analysis of approximately 1000 bp (between positions −970 and +175) in the GPC3 promoter. DNase I digestion of DNA leaves ligatable 5′-phosphorylated breaks, but the 3′-ends are free hydroxyl groups. To avoid nonspecific priming of these 3′-OH ends [22], we performed DNA denaturation, primer 1 hybridization and primer 1 extension at a higher temperatures using the thermostable Pfu exo$^-$ DNA polymerase (Stratagene, La Jolla, CA, USA). One μg of treated DNA was used for each sample. The chemically cleaved G, A, T+C and C samples done on purified genomic DNA from human peripheral blood leukocytes were included along with the other samples in the LMPCR assays as sequence markers. For primer 1 extension, the samples were denatured at 98 °C for 3 min, annealed at a temperature around the Tm of the first primer for 5 min and extended at 75 °C for 15 min on a Biometra thermocycler using the Pfu exo$^-$ DNA polymerase (1.5 U). The double-strand linker was then ligated to blunted ends with T_4 DNA ligase (3.25 U/reaction, Roche Diagnostics, Laval, QC, Canada), at 18 °C for about 15 h. After the precipitation, the DNA fragments were amplified by conventional PCR using Taq DNA polymerase (0.03 U/μL) or Pfu exo$^-$ DNA polymerase (0.07 U/μL) with the 25-mer of the linker and the second primer (Table 1). The resulting PCR fragments were phenol/chloroform extracted, ethanol precipitated and separated on an 8% polyacrylamide gel (65 cm long) containing 7 M urea and electroblotted onto a nylon membrane and fixed by UVC crosslinking [19]. Primer extension was used to make single stranded hybridization probes by utilizing the second primer. Hybridization of the membrane was done overnight at 65 °C.

2.3. DNA sequence analysis

To identify putative TFs, the sequence of the promoter region was searched for the presence of consensus sequences for TF binding sites using Matinspector and the TRANSFAC matrices at "optimized" settings ("thresholds (selection) that minimize false positives for each individual matrix"; see details on the Genomatix website: http://www.genomatix.de/software_services/software/MatInspector/MatInspector_stb.html).

2.4. Electromobility shift assays (EMSA)

Nuclear extracts were prepared as described in Dignam et al. [23]. The labeling reaction mix contained 50 ng (350 nM) of double-stranded oligo probe (sense strands: −318/−271: GCGGACGGCTGCTGGGAAGCCAATCAGCG-CGCTCGAGCCTGCAGCCCC, −400/−341: GGGAAAAGCCCTCCAGGC-TGTAGGCCAATGAGCGGCGGGAAGGAGGAGTGAGGCTGGGGA, NFY: ATTTTTCTGATTGGTTAAAAGT, NFY-mut: ATTTTTCTGATTaaT-TAAAAGT, OCT: CCTCTTGGATTTGCATATGGGCTG), 25 μCi of [γ-^{32}P] ATP (6000 Ci/mM), 10 U of T4 polynucleotide kinase (Invitrogen, Burlington, ON, Canada), 1× kinase forward exchange buffer (Invitrogen, Burlington, ON, Canada), in a final volume of 10 μl. The mix was incubated at 37 °C for 10 min. The reaction was stopped by the addition of 1 μl of 500 mM EDTA solution. The volume was completed to 100 μl with TE. The labeled probes were purified on a G-25 column (Amersham Biosciences, Piscataway, NJ, USA). For the binding reaction, the mixtures contained nuclear protein extract from SK-N-FI (25 μg), SJNB-7 (25 μg) cells, 12% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 62.5 ng/μl of Poly dI–dC, 1 mM DTT, 1 mM EDTA, in a total volume of 16 μl. The extract premix was incubated at 4 °C for 20 min. The probe premix contained 0.5 ng (4.4 nM) of oligo probe, 12% glycerol, 20 mM HEPES (pH 7.9), 50 mM KCl, 4 mM $MgCl_2$, 1 mM DTT, 1 mM EDTA in a final volume of 8 μl. The probe premix was incubated at 4 °C for 20 min. 16 μl of the extract premix and 8 μl of the probe premix were mixed together and incubated at 4 °C for 2 h. For competition assays, we added a 100-fold molar excess of unlabeled