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Promoter paper

The role of Sp1 and Sp3 in the constitutive *DPYD* gene expression

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Abstract

Dihydropyrimidine dehydrogenase (DPD), the initial and rate-limiting enzyme in the 5-fluorouracil (5-FU) catabolic pathway, has been implicated as one of the factors determining the efficacy and toxicity of the anticancer agent 5-FU. Studies have attributed variation in DPD activity partially to alterations at the transcriptional level of *DPYD* gene. We investigated the transcription factors implicated in the constitutive expression of DPYD by utilizing a 174-bp fragment of the *DPYD* promoter region in which three consensus Sp protein binding sites (SpA, SpB and SpC) were predicted. The binding of Sp1 and Sp3 transcription factors to this region was detected by electrophoretic mobility shift and chromatin immunoprecipitation assays. By ectopically expressing human Sp1 and Sp3 in Sp-deficient Drosophila S2 cells, we demonstrated that Sp1 is a strong activator, while Sp3 by its own is a weak activator of the *DPYD* promoter. Moreover, Sp3 may serve as a competitor of Sp1, thus decreasing the Sp1 induced promoter activity. SpA, SpB and SpC sites are all Sp1 inducible. In the full activation of the *DPYD* promoter in human cell lines, the SpB site is essential; the SpC site works cooperatively with SpB, while SpA has minor promoter activity. These studies provide further insight into the molecular mechanisms underlying the heterogeneity of DPD activity, and may facilitate the efficacy and safety of 5-FU-based chemotherapy.

Keywords: Sp1; DPYD; Dihydropyrimidine dehydrogenase; Promoter

1. Introduction

5-fluorouracil (5-FU) is one of the most widely prescribed cancer chemotherapy drugs for the treatment of several malignancies including carcinomas of the colon, breast, skin and head and neck [1,2]. It blocks DNA synthesis through inhibiting thymidylate synthase (TS), which disrupts the intracellular nucleotide pools [3]. More than 80% of the administered dose of 5-FU is rapidly catabolized to inactive metabolites, which indicates a potentially critical role of this catabolic pathway in 5-FU efficacy and clearance [3–5]. Specifically, the initial and rate-limiting enzyme in the 5-FU catabolic pathway is dihydropyrimidine dehydrogenase (DPD) [2]. It is now well established that the variation in DPD enzyme activity is responsible for much of the observed interpatient and intrapatient variability in the clinical pharmacokinetics of 5-FU [6]. Furthermore, elevated DPD activity has been suggested as a determinant of decreased sensitivity to 5-FU [7], while DPD deficiency is often accompanied by severe and life-threatening toxicity [8]. Hence, an understanding of the mechanisms controlling the expression of DPD has become important in improving 5-FU-based chemotherapy [5].

Research aimed at the prediction of individual response to 5-FU using DPD as a marker has been performed extensively, and has directed efforts towards identifying the factors modulating DPD activity. For instance, mutations in the dihydropyrimidine dehydrogenase gene (*DPYD*) which result in dysfunction of the DPD protein have been identified as an important mechanism responsible for DPD deficiency. In particular, 13 such mutations have been reported [3]. It is important to note, however, that

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genetic polymorphisms in *DPYD* alone cannot adequately explain all the cases of 5-FU toxicity associated with reduced DPD activity. Therefore, effort has been made to identify other mechanisms regulating DPD enzyme levels. The factors proposed so far include cell density/growth [9,10], circadian rhythm [11,12], tumorigenesis [7,13–16], and dietary nutrition supplement [17]. Other studies have investigated the molecular levels at which *DPYD* expression is regulated. A correlation between *DPYD* mRNA level and enzyme activity has been reported, and suggests that the transcriptional regulation is an important mechanism leading to variability in DPD protein levels [1,18–20]. However, in contrast to the wide recognition of DPD as a biomarker in 5-FU-based cancer chemotherapy, the regulatory mechanisms in *DPYD* transcription are poorly understood.

Investigations into the transcriptional regulation of *DPYD* have cloned and characterized up to 3 Kb of the promoter region, and DNA elements that contribute to both inducible and constitutive expression have been identified within this region [21,22]. Ukon and colleagues reported the induction of *DPYD* expression by phorbol 12-myristate 13-acetate (PMA) in which the binding of AP1 to the DNA element located between −290 and −280 is involved [22]. The constitutive expression of *DPYD* has also been studied. By using the luciferase reporter assay in HEK293 and HeLa human cell lines, our laboratory reported that the promoter region downstream of −121 has full promoter activity in the constitutive expression of *DPYD* [21]. However, the transcription factors involved in this regulation have thus far not been elucidated.

In the current study, we investigated the role of Sp1 and Sp3 as transcription factors implicated in *DPYD* constitutive expression. Our data show the binding of Sp1 and Sp3 to the promoter region, as well as their differentiated function in *DPYD* promoter activation. Furthermore, the analysis on the individual Sp protein binding sites revealed their different roles in Sp1-dependent *DPYD* gene expression. This study provides the basis to further understand the variability in DPD protein levels. This may have implications in understanding both the physiological and pathological (neoplastic) control of DPD expression, which may in-turn be applied to increase the efficacy of 5-FU and lead to strategies for the individualization of 5-FU-based chemotherapy.

2. Materials and methods

2.1. Cell culture

The human cervical cancer cell line HeLa and the human embryonic kidney epithelium cell line HEK293 were obtained from ATCC and maintained in DMEM and DMEM/F12 50/50 mix, respectively with 10% FBS (HyClone) and without antibiotics. The cells were incubated at 37 °C in an atmosphere of 5% CO_2. Drosophila Schneider line 2 (S2) cells were kindly provided by Dr. Douglas Ruden (University of Alabama at Birmingham, Birmingham, USA), and were maintained at room temperature in Schneider's Drosophila medium (Life Technologies, Inc.) supplemented with 10% FBS (HyClone).

2.2. Plasmids

DPYD promoter-luciferase reporter constructs were described previously [21]. The human Sp1 expression plasmid (pPacSp1) and its control vector (pPac0) were gifts from Dr. Robert Tjian (University of California at Berkeley, Berkeley, USA) [23]. The human Sp3 expression plasmid (pPacSp3 K/R) and pPacRL (renilla luciferase) plasmid were kindly provided by Dr. Guntram Suske (Philipps-Universität Marburg, Marburg, Germany) [24]. The pRL-TK plasmid (Promega) and pPacRL plasmid were used as the internal control for transfections in human cell lines and S2 cells, respectively.

2.3. Transient transfection

The transfection was performed using FuGene 6 (Roche Applied Science) at the DNA to FuGene 6 ratio of 1:3. For transient transfection in Drosophila S2 cells, 2×10^5 cells were seeded in each well of the 6-well plate 24 h prior to the transfection. Subsequently, 1 μg of reporter, variable amounts of pPacSp1 or pPacSp3 K/R, and 0.05 μg of pPacRL plasmid DNA were used for each of the transfections. Variable amounts of the pPac expression plasmids were adjusted with the pPac0 plasmid DNA so that equal amount of DNA was used in each of the transfections. For the human cell lines, the cells were seeded at 20–30% confluence in 24-well plates 24 h prior to transfection. For each of the transfections, 0.2 μg of reporter and 0.02 μg of pRL-TK were utilized. The cells were then incubated for an additional 48 h followed by the luciferase activity assay.

2.4. Luciferase activity assay

The luciferase activity was assayed using the dual-luciferase reporter assay system (Promega) and the Turner 20/20 luminometer (Turner Designs, CA, USA) as directed by the manufacturers.

2.5. Electrophoretic mobility shift assay (EMSA)

DNA oligonucleotide corresponding to the promoter region from −174 to +10 was used as the probe in the EMSA. This region was PCR amplified from the *DPYD* promoter-luciferase construct Z59 (see Fig. 1A) with fast start Taq DNA polymerase in GC rich mixture (Roche Applied Science). Primers used were as follows: Forward-, 5′-acttacgaattctccctccctcccttctgcttgc-3′; Reverse-, 5′-acttacgaattccggagcgcgagtcgaaaacagg-3′. After 30 cycles at 95 °C for 30 s, 60 ° for 30 s and 72 °C for 30 s, the PCR product was gel purified using the Qiaquick gel extraction kit (Qiagen). The sequence of the PCR product was verified by cloning 1 μl of the product into pGEM-T vector (Promega), followed by sequencing. The rest of the product was digested with *Eco*RI and purified again with the Qiaquick PCR purification kit (Qiagen). The oligonucleotide with *Eco*RI overhangs was then labeled with α-^{32}P-deoxyadenosine 5′-triphosphate (PerkinElmer Life and Analytical Sciences) by DNA polymerase I large (Klenow) fragment (Promega) and purified by using a G25 Spin column. A reaction mixture, made up in a total volume of 19 μl, consisted of 5% glycerol, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris–HCl (pH 7.5), 0.05 μg of poly (dI-dC) and 3.35 μg of HeLa nuclear extract (Promega, in vitro transcription grade) was pre-incubated at 22 °C for 10 min. Subsequently, 0.035 pmole of ^{32}P-labeled oligonucleotide (12,000 cpm) was added and reaction was incubated for an additional 20 min. For competition experiments, unlabeled double-stranded oligonucleotides as listed in Fig. 1C were added to the pre-incubation mixture; for super shift analysis, Sp1 antibody (Santa Cruz, CA, USA), Sp3 antibody (Active Motif, CA, USA) or pre-immune rabbit IgG were added. Bound and free DNA were resolved by electrophoresis through a 5% polyacrylamide (acrylamide:bisacrylamide at 37.5:1) gel at 350 V in 0.5X TBE at 4 °C. Gels were exposed to Fuji medical X-ray film with intensifying screens at −80 °C.

2.6. Chromatin immunoprecipitation (ChIP)

HeLa cells were grown for 2 days until they reached ~95% of confluency. ChIP was performed as described previously with modifications [25]. Briefly, chromatin was cross-linked using 1% formaldehyde and then sheared to an average fragment size of 500 bp. After centrifugation, 0.2 A_{260} units of the supernatant was used as input, and the rest was diluted 1:5 with dilution buffer (1.2 mM EDTA, 167 mM NaCl, 16.7 mM Tris–HCl (pH 8.1), 1.1% Triton X-100, and 0.01% SDS). A portion of the diluted fraction (18 A_{260} units) was subjected to immunoprecipitation overnight by Sp1 (UPSTATE), Sp3 (Santa