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Original article

DNMT1: An emerging target in the treatment of invasive urinary bladder cancer

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Abstract

Objectives: More than 14,000 people die from invasive urothelial carcinoma (iUC) of the urinary bladder each year in the USA, and more effective therapies are needed. Naturally occurring canine iUC very closely resembles the disease in humans and serves as a highly relevant translational model for novel therapy of human iUC. Work was undertaken to identify new targets for anticancer therapy in dogs with the goal of translating successful therapeutic strategies into humans with iUC.

Materials and methods: Microarray expression analyses were conducted on mRNA extracted from canine normal bladder ($n = 4$) and iUC tissues ($n = 4$) using Genome Array 1.0 and analyzed by GeneSpring GX 11, with the stringency of $P < 0.02$ and a ≥2-fold change. The genes thus identified were further analyzed for functional and pathway analysis using Protein ANalysis THrough Evolutionary Relationships (PANTHER) Classification System. In selecting genes for further study, consideration was given for evidence of a role of the gene in human iUC. From these analyses, DNA methyltransferase 1 (DNMT1) was selected for further study. Immunohistochemistry (IHC) of canine normal bladder and iUC tissues was performed to confirm the microarray expression analyses. The effects of targeting DNMT1 in vitro was assessed through MTT assay and Western blot of canine iUC cells treated with 5-azacitidine (5-azaC) and trichostatin A (TSA).

Results: DNMT1 was expressed in 0 of 6 normal canine bladder samples and in 10 of 22 (45%) canine iUC samples. The proliferation of canine iUC cells was inhibited by 5-azaC (at concentrations ≥5 μm) and by TSA (at concentrations ≥0.1 μm). Western blot results were supportive of DNMT1-related effects having a role in the antiproliferative activity.

Conclusions: Microarray expression analyses on canine tissues identified DNMT1 as a potentially "targetable" gene. Expression of DNMT1 in canine iUC was confirmed by IHC, and in vitro studies confirmed that drugs that inhibit DNMT1 have antiproliferative effects. These findings are similar to those recently reported in human iUC and are also in line with results of a preclinical (prehuman) trial of 5-azaC in dogs with naturally occurring iUC. DNMT1 has excellent potential as a target for iUC therapy in humans.

Keywords: Canine; Invasive urinary bladder cancer; Transitional cell carcinoma; DNA methyl transferase; Epigenetic modifications; Gene chip; Cell lines

1. Introduction

In 2011, more than 14,000 people in the USA died of invasive urothelial carcinoma (iUC), also referred to as invasive transitional cell carcinoma of the urinary bladder. Research to improve the prevention and treatment of iUC is crucially needed [1]. Research to test new therapies for iUC

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would benefit from a highly relevant animal model, which mimics the human condition at the molecular, cellular, and clinical level. The study of new interventional strategies in dogs with naturally occurring iUC and selection of the most promising approaches for subsequent human clinical trials represents 1 promising strategy [2]. Naturally occurring iUC in dogs closely mimics iUC in humans in regards to histopathology, molecular features, sites of metastasis, prognostic factors, and response to chemotherapy [2]. There are over 70 million dogs in the USA, and approximately 25% of older dogs develop cancer [2]. Even though iUC comprises only 2% of canine cancer, there are thousands of dogs that develop iUC yearly in the USA. The compressed life span of dogs allows study of the cancer process from earliest changes until death in a relatively short period. There is already evidence that new treatment approaches for iUC that are successful in dogs hold promise in humans with cancer [2–4].

Micro array expression analyses were performed to identify potential new therapeutic targets. In selecting genes for clinical relevance for canine bladder cancer based on microarray data analyses, consideration was given for evidence of a role for those same genes in human iUC. From these analyses, DNA methyltransferase 1 (DNMT1) was selected for further study. Selection of the gene was based on: (1) differential up-regulation of the gene in iUC compared with normal urothelium in dogs; (2) support from prior studies for a biologically important role of the gene in cancer development and progression; (3) evidence from the literature that the same gene is up-regulated in human iUC and is expected to be important in human cancer, and (4) availability of compounds that could target the gene, which would have potential antitumor activity.

DNMT1 is a key enzyme that methylates CpG islands located near the regulatory regions of genes, which affects transcription of specific genes involved in cancer development and progression [5,6]. Epigenetic events of importance in cancer include DNA methylation and histone tail modifications via acetylation and methylation [7,8]. DNA methyl transferases (DNMTs) are enzymes that catalyze the methylation of DNA [5–9]. DNMT1, DNMT2, DNMT3a, and DNMT3b have been identified in mammalian cells [5,6,8–11]. Despite their genetic homology, functional differences have been ascribed to these enzymes [12]. The activity of DNMTs is closely tied to the activity of histone deacetylases (HDACs) and histone methylases, which change the structure of histones into condensed chromatin state [8,13]. Gene methylation and changes in histone structure regulate transcription through similar pathways [14]; both of these modifications lead to the silencing of tumor suppressor genes [15]. DNMT1 up-regulation has been reported in several forms of human cancer, including iUC [7,8,16,17]. Compounds that inhibit DNMT1 are available, and these compounds, especially when given with HDAC inhibitors, have been shown to reverse gene methylation, thereby causing reactivation of key tumor suppressor genes while suppressing the transcription of some other key genes [7,8,18–21]. Drugs that inhibit DNMT1 are being evaluated in people with acute myelodysplastic syndrome, myeloma, and solid tumors [22–25], but these drugs have not yet been studied in humans with iUC. Recently, in a preclinical phase I trial in dogs with naturally occurring iUC, the effects of 5-azacitidine were determined in 19 dogs [26]. Of 18 dogs evaluable for tumor response, partial remission, stable disease, and progressive disease were observed in 4 (22%), 9 (50%), and 5 (28%) dogs, respectively. In the work described here, the potential role of DNMT1 as a target for iUC treatment and the extent to which this treatment approach could be studied in canine iUC were assessed. The ultimate goal is to utilize studies in canine iUC to facilitate the development of improved therapy for iUC in humans, as well as in dogs.

2. Materials and methods

2.1. Overview

The work included: (1) microarray expression profile analysis to determine genes differentially expressed between canine iUC and normal canine bladder, (2) immunohistochemical detection of DNMT1 protein in bladder tissues from dogs with iUC and from normal dogs, and (3) in vitro assays to determine the effects of DNMT1 targeted drugs in the proliferation and protein expression of canine iUC cells. The drugs studied consisted of the demethylating agent 5-azacitidine ("5-azaC") (Vidaza; Celgene, Summit, NJ), which acts directly to inhibit DNMT1, and the HDAC inhibitor, trichostatin A (TSA) (Sigma, St. Louis, MO), which facilitates the action of 5-azaC by allowing the relaxation of the chromatin/transcriptional machinery [7,8,14,26]. Since 5-azaC and TSA have been demonstrated to induce cell cycle arrest and or apoptosis in other human cancers [27–29]; changes in the expression of proteins that mediate apoptosis [poly (ADP-ribose) polymerase (PARP), survivin] and cell cycle regulatory proteins (p16, p21, pRb, cyclin D1) were assessed to identify potential mechanisms of action of the drugs. Changes in DNMT1 protein levels were also assessed.

2.2. Microarray expression profiling analysis

Gene expression profiling was performed using the Genome Array 1.0 (Affymetix canine 1.0 with 23,913 well-annotated RefSeq transcripts; Affymetix, Santa Clara, CA). Labeling, hybridization, and scanning of the microarray were performed at the Center for Medical Genomics (CMG), Indiana University, Indianapolis, IN. Total RNA was extracted from freshly isolated full thickness normal canine bladder ($n = 4$) and iUC samples before therapy ($n = 4$) using Trizol (Invitrogen, Carlsbad, CA) and purified using RNAeasy (Qiagen, Valencia, CA). Fresh tumor sam-