Research Article

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The therapeutic effect of histone deacetylase inhibitor PCI-24781 on gallbladder carcinoma in BK5.erbB2 mice

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Background & Aims: Gallbladder carcinoma (GBCa), a type of biliary tract cancer (BTC), has proven challenging to treat, demonstrating the need for more effective therapeutic strategies. In our current study, we examined the therapeutic effects of the histone deacetylase (HDAC) inhibitor PCI-24781 against GBCa that developed in BK5.erbB2 mice.

Methods: PCI-24781 [50 mg/kg/day] and control solutions were administered to BK5.erbB2 mice for 4 weeks. The therapeutic effect of PCI-24781 was evaluated by ultrasound biomicroscopy (USBM) throughout the experiment and histological analyses at the end of the experiment. To investigate potential mechanisms underlining the therapeutic effects of PCI-24781 on GBCa in BK5.erbB2 mice, PCI-24781-treated gallbladders were subjected to Western blot and RT-PCR analysis. The inhibitory effect of PCI-24781 on the growth of BTC cells was compared to the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA) and gemcitabine. To study the role of miRNAs in GBCa tumorigenesis, the expression profile of 368 miRNAs in GBCas from BK5.erbB2 (both treated and untreated) and wild type mice was analyzed.

Results: Treatment of BK5.erbB2 mice with PCI-24781 for 1 month prevented 79% of GBCa cases from progression and showed a clinical effect in 47% of cases. We also confirmed a potent inhibitory effect on tumor cell growth in human BTC cell lines treated with PCI-24781. This effect was associated with downregulation of *ErbB2* mRNA and ErbB2 protein/activity and upregulation of acetylated histone and acetylated tubulin. Treatment with PCI-24781 resulted in decreased expression of Muc4, an intramembrane ligand for ErbB2, in BTC cells. PCI-24781 had more effects on growth inhibition of BTC cells than SAHA. In addition, PCI-24781 effectively inhibited the growth of gemcitabine-resistant cells. miRNA profiling revealed that the expression of several miRNAs was significantly altered in GBCa in the BK5.erbB2 mouse compared to normal gallbladder, including upregulated miR21, which was downregulated by PCI-24781.

Conclusions: These results indicate that PCI-24781 potently inhibits the growth of BTC cells by decreasing ErbB2 expression and activity as well as regulating altered miRNA expression. PCI-24781 may have a potential value as a novel chemotherapeutic agent against human BTC in which ErbB2 is overexpressed.

Cancer

Introduction

GBCa has proven challenging to treat due to both its poor sensitivity to conventional therapies and its inability to be detected at an early stage. These difficulties lead to a poor overall prognosis [1] and demonstrate the need for better therapeutic modalities. Overexpression of ErbB2 has been reported in a significant percentage of human BTC and is believed to be one of the major mechanisms underlying BTC carcinogenesis [1–5]. The established frequency of ErbB2 overexpression in all BTCs ranges from 6.2% to 65.0% [1,4,5], which may be due to differences in experimental methods, the use of different antibodies for immunostaining, or the different criteria used for evaluation. Other evidence for the involvement of ErbB2 in the development of BTC malignancies comes from chemically-induced animal models using furan [6] and transfected cell model [2].

We previously reported on the development of GBCa in transgenic mice where expression of a rat *ErbB2* cDNA is targeted to the basal layer of epithelial tissues by the bovine keratin 5 (*BK5*) promoter (BK5.erbB2 mice) [7]. GBCa develops in approximately 80% of these transgenic mice within 2 months of age. Similarities between GBCa in BK5.erbB2 mice and humans include histopathological and molecular characteristics [7,8].

HDACs contribute to the regulation of a limited number of genes involved in cell growth, differentiation, and survival [9]. It has been hypothesized that aberrant patterns of histone acetylation maintain the transformed state of human tumors, a

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Abbreviations: GBCa, gallbladder carcinoma; HDAC, histone deacetylase; EGFR, epidermal growth factor receptor; BTC, biliary tract cancer; BK5, bovine keratin 5; BrdU, bromodeoxyuridine; USBM, ultrasound biomicroscopy; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling; SAHA, suberoylanilide hydroxamic acid; miRNA, microRNA.

state that can be reversed by inhibiting these HDACs. Changes in expression of HDACs have been reported in various cancers [9].

More than 10 different HDAC inhibitors, including a broad-spectrum phenylhydroxamic acid HDAC, PCI-24781 (Pharmacyclics, Sunnyvale, CA), are currently undergoing clinical trials [10].

In this study, we examined the therapeutic effects of PCI-24781 against GBCa that develops in BK5.erbB2 mice. To elucidate the mechanism of these inhibitory effects, the altered expression and activity of ErbB2, its downstream signaling molecules, Muc4, and the expression profile of miRNAs were also determined after treatment.

Materials and methods

Human BTC cells and chemicals

Human BTC cell lines TGBC and Sk-ChA-1 [11] were provided by Dr. Takeshi Todoroki (Tsukuba University, Ibaraki, Japan). PCI-24781 was provided by Pharmacyclics, Inc. (Sunnyvale, CA).

Animals and treatment protocols

BK5.erbB2 mice are maintained at the University of Texas M.D. Anderson Cancer Center, Science Park – Research Division. Twenty-eight candidate BK5.erbB2 mice were screened for the presence of GBCas by USBM (Visual Sonics, Toronto, Canada) at 2 months of age in order to ensure that each group had an initial GBCa incidence of 100%. PCI-24781 (50 mg/kg/0.2 ml/day, i.p. injection twice a day), dissolved in 50 mM sodium lactate buffer (pH 4.2), was administered to 19 BK5.erbB2 mice, 5 consecutive days per week for 4 weeks. The same solution without PCI-24781 was delivered to 9 BK5.erbB2 mice as control. Animals were monitored twice a week to evaluate systemic toxicity and body weight. The size of tumors in mice was monitored by USBM every 2 weeks. Feeding was stopped 24 h prior to USBM analysis for gallbladder volume maximization. The determination of the tumor size by USBM was performed as a blinded experiment. All experiments were carried out with strict adherence to institutional guidelines in order to minimize distress in animals.

Histological analysis

After being fixed in formalin and embedded in paraffin, 5 μm serial sections (28 μm between each section) of biliary tract tissue were cut and stained with hematoxylin and eosin (H&E). Utilization of bromodeoxyuridine (BrdU) as a proliferation marker was performed as previously described [8].

Immunofluorescence staining

The expression and localization of ErbB2, phosphorylated ErbB2 (p-ErbB2), EGFR, and p-EGFR were determined by immunofluorescence on sections of gallbladders as described previously [7]. Sections were analyzed using a laser confocal microscope (Zeiss 510 Meta).

Cell growth

Growth of human BTC cells treated with PCI-24781, SAHA, or gemcitabine was determined with the Cell Proliferation Assay Kit (Promega, Madison, WI) according to the manufacturer's protocol. Each experiment was performed at least 3–4 times.

Western blot analysis

Epithelial cell lysates were prepared from three pooled mouse gallbladders, as described previously [12]. Anti-Muc4 antibody was kindly provided by Dr. Surinder Batra (University of Nebraska). All experiments were performed in triplicate and repeated at least twice.

Evaluation of the therapeutic efficacy of PCI-24781

The final diagnosis of GBCa tumors was based on images generated by USBM and histological analyses. The criteria for evaluation of therapeutic effects are as follows: Complete Response (CR), complete disappearance of a previous tumor; Partial Response (PR), >30% reduction of all the measurable lesions in the tumor; Minimum Change (MC), <30% change in the measurable size of the tumor compared with the original tumor; Progressive Disease (PG), >30% increase in the size of all measurable lesions in the original tumor or the appearance of new lesion(s).

Detection of mRNA levels by Real Time PCR

ErbB2 mRNA transcript levels were assessed as previously described [13].

Primary culture of gallbladder epithelial cells from BK5.erbB2 and wild type mice

The long-term cultures of gallbladder epithelial cells from both BK5.erbB2 and wild type mice were prepared as previously described [14].

MicroRNA microarray

To compare the expression of miRNA in GBCa to that of control gallbladders, RNA from 8 GBCas from BK5.erbB2 mice and 30 gallbladders from wild type mice was isolated. In addition, to study the effect of PCI-24781 on the expression of miRNAs in GBCa, the expression of miRNAs in GBCas treated with PCI-24781 was compared to that of untreated GBCas. A microarray was performed and expression profiles of 368 miRNAs were analyzed at the University of Texas M.D. Anderson Cancer Center, Science Park Molecular Biology Core. The ratios of miRNA were the average value of three independent experiments.

Detection of plasma concentration of PCI-24781 in BK5.erbB2 mice

The plasma concentration of PCI-24781 in BK5.erbB2 mice was measured by administering PCI-24781 (50 mg/kg/day in 50 mM sodium lactate buffer, pH 4.2) i.p. to these mice twice daily for 2 weeks. Blood samples were collected 4 h after the last injection and plasma was assayed for PCI-24781 by liquid chromatography with tandem mass spectrometry as previously described [10].

Statistical analysis

All the data are expressed as mean ± SD. Statistical significance was determined by the Mann–Whitney U-test and the Fisher's exact test. $p < 0.05$ was considered to be significant.

Results

Therapeutic efficacy of PCI-24781 on GBCa in BK5.erbB2 mice

All mice were screened for GBCa by USBM at the start of treatment. Serial sections stained with H&E were analyzed to verify the therapeutic evaluation determined by USBM. In the control group (n = 9), seven mice (78%) were diagnosed as Progressive Disease (PG) and only two (22%) were diagnosed as Minimum Change (MC) (Fig. 1A). While no diagnosis of Complete Response (CR) was made in the treated group, nearly half (47%) of the treated mice were diagnosed as Partial Response (PR). Six cases (32%) from this group were diagnosed as MC and only four cases (21%) were diagnosed as PG (Fig. 1A). Thus, nearly 80% of treated mice showed either therapeutic efficacy (PR) or prevention from progression (MC). These effects are statistically significant compared with the control group. None of the treated BK5.erbB2 mice showed any signs of toxicity, neurogenic abnormalities or significant difference in body weight gain.