BASIC AND TRANSLATIONAL—ALIMENTARY TRACT

Pan-Histone Deacetylase Inhibitor Panobinostat Sensitizes Gastric Cancer Cells to Anthracyclines via Induction of CITED2

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BACKGROUND & AIMS: Chemotherapy modestly prolongs survival of patients with advanced gastric cancer, but strategies are needed to increase its efficacy. Histone deacetylase (HDAC) inhibitors modify chromatin and can block cancer cell proliferation and promote apoptosis. **METHODS:** Gastric cancer cell lines were incubated with the HDAC inhibitor LBH589 (Panobinostat, Novartis, Germany); levels of proliferation, apoptosis, histone acetylation, and gene expression were determined. We identified factors downstream of HDAC that regulated chemoresistance. The effects of combination chemotherapy of HDAC inhibitors and anthracyclines were studied in CEA424/SV40 T-antigen (*CEA/Tag*) transgenic mice, which develop gastric tumors. We analyzed gastric tumor samples from patients using immunohistochemistry. **RESULTS:** HDAC2 was expressed in human gastric cancer cell lines and tumor samples, as well as in gastric tumors from *CEA/Tag* mice, compared with non-neoplastic gastric tissue. LBH589 inhibited proliferation of cancer cells in vitro. LBH589 down-regulated expression of genes that mediate anthracycline resistance by activating expression of Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain 2 (CITED2), a gene that mediates sensitivity to chemotherapeutics. Preincubation of cells with an HDAC inhibitor and overexpression of CITED2-sensitized gastric cell lines to anthracycline-mediated cell death. In *CEA/Tag* mice, LBH589 induced tumor-cell expression of CITED2 and increased the efficacy of anthracycline to reduce tumor growth. Levels of CITED2 were increased in gastric tumor samples from patients who had complete responses to epirubicin. **CONCLUSIONS: The HDAC inhibitor LBH589 can overcome the resistance of mouse gastric cancer cells to anthracyclines by inducing expression of CITED2. Levels of CITED2 in gastric tumors correlate with patients' response to epirubicin. LBH589 might be used to increase the response of patients to anthracyclines.**

Keywords: Cancer Treatment; Stomach; Chemotherapeutic Agent.

Gastric cancer is the fourth most common cancer and the second leading cause of cancer-related deaths worldwide.[1] In patients with advanced disease, the efficacy of systemic chemotherapy still is limited and toxicity of combination chemotherapy is substantial. Furthermore, drug resistance is a frequent observation in gastric cancer as in other tumors, although the biology of drug resistance is largely unknown.[2]

Alterations in epigenetic regulation are frequent events in cancer development and play an important role in gastric cancer pathogenesis. Thus, changes in DNA methylation and histone modifications may lead to aberrant gene expression profiles and result in silencing of tumor-suppressor genes.[3] Accordingly, high expression levels of histone deacetylase (HDAC) class I enzymes have been reported in different types of tumors, including gastric cancer.[4] In addition, other studies described a decrease in acetylated histones and an overexpression of HDAC2 in gastric cancer patients.[5] These findings indicate that high expression of HDAC class I may be an important alteration in gastric carcinogenesis and could serve as a potential target for epigenetic therapy.

In the present study we examined the functional role of HDAC inhibition in gastric cancer. Our findings indicate that HDAC inhibition through the Pan-HDAC inhibitor LBH589 (Panobinostat, Novartis, Germany) exerts potent anticancer activity in combination with epirubicin in gastric cancer in vivo and in vitro. We believe that the analysis of the complex interaction of HDAC inhibition and che-

Abbreviations used in this paper: cDNA, complementary DNA; CEA/Tag, transgenic CEA424/SV40 T-antigen mouse line; ChIP, chromatin immunoprecipitation; CITED2, Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyterminal domain 2; ECF, epirubicin, cisplatin, and fluorouracil; Epi, epirubicin; EV, empty vector; HDAC, histone deacetylase; IC50, half-maximal inhibitory concentration; mRNA, messenger RNA; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; pCITED2, CITED2 expression plasmid; siRNA, small interfering RNA.

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motherapy may provide an interesting basis for the development of individualized treatment strategies in this cancer.

Materials and Methods

Cell Culture

The gastric cancer cell lines AGS, SNU-1, SNU-5, NCI-N87, KATO-III (from the American Type Culture Collection, MD), and MKN-7 and MKN-45 (from Japan Cell Bank) were maintained at 37°C in a humidified atmosphere of 5% CO_2 and 95% air in either Dulbecco's modified Eagle medium (AGS) or RPMI 1640 with 10% (vol/vol) fetal calf serum (all from Life Technologies, Inc, Darmstadt, Germany).

Patient Samples

Human gastric cancer (tumor) and corresponding normal tissues were collected before therapy as described previously.[6] Analysis of human tumor samples was conducted in agreement with ethical guidelines of the Technical University of Munich and the study was approved by the Human Subjects Committee of the Technical University of Munich. Tissue array ST843 of gastric cancer was purchased from US Biomax, Inc (Rockville, MD).

Animals

Transgenic CEA424/SV40 T-antigen (*CEA/Tag*) mice were described previously and maintained on a pure C57BL/6 background.[7] Details of experimental studies in transgenic mice are provided in the Supplementary Table 1 and in the Supplementary Materials and Methods section. Animal studies were performed according to regulatory standards and approved by the government of Bavaria.

Gene Expression Microarray

Total RNA (1 μg) from LBH589-treated (100 nmol/L for 24 h) and 5% dextrose-treated AGS cells was labeled using the One-Cycle complementary RNA labeling kit (Affymetrix, High Wycombe, United Kingdom) and hybridized to HGU133 Plus 2.0 arrays (Affymetrix).[8,9] Gene signatures were identified using gene set enrichment analysis.[10,11] Microarray data can be accessed at http://www.ncbi.nlm.nih.gov/geo/ (accession number: GSE36444).

DNA Constructs

The complementary DNA (cDNA) of the human Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxyterminal domain 2 (*CITED2*) gene (Accession number: NM_001168389) was cloned into the pcDNA3 plasmid (gift of Jens Siveke, Technical University of Munich). The promoter sequences of human *FOXM1* (1507–53 bp downstream of ATG) and *HMGB2* (1100–43 bp downstream of ATG) were cloned into pGL3-basic-luciferase vector (Promega, Mannheim, Germany). Silencer select predesigned and validated small interfering RNA (siRNA) against HDAC1 (s73) and HDAC2 (s6493) and Cy3-labeled negative control siRNA were purchased from Life Technologies.

Protein Analysis

Immunohistochemistry, H&E, and Elastica van Gieson staining was performed as previously described.[4,6] Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of cell lysates and tissue lysates were generated, and Western blotting was performed as previously described.[4,6]

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as recommended in the manufacturer's protocol (ChIP assay kit; Upstate Biotechnology, Temecula, CA). More details are provided in the Supplementary Materials and Methods section.

Cellular Assays

To analyze cell viability, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) conversion was measured with colorimetric MTT assays as suggested by the manufacturer (Roche Diagnostics, Mannheim, Germany). Cells were seeded at a density of 10,000 cells per well in a 96-well format and were treated with different drug concentrations. A half-maximal inhibitory concentration (IC50) analysis was performed in a range of 0–5000 nmol/L for LBH589. Cells were stained with annexin and propidium iodide (BD Pharmingen, Heidelberg, Germany), and apoptotic cells were identified by flow cytometry analysis according to the manufacturer's recommendations. In addition, colony formation was assessed by treating 5000 AGS or MKN-45 cells for 24 hours with 10, 50, 100, and 200 nmol/L LBH589. After medium change, cells were grown for 2 weeks, fixed with methanol, and stained with 0.5% (vol/vol) crystal violet. Cell colonies were analyzed with ImageJ (National Institutes of Health, Bethesda, MD) software. Transient transfection of expression and reporter plasmids was performed with Turbofect (Fermentas, St. Leon-Rot, Germany) according to the manufacturer's protocol. Luciferase activity was measured with Dual Glow Luciferase Assay (Promega). The HDAC enzymatic activity assay was purchased from Cayman Chemicals Company (Ann Arbor, MI) and performed according to the manufacturer's instructions. Nuclear extracts of AGS and MKN-45 cells were treated with 10, 50, and 100 nmol/L of LBH589. Combination treatment of cells was started with 12 hour pre-incubation with 50 nmol/L LBH589 for AGS cells or 100 nmol/L LBH589 for MKN-45 cells followed by 48-hour incubation with 0.1, 0.5, or 1 μmol/L of doxorubicin or epirubicin. For comparison, cells were pretreated for 12 hours with vehicle control and incubated for 48 hours with 0.1, 0.5, or 1 μmol/L doxorubicin or epirubicin.

Statistics

Results are means ± standard error from at least 4 individual animals per genotype or 3 independent experiments from different cell passages. P values ($<.05$) were calculated using the Student t test or the Mann–Whitney U test. GraphPad Prism software (La Jolla, CA) was used to analyze the data.

Results

Expression of HDAC2 in Gastric Cancer Tissue, Cell Lines, and Mouse Model

First, we determined HDAC2 expression in human gastric cancer cell lines, patient tumor tissue samples, and in a gastric cancer transgenic mouse model (*CEA/Tag*). Increased levels of *HDAC2* messenger RNA (mRNA) were observed in all cell lines (fold-change ranging from 6.08 ± 0.86 for MKN-7 up to 29.32 ± 3.11 for SNU-1) compared with healthy control stomach tissue (Figure 1*A*). Protein expression of HDAC2 was confirmed in gastric cancer cell lines by Western blot (Figure 1*B*). Patient samples of gastric cancer (tumor) and corresponding normal tissue (n = 19) also were analyzed by Western blot analysis