HIGHLY EXPRESSED GENES IN RAPIDLY PROLIFERATING TUMOR CELLS AS NEW TARGETS FOR COLORECTAL CANCER TREATMENT

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TRANSLATIONAL RELEVANCE

The identification of novel therapeutic targets would significantly improve the clinical management of colorectal cancer patients. Because rapidly proliferating tumors are associated with poor patient prognosis, here we assessed the growth rates of a panel of 52 colorectal cancer cell lines and used microarray analysis to identify a subset of 966 genes with high expression levels in colorectal tumor cells with faster growth. As a proof of concept, we then demonstrated that similarly to the thymidylate synthase inhibitor 5-fluorouracil, a well-established therapeutic agent, pharmacological inhibition of protoporphyrinogen oxidase (PPOX), a gene with significantly higher expression levels in rapidly proliferating tumor cells, resulted in reduced growth in vitro and in a preclinical xenograft model. Importantly, PPOX and other genes highly expressed in rapidly growing tumor cells could constitute novel therapeutic targets for colorectal cancer patients.
ABSTRACT

Purpose: The clinical management of colorectal cancer patients has significantly improved due to the identification of novel therapeutic targets such as EGFR and VEGF. Because rapid tumor proliferation is associated with poor patient prognosis, here we characterized the transcriptional signature of rapidly proliferating colorectal cancer cells in an attempt to identify novel candidate therapeutic targets.

Experimental Design: The doubling time of 52 colorectal cancer cell lines was determined and genome-wide expression profiling of a subset of these lines was assessed by microarray analysis. We then investigated the potential of genes highly expressed in cancer cells with faster growth, as new therapeutic targets.

Results: Faster proliferation rates were associated with microsatellite instability and poorly differentiated histology. The expression of 1,290 genes was significantly correlated with the growth rates of colorectal cancer cells. These included genes involved in cell cycle, RNA processing/splicing and protein transport. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and protoporphyrinogen oxidase (PPOX) were shown to have higher expression in faster growing cell lines and primary tumors. Pharmacological or siRNA-based inhibition of GAPDH or PPOX reduced the growth of colon cancer cells in vitro. Moreover, using a mouse xenograft model, we show that treatment with the specific PPOX inhibitor acifluorfen significantly reduced the growth of 3 of the 7 (42.8%) colon cancer lines investigated.

Conclusion: We have characterized at the transcriptomic level the differences between colorectal cancer cells that vary in their growth rates, and identified novel candidate chemotherapeutic targets for the treatment of colorectal cancer.
INTRODUCTION

Colorectal cancer is one of the three most prevalent types of cancer in the Western world and accounts for over 1.2 million new cases and 600,000 deaths every year worldwide (1). The genetic and epigenetic defects and the sequence of how these events accumulate during tumor progression are well characterized. Our current knowledge of the molecular mechanisms underlying the development of colorectal cancer is the result of extensive investigation in previous decades and the new light shed by the more recent genome-wide efforts such as The Cancer Genome Atlas –TCGA project (2) or the Encyclopedia Of DNA Elements –ENCODE (3).

Since the late 1990’s when expression microarray analysis became popular in the field, it was apparent that many genes were involved in the regulation of the cell cycle (4,5). Since a major hallmark of cancer is uncontrolled rapid proliferation, it was not surprising to find that many of the genes that control cell cycle progression were deregulated in the different tumor types investigated, compared to the corresponding normal tissue (4,6). However, despite some early studies (7,8), the genes with higher expression in rapidly proliferating tumor cells compared to slowly cycling tumors are not as well characterized. This is of considerable clinical relevance since it has been repeatedly observed that rapid tumor proliferation is associated with poor patient prognosis (9–12). Moreover, some of the most widely used chemotherapeutic agents for various types of cancer are inhibitors of proteins that are involved in cell proliferation, such as hydroxyurea, methotrexate and doxorubicin/etoposide, which target ribonucleotide reductase, dihydrofolate reductase and topoisomerase II, respectively. Notably, 5-fluorouracil (5-FU), the gold standard agent for the treatment of colorectal cancer patients for over five decades (13), targets thymidylate synthetase, an important gene required for cell proliferation. In addition to 5-FU, the therapeutic options currently approved for the treatment of colorectal cancer are limited, and include irinotecan, oxaliplatin and the targeted agents cetuximab/panitumumab, bevacizumab and regorafenib. When used as single agents, the response rates for these drugs is below 30% and there is a clear need for the improvement of the clinical management of these patients.
that the identification of new therapeutic targets and novel agents would bring about.

In this study we used a panel of 52 colorectal cancer cell lines to investigate different features associated with the growth rates of these cells. We found that higher proliferation rates in colorectal cancer cells were associated with a microsatellite instable (MSI) phenotype and poor differentiation. In addition, we used microarray analysis of a subset of 31 of these cell lines to determine the expression signature of rapidly proliferating tumor cells. Moreover, we identified protoporphyrinogen oxidase (PPOX) as a novel chemotherapeutic target candidate, and using chemical inhibitors or siRNA-based knockdown we confirmed that targeting of PPOX in vitro and in vivo significantly interferes with tumor growth.
MATERIAL AND METHODS

Cell culture and primary tumor samples. A total of 52 colorectal cancer cell lines were used: Caco2, Colo201, Colo205, Colo320, DLD1, HCT116, HCT15, HCT8, HT29, HUTU80, LoVo, LS1034, LS174T, LS513, RKO, SKCO1, SNUC2B, SW1116, SW403, SW48, SW480, SW620, SW837, SW948, T84, and WiDr were purchased from ATCC (Manassas, VA). HDC108, HDC111, HDC114, HDC133, HDC15, HDC54, HDC75, HDC8, HDC87, and HDC9 were a kind gift from Dr. Johannes Gebert (Institute of Pathology, University Hospital Heidelberg, Heidelberg, Germany). HT29-cl16E, HT29-cl19A, HCC2998, KM12, and RW2982, were a kind gift from Dr. L.H. Augenlicht (Albert Einstein Cancer Center, Bronx, NY). LIM1215 and LIM2405 were obtained from the Ludwig Institute for Cancer Research in Melbourne. ALA, Co115, FET, Isreco1, Isreco2, Isreco3, and TC71 were a kind gift from Dr. Hamelin, Paris, France. GP5D and VACO5 were a kind gift from Dr. L.A. Aaltonen (Biomedicum Helsinki, Finland).

All lines were obtained more than six months before the beginning the experiments in this study and maintained in MEM (Life Technologies, Carlsbad, CA) supplemented with 10% fetal bovine serum, 1x antibiotic/antimycotic (100 units/ml streptomycin, 100 units/ml penicillin, and 0.25 μg/ml amphotericin B), 1x MEM Non-Essential Amino Acids Solution, and 10 mM HEPES buffer solution (all from Life Technologies, Carlsbad, CA). All lines were tested to be negative for mycoplasma contamination (PCR Mycoplasma Detection Set, Takara). Cell lines were cultured until they reached 70%-80% confluence and the medium was changed 8h before harvesting the cultures for RNA extraction.

The cell lines used were not authenticated, but possible cell line cross-contamination was investigated by clustering analysis of genome-wide mRNA expression microarray data at the time of these experiments.

The data from primary tumor samples used in this study were obtained from The Cancer Genome Atlas (TCGA). mRNA expression levels (Illumina RNAseq and Agilent microarray G4502A) and haematoxylin and eosin-stained high resolution images of formalin-fixed, paraffin-embedded sections of primary tumors were downloaded from the TCGA data portal (https://tcga-data.nci.nih.gov/tcga/). For light microscopy quantification of mitotic cells in
these tumors, three random fields were selected, and the total number of cells (>500) and mitotic cells was scored blinded from the sample identity.

**Doubling time.** To determine the doubling time of each cell line, cells were seeded in seven 96-well plates. Seeding densities varied from 1x10^3 to 1.5x10^4 cells/well to ensure control cell densities did not exceed 80% confluence at the completion of the experiment. The plates were fixed with trichloroacetic acid (final concentration 10% w/v) at 24h intervals for seven days. Plates were washed with tap water, air dried and stained with 0.4% (w/v) sulforhodamine B (SRB) for 30 min. Excess SRB was washed out with 1% acetic acid and the plates were air dried. Cell-bound SRB was solubilized with 10mM Tris buffer pH=10 and absorbance was measured at 590nm using a microplate reader (Sunrise, Tecan). The doubling times were calculated using Prism V.5.01 (GraphPad, San Diego, CA). All experiments were carried out at least 3 times with eight replicates each time.

As an independent approach to assess cell growth, the Roche xCELLigence System was used for real-time monitoring of cell proliferation (14). Cell lines were seeded in quadruplicate at a density of 5000 cells/well in an E-Plate 96 (Roche Diagnostics, GmbH). The Real-Time Cell Analyzer MP instrument (Roche Diagnostics, GmbH), together with the E-Plate 96, was placed in a cell culture incubator maintained at 37°C with 5% CO₂, and continuous electrical impedance measurements were taken hourly for eight days. Doubling times were calculated using Cell Index data from the exponential growth phase for each cell line, with RTCA software version 1.2.1.

**Growth inhibition assay.** The dose resulting in 50% growth inhibition (GI₅₀) in the presence of 5-FU, acifluorfen, sodium iodoacetate, oxadiazon (all from Sigma-Aldrich, St. Louis, MO) or CGP 3466B maleate (Tocris, UK), compared to the corresponding control, was determined as described (15,16). Briefly, 5x10^3 cells per well were seeded in 96-well plates. Twenty-four hours after seeding, cells were treated with 5-FU (0, 0.01, 0.1, 0.5, 1, 2.5, 5, 10, 25, 50, 100, and 500 μM), acifluorfen (0, 5, 25, 100, 200, 300, 400, 500, 750, 1000, 2000, and 3000 μM), Na iodoacetate (0, 0.01, 0.1, 1, 2.5, 5, 7.5, 10, 20, 30, 60, and 120 μM), oxadiazon (0, 25, 50, 100, 200, 300, 400, 500, 600, 750, 1000,
and 1250 μM) or CGP 3466B maleate (0, 5, 10, 25, 50, 75, 100, 125, 250, 500 and 750 μM) for 72 h. Cells were fixed with trichloroacetic acid and stained with sulforhodamine B, as described above. One plate of each cell line was fixed to assess cell number at the time when drug treatment started. The GI50 was calculated as described previously (17,18). These experiments were carried out at least three times in quadruplicates.

**Apoptosis and cell cycle analysis.** Two hundred thousand cells were seeded in triplicate in 6-well plates. Control wells reached a confluence of approximately 80% at the completion of the experiment. 24h after seeding, cells were treated with 0, 10, 20, or 30 μM sodium iodoacetate or 0, 400, 800, 1200 μM acifluorfen (both Sigma-Aldrich, St. Louis, MO) for 72 h. Both, floating and adherent cells, were harvested, washed with cold PBS, and resuspended in 50 μg/ml propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells were stained for 2h at 4°C, and 10,000 cells were analyzed for DNA content using a FacsCalibur Flow Cytometer (Becton Dickinson, Franklin Lakes, NJ). The percentage of cells with a subdiploid DNA content was quantified using WinList 2.0 (Verity Software House, Topsahm, NE). The cell cycle profile was established using the ModFit 2.0 (Verity Software House, Topsahm, NE).

**Protein extraction and Western blot.** Seven hundred and fifty thousand cells were seeded in 6-well plates. 24h after seeding, cells were treated with 0, 10 or 20μM sodium iodoacetate or 0, 400 or 800μM acifluorfen for 24h. Cells were harvested, washed with cold PBS, and cell pellets resuspended in 0.1 ml lysis buffer (25mM Heps, pH=7.5, 150mM NaCl, 5mM MgCl₂, 1% NP-40, 1mM DTT, 10% glycerol and protease inhibitors). Aliquots of the cleared supernatant containing total protein (25 μg) were loaded on a 15% acrylamide gel. After gel electrophoresis, proteins were transferred to a PVDF membrane and probed with rabbit polyclonal anti-cleaved PARP (Asp214) antibody (#9541, Cell Signaling Technology; 1:2000), mouse monoclonal anti-β-tubulin antibody (T4026, Sigma-Aldrich; 1:1000), or rabbit polyclonal anti-actin antibody (Santa Cruz Biotechnology, H-300; 1:1000).

**Clonogenic assay.** Five hundred HCT116 or DLD1 cells were seeded in triplicate in 6-well plates. 24h after seeding, cells were treated with 0 or 15 μM
sodium iodoacetate or 0 or 1200 μM acifluorfen for 9 h. The medium containing the drug was washed off and replaced with fresh medium without drug. Colony formation was monitored over the following 2–3 weeks. Cultures were stained with 1% crystal violet for 30 min, washed with distilled water, air dried, and the number of colonies was determined blinded from the sample identity. Each cell line was assayed three times, each time in triplicate.

RNA extraction and quantitative RT-PCR. Cell cultures were harvested at 70-80% confluence and total RNA was extracted using TRizol Reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Total RNA (2μg) was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Life Technologies, Carlsbad, CA), and relative mRNA levels of PPOX, GAPDH, TYMS, CALCOCO2, CBX5 and SMAD4 were assessed by Real-Time PCR using SYBR Green Master Mix (Life Technologies, Carlsbad, CA). 18S rRNA (Taqman Master Mix, Life Technologies, Carlsbad, CA) was used as a standardization control for the 2^-ΔΔCt method as described before (19). The primers used were TYMS-qPCR-forward 5'-ACA CAC TTT GGG AGA TGC AC-3', TYMS-qPCR-reverse 5'-GGT TCT CGC TGA AGC TGA AT-3', PPOX-qPCR-forward 5'-GGC GCT GGA AGG TAT CTC TA-3', PPOX-qPCR-reverse 5'-CTG AAG CTG GAA TGG CAC TA-3', GAPDH-qPCR-forward 5'-ACC CAC TCC TCC ACC TTT GAC-3', GAPDH-qPCR-reverse 5'-CAT ACC AGG AAA TGA GCT TGA CAA-3', SMAD4-qPCR-forward 5'-AAA ACG GCC ATC TTC AGC AC-3', SMAD4-qPCR-reverse 5'-AGG CCA GTA ATG TCC GGG A-3', CALCOCO2-qPCR-forward 5'-GAA AGA GAG ATT GGA AGA AA-3', CALCOCO2-qPCR-reverse 5'-AGG TAC TTG ATA CGG CAA AGA AA-3', CBX5-qPCR-forward 5'-ACC CAG GGA GAA GTC AGA AA-3', CBX5-qPCR-reverse 5'-CGA TAT CAT TGC TCT GCT CTC T-3', 18S-qPCR-forward 5'-AGT CCC TGC CCT TTG TAC ACA-3', 18S-qPCR-reverse 5'-GAT CCG AGG GCC TCA CTA AAC-3', and 18S-Probe 5'-[6FAM]-CGC CCG TCG CTA CTA CCG ATT GG-[TAM]-3'.

Microarray mRNA expression analysis. All cell lines were cultured as described above. Total RNA was extracted with TRizol Reagent (Life Technologies, Carlsbad, CA) and then labeled and hybridized to Affymetrix HG-
U133 Plus 2.0 chips as previously described (19). The mRNA levels were calculated after RMA (Robust Multichip Average) normalization as described (20). Clustering analysis was done with dChip software (21). Microarray data has been deposited at ArrayExpress (E-MTAB-2971).

Given that, for many genes, the relation between expression and growth rate was monotonic but not linear, a Spearman's rank correlation was used to identify genes whose expression was associated with growth rates across a panel of 31 colorectal cancer cell lines. The Benjamini-Hochberg procedure was used to correct for multiple hypothesis testing (p<0.1). To investigate whether there were gene sets with significant enrichment in the number of genes with expression/proliferation correlations, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) v6.7 (22). A Fisher's exact test was used to identify significantly enriched categories of genes associated with cell growth. The Benjamini-Hochberg procedure was used to correct for multiple hypothesis testing (p<0.05).

**RNAi knockdown of PPOX and GAPDH.** HCT116 cells (2x10⁵) were seeded in 6-well plates and 24h later they were transfected with control On-TARGET plus non-targeting siRNA, or siRNA pools against GAPDH or PPOX (D-001810-10-05, D-001830-10-05 or L-008383-00-0005, respectively; Dharmacon, Lafayette, CO) using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). Expression levels and cell numbers were assessed 72h after transfection as described above.

**Drug effects *in vivo* using a xenograft model.** Six to seven weeks old female and male NOD/SCID mice were purchased from Charles River (Wilmington, MA). The mice were maintained under sterile conditions and the experiments were carried out under observance of the protocol approved by the ethical committee for animal experimentation of the University Hospital Vall d’Hebron, Barcelona. The tumors were established by subcutaneous injection of 2x10⁵ DLD1, Isreco1 or HCC2998 cells, 2.5x10⁶ HCT116 cells, 1x10⁶ HT29 cells, 3x10⁶ RKO cells, and 5x10⁶ T84 cells, all resuspended in 100μl sterile PBS. When the tumors reached a volume of about 80mm³, the animals were randomized to groups treated with vehicle (PBS), 5-fluorouracil, acifluorfen or...
sodium iodoacetate (50mg/kg, 168mg/kg and 18.4mg/kg, respectively) three times per week intraperitoneally. The long (L) and short (S) axis of the tumor were measured with a caliper five times a week. The tumor volume was calculated using the formula: \( V = L \times S^2 \times 0.52 \).

**Determination of the grade of differentiation of cell lines in a xenograft model.** Six to seven weeks old male NOD/SCID mice were purchased from Charles River (Wilmington, MA), and experiments carried out under observance of a protocol approved by the Institute’s oversight committee for animal experimentation. Tumors were established by subcutaneous injection of \( 5 \times 10^6 \) cells in 200μl of a 1:1 PBS:matrigel solution into the right flank. When the tumors were >1000mm\(^3\), they were formalin-fixed, paraffin embedded and Hematoxylin and Eosin stained sections were used to score tumor grade by an experienced pathologist blinded from the sample identity.
RESULTS

Proliferation of colorectal cancer cell lines. Significant variability has been observed in the growth rates of colorectal tumors (9–12). Here, we thoroughly characterized the growth rates of a large panel of human colorectal cancer cell lines derived from colorectal tumors. The doubling time of these 52 cell lines was initially determined using an indirect sulforhodamine B (SRB) assay to quantify the total protein content in cell line cultures at 24h intervals over one week. Cell line growth demonstrated the expected lag phase before reaching an exponential growth phase followed by a growth plateau (Figure 1A). Significant variability was observed in the doubling time during the exponential growth phase of this panel of cell lines (Figure 1B and Supplementary Table 1). For a subset of 22 lines, we validated these results using an independent technique based on electrical impedance as the readout for real-time noninvasive cell growth monitoring (xCELLigence, Roche Diagnostics, Mannheim, Germany), and we found good correlation between the doubling time calculated through both approaches (Pearson’s r=0.66, p=0.0007; Figure 1C).

Inactivation of mismatch repair genes results in the accumulation of mutations throughout the genome which manifests as microsatellite instability (MSI) in approximately 15% of colorectal tumors (23). However, the majority of colorectal tumors show no microsatellite instability and instead display chromosomal instability with large chromosomal abnormalities, and are referred to as microsatellite stable (MSS) or chromosomal instable (CIN) tumors. We found here that cell lines with microsatellite instability grew significantly faster than microsatellite stable lines (Figure 2A). A subset of 27 of these cell lines were grown as subcutaneous xenografts in immunodeficient mice, and the histological grade of the tumors formed was determined. Microsatellite instable tumors have been shown to be associated with high tumor grade (23). In good agreement, higher tumor grade was found to be associated with an MSI phenotype in these cell lines (Chi^2, p<0.05), and faster growth was observed in cell lines that generated high grade tumors when grown as xenografts, compared to lines generating low/moderate grade tumors (Figure 2B). No associations were found between cell line doubling time and the mutational
status of the genes most frequently mutated in colorectal tumors, such as \textit{BRAF}, \textit{KRAS}, \textit{TP53}, \textit{APC}, \textit{PIK3CA}, \textit{SMAD4}, \textit{TCF7L2} and \textit{CTNNB1} (\textit{Supplementary Figure 1}).

\textbf{Expression profiling of colorectal cancer cell lines with different growth rates.} High proliferation rates in colorectal tumors have been previously associated with poor patient prognosis (9–12), and although the molecular mechanisms regulating the progression of tumor cells through the different phases of the cell cycle are well characterized, the key rate-limiting steps are not fully understood. Here we used microarray analysis to perform global gene expression profiling on a subset of these colorectal cancer cell lines (n=31) to investigate the molecular mechanisms underlying the differences in growth rates.

For this analysis, we considered genes with expression levels significantly above background in 23 of the 31 cell lines investigated (>75%). Of the 11,512 genes investigated, the expression of 1,290 (11.2%) was significantly correlated with the doubling time of these cell lines (966 negatively and 324 positively correlated; Spearman’s correlation, BH FDR<0.1 for at least one probe; \textbf{Figure 3A}; Table 1 and \textit{Supplementary Table 2}). The expression levels of six of these genes were independently assessed using quantitative real-time RT-PCR and a significant correlation was observed with mRNA levels quantified by microarray analysis (\textit{Supplementary Figure 2}).

Among the genes whose expression was found to be significantly correlated with the doubling time of the cell lines were multiple genes known to be key cell cycle regulators, including multiple cyclins (A2, B1, B2, E2, F, I, and T2), cyclin-dependent kinases (CDKs; 1, 2, 9 and 13), the CDK inhibitor 2D (p19) and the cell division cycle (CDC) proteins 5L, 6, 14B, 25C, 27, and 37 (\textbf{Figure 3B} and \textit{Supplementary Table 2}). Consistently, functional group enrichment analysis also identified groups of genes that have long been known to participate in cell cycle regulation both in normal and tumor cells, such as Gene Ontology biological process categories involved in cell cycle, mitosis, RNA processing and DNA metabolic process (\textit{Supplementary Table 3}). In addition, other groups of functionally related genes whose expression levels are associated
with growth rates included RNA splicing, protein transport and ubiquitin-dependent protein catabolic process (Supplementary Table 3).

**Identification of new candidate therapeutic targets.** High rates of proliferation are associated with poor patient prognosis and at least some of the genes with higher relative expression in the tumors with faster growth are likely to be necessary to sustain rapid proliferation. We therefore hypothesized that targeting these genes could impair tumor growth. Genome-wide microarray analysis of the panel of 31 colorectal cancer cell lines investigated identified 966 genes with significantly higher expression in rapidly proliferating tumor cells (genes with negative Spearman’s r in Supplementary Table 2). Importantly, thymidylate synthase (TYMS), the direct target of the well-established chemotherapeutic agent 5-fluorouracil (5-FU), was among the top 50 genes with highest negative correlation between doubling time and gene expression (Figure 4A). Because of the availability of chemical inhibitors, we selected two additional genes, protoporphyrinogen oxidase (PPOX) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), that showed significant negative correlations between gene expression and the doubling time of colorectal cancer cell lines (Figure 4B-C). Importantly, the levels of expression of PPOX and GAPDH were significantly correlated with the rates of proliferation (percentage of mitotic cells) in a cohort of 36 primary colorectal tumors (Supplementary Figure 3). No associations were observed between PPOX/GAPDH mRNA levels and tumor size, site, pathological T/N/M, venous invasion, patient age, gender or overall survival (Cox regression p>0.56) in an extended cohort of 433 colorectal primary tumors (Supplementary Table 4). A modest reduction in PPOX levels was observed in late stage tumors (Supplementary Table 4).

**Inhibition of PPOX and GAPDH reduces the growth of colorectal cancer cells in vitro.** We then used 5-FU, acifluorfen and Na iodoacetate, specific chemical inhibitors of TYMS, PPOX and GAPDH, respectively, to investigate whether their activity is necessary for the growth of colon cancer cells. As expected, treatment with 5-FU, a chemotherapeutic agent clinically used for the treatment of colorectal cancer, efficiently inhibited the growth of colon cancer.
cells (Figure 4D). Similarly, acifluorfen and Na iodoacetate treatment resulted in a dose-dependent inhibition of the growth of colon cancer cells (Figure 4E-F), that was not dependent on the growth rates of the cell lines (Supplementary Figure 4). Moreover, both acifluorfen and Na iodoacetate significantly reduced the long term (>2 weeks) clonogenic capacity of colon cancer cells after short term (9h) treatment, suggesting that these agents could cause cell death in addition to growth inhibition (Supplementary Figure 5A-B). Consistently, flow cytometry analysis of propidium iodide-stained cells after acifluorfen or Na iodoacetate treatment revealed the presence of a significant proportion of cells with a sub-diploid amount of DNA (Figure 4G-I and Supplementary Figure 5C-D), and PARP cleavage (Supplementary Figure 5E-F), indicating that these agents induced apoptotic death in colon cancer cells. In addition, acifluorfen treatment was also associated with an arrest of the cell cycle in the G0/G1 phase (Figure 4G-I, Supplementary Figure 6).

Treatment of colon cancer cells with two additional chemically unrelated inhibitors of PPOX and GAPDH (oxadiazon and CGP 3466B maleate, respectively) also resulted in a dose-dependent growth inhibition of colon cancer cells (Supplementary Figure 7A-B). Moreover, RNAi-based knock down of PPOX and GAPDH also interfered with the growth of colon cancer cells (Supplementary Figure 7C-F), further indicating that PPOX and GAPDH are necessary for proliferation of colon cancer cells.

**PPOX inhibition reduces the growth of colon cancer cells in a xenograft model.** The *in vitro* experiments above suggested that PPOX and GAPDH could constitute novel therapeutic targets for colorectal cancer. To further investigate this possibility, we used a xenograft model in NOD/SCID immunodeficient mice. DLD1 and HCT116 cells were subcutaneously injected into the flanks of 24 animals and when the tumors reached a volume of 80mm³ the animals were randomized to a control group, or groups treated with acifluorfen, Na iodoacetate or 5-FU. As expected, 5-FU treatment reduced the growth of these colon cancer cell lines (Figure 5A-B). Although treatment with the GAPDH inhibitor Na iodoacetate did not have any effect on the growth of these cell lines, systemic administration of the PPOX inhibitor acifluorfen
resulted in a significant inhibition of the growth of DLD1 cells (Figure 5A-B). The average weight changes of control mice and 5-FU, Na iodoacetate or acifluorfen treated animals are shown in Figure 5C. To further investigate the sensitivity of colon cancer cell lines to acifluorfen and Na iodoacetate, additional cell lines were subcutaneously injected into immunodeficient NOD/SICD mice that were treated with these agents. While Na iodoacetate did not significantly affect the growth of these additional cell lines, the growth of T84 and Isreco1 cells was significantly reduced in animals treated with acifluorfen (Supplementary Figure 8). Collectively, these results indicate that PPOX could constitute a novel therapeutic target for the treatment of colon cancer.
DISCUSSION

Significant variability has been reported in the rates of proliferation of colorectal cancer tumors, and faster proliferation is associated with poor patient prognosis (9–12). In primary colorectal tumors, an association has been reported between high grade (poorly differentiated) (24,25) or microsatellite instability (26) and faster proliferation rates. Here we show that cell lines that form high grade tumors when grown as xenografts or have microsatellite instability proliferate significantly faster than cell lines forming low grade (differentiated) tumors or microsatellite stable lines. These results indicate that the proliferative profile of the cell line panel used here closely recapitulates the characteristics of primary colorectal tumors. This is consistent with our recent findings demonstrating that the mutational landscape of colorectal cancer cell lines closely resembles that of primary colorectal cancers (27), and collectively establish cell lines as suitable models for the investigation of this disease. Interestingly, the mutational status of the genes most frequently mutated in colorectal tumors did not correlate with the growth rates of colon cancer cell lines, suggesting that these common genetic changes, when considered individually, do not have a consistent effect on the proliferation rates of colon cancer cells. However, the expression of 11.2% of the genes investigated was associated with the growth of these cell lines, indicating that changes in proliferation are fine-tuned at the transcriptional level in colon cancer cells.

Although several pioneer studies identified genes periodically expressed during different phases of the cell cycle (4,5), limited progress has been made regarding the identification of genes with differential expression patterns in tumors with high and low proliferation rates (7,8). Consistent with previous studies using a small number of cancer lines from nine different tumor types (NCI60 set containing 7 colon cancer cell lines) (7,8) we found that genes involved in cell cycle, RNA and protein synthesis are closely correlated with the growth rates of colon cancer cells. Here we found that analysis of a larger set of colon cancer lines (n=31) that widely vary in their growth rates did not confirm some of the findings made on the NCI colon cancer cells, such as cholesterol metabolism, iron metabolism and fatty acid metabolism. However, we found
additional groups of functionally related genes significantly correlated with the growth rates of colorectal cancer cells including several categories related with protein metabolism, such as translation, protein transport and cellular protein catabolic process (Supplementary Table 3).

There are currently a limited number of chemotherapeutic agents approved for their routine use in the fight against colorectal cancer, namely the antimetabolite 5-Fluorouracil (5-FU), the platinum compound oxaliplatin and the topoisomerase I inhibitor irinotecan, in addition to the targeted agents cetuximab, panitumumab, bevacizumab and regorafenib. However, the response rate to each of these drugs used as single agents is below 30% and the identification of novel therapeutic targets and the subsequent development of new chemotherapeutic agents would likely improve the survival of these patients. Here we hypothesized that inhibition of genes highly expressed in rapidly proliferating colorectal cancer cells can interfere with tumor growth, and these genes are therefore good candidate chemotherapeutic targets. In support of this hypothesis, we found that the direct target of 5-FU, thymidylate synthetase, was among the genes showing a highly significant correlation between its expression level and the rate of tumor cell growth. As a proof of concept, we selected two additional genes with high expression levels in rapidly proliferating colorectal cancer cells (GAPDH and PPOX) with known specific inhibitors for the encoded proteins (Na iodoacetate and oxadiazon or acifluorfen and CGP 3466B maleate, respectively) and found that, as the TYMS inhibitor 5-FU, GAPDH and PPOX inhibitors significantly reduced the growth of colon cancer cells at micromolar concentrations. Moreover, using a preclinical subcutaneous xenograft model, we could demonstrate that at least the PPOX inhibitor acifluorfen was able to inhibit the growth of colon cancer cell lines (3 of 7; 42.3%). Protoporphyrinogen oxidase (PPOX) catalyzes the 6-electron oxidation of protoporphyrinogen IX to form protoporphyrin IX, the penultimate reaction of heme biosynthesis. Heme plays critical roles in multiple processes involving oxygen metabolism. This includes proteins that transport or store oxygen such as hemoglobin and myoglobin, but is also important in mitochondrial respiratory chain complexes, in cytochrome P450s and in other enzymes that use or detoxify oxygen such as peroxidases and catalases (28). Our findings are
consistent with the observation that inhibition of heme synthesis significantly reduced proliferation in lung cancer cells (29).

It has long been known that most cancer cells predominantly produce energy by a high rate of glycolysis and lactate production, an observation known as the Warburg effect (30). Therefore, it has been suggested before that differences in the metabolisms of tumor cells could offer a therapeutic window (31,32). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) catalyzes the sixth step of glycolysis, the conversion of glyceraldehyde 3-phosphate to D-glycerate 1,3-bisphosphate. Recently, GAPDH has been shown to translocate to the nucleus and to be implicated in several non-metabolic processes, including transcriptional regulation and apoptosis (33,34). We show here that inhibition of GAPDH efficiently reduces the growth of colon cancer cells in vitro. Interestingly, although the GAPDH inhibitor Na iodoacetate has been shown to reduce the growth of Ehrlich ascites carcinoma (EAC) cells and xenografts of colon cancer cells at doses similar or lower than the one used in vivo in this study (35,36), no significant effects were observed here on the growth of subcutaneous xenografts of four different colon cancer cell lines. However, no toxicity was observed at the doses used, and based on the in vitro effects observed, it remains possible that Na iodoacetate treatment at higher doses and/or in other tumor cell lines, may interfere with tumor growth.

Importantly, we provide here a list of 966 genes that have significantly higher expression in the tumor cell lines with higher proliferation rates. The two genes that were further investigated in this study were selected because of the availability of specific chemical inhibitors. However, genetic inactivation of selected genes with significant associations between expression and tumor growth could be used to identify the most promising therapeutic targets, for which novel specific inhibitors could then be developed.

In summary, we found that the proliferation of colorectal cancer cells is significantly associated with higher tumor grade and an MSI phenotype. In addition, microarray transcriptomic analysis of a panel of 31 colorectal cancer cell lines shed new light on the molecular mechanisms regulating the uncontrolled proliferation of colorectal cancer cells. Moreover, we demonstrate
that genes with high expression in rapidly proliferating tumor cells are good candidates for therapeutic targeting. As a proof of concept, we demonstrate that acifluorfen inhibits the growth of colorectal cancer cells \textit{in vitro} and \textit{in vivo}, identifying PPOX as a novel candidate chemotherapeutic target for the treatment of colorectal cancer.
REFERENCES


FIGURE LEGENDS

Figure 1: Growth of colorectal cancer cell lines. (A) For a panel of 52 colorectal cancer cell lines, cells were seeded in seven 96 well plates, harvested daily for 7 consecutive days and cell number assessed indirectly using sulforhodamine B (SRB) staining and colorimetric quantification. Representative cell lines with rapid and slow growth are shown. (B) Histogram showing the doubling time of all 52 cell lines used in this study determined with the SRB assay (average of three experiments ± SEM). (C) The growth of a subset of 22 of these cell lines was assessed using electrical impedance as the readout for the number of cells (xCELLigence). The doubling time was calculated with both techniques and the correlation of the results obtained with both methods is shown.

Figure 2: The doubling time of colorectal cancer cell lines is associated with microsatellite instability and tumor grade. (A) Colorectal cancer cell lines with an MSI phenotype showed significantly faster growth (lower doubling time) compared to lines without MSI phenotype. (B) Cell lines growing as poorly differentiated (grade 3) tumors in subcutaneous xenografts in immunodeficient mice had faster growth than cell lines displaying moderately/highly differentiated histology (grade 1 and 2). N: number of cell lines. The mean ± SEM is shown. Asterisks indicate Student’s t-test p<0.05.

Figure 3: Associations between gene expression and growth of colorectal cancer cells. (A) Clustering analysis of the 40 genes (rows) whose expression is best correlated with the doubling time of a panel of 31 colorectal cancer cell lines. Cell lines (columns) are ordered by increasing doubling times. Genes with relative expression levels above or below the mean are shown in red and blue, respectively (color scale is shown at the bottom). (B) Cell cycle KEGG pathway showing genes with expression levels significantly correlated with the growth of a panel of 31 colorectal cancer cell lines. Genes are represented by rectangular boxes. Green: higher relative levels in rapidly proliferating cells (Spearman’s correlation, FDR<0.1); red: lower levels in rapidly proliferating cells (Spearman’s
correlation, FDR<0.1); and grey: present on the chip, but not significantly correlated.

**Figure 4: Genes highly expressed in rapidly proliferating colon cancer cells as novel candidate therapeutic targets.** The expression of thymidylate synthase (TYMS; A), the direct target of the chemotherapeutic agent 5-FU, as well as glyceraldehyde 3-phosphate (GAPDH; B) and protoporphyrinogen oxidase (PPOX; C) were negatively correlated with the doubling time of a panel of 31 colorectal cancer cell lines. Inhibition of TYMS with 5-FU (D), GAPDH with Na iodoacetate (E) and PPOX with acifluorfen (F) resulted in a dose dependent inhibition of the growth of different colorectal cancer cell lines. The GI50 of the cell lines tested is shown. Panels (G-I) show the effects of acifluorfen and sodium iodoacetate treatment on the cell cycle of colon cancer cells. Cells were stained with propidium iodide and analyzed by flow cytometry.

**Figure 5: Effects of GAPDH and PPOX inhibition on tumor growth using a xenograft model.** Groups of NOD/SCID immunodeficient mice (n=6 per group) with DLD1 and HCT116 cells as subcutaneous xenografts were treated (i.p.) three times per week with acifluorfen (168mg/kg), Na iodoacetate (18.4mg/kg), 5-fluorouracil (50mg/kg) or vehicle PBS, starting when the tumors reached approximately 80mm³. Arrowheads in the X-axis indicate treatment times. Tumor size was monitored over time for DLD1 (A) and HCT116 (B) cells. Percentage animal weight gain/loss after drug treatment is shown in panel (C). Asterisk indicate statistically significant differences (Student’s T-test, p<0.05) in the mean tumor size in the control (PBS) group and treatment groups (5-FU or acifluorfen). The mean ± SEM is shown.
Table 1: Top 20 probes with highest correlation coefficient (positive and negative) between gene expression and doubling time in a panel of 31 colorectal cancer cell lines. TMY5, GAPDH and PPOX are also shown.

<table>
<thead>
<tr>
<th>ProbeSet ID</th>
<th>Gene Symbol</th>
<th>Gene Name</th>
<th>Spearman's Rho</th>
<th>Spearman's p val</th>
<th>BH (FDR) adjusted p-value</th>
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Figure 1

A

B

C

Pearson $r = 0.66$

$P$ value $= 0.0007$
Figure 2

A

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Figure 3

A

B

Cell cycle

Growth factor

Growth factor withdrawal

MAPK signaling pathway

DNA damage checkpoint

ARF

p300

DNA-PK

ATM/ATR

Mdm2

Rb

p53

Apoptosis

Sensin

Rb

Mdm2

Bub1

Bub3

ApoC2

Cdc20

Cdc16

UBQ

MCM

CCRF

DNA synthesis

DNA

S-phase proteins, CyclE

S-phase proteins

DNA biosynthesis

Molecular interaction or relation

Binding/association

Inhibition

Dissociation

Indirect effect

Another map/pathway

Higher expression in rapidly proliferating cells

No significant association with growth rates

Lower expression in rapidly proliferating cells

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Figure 4

(A) TYMS
Spearman’s r = -0.68
p < 0.0001

(B) GAPDH
Spearman’s r = -0.55
p = 0.0013

(C) PPOX
Spearman’s r = -0.60
p = 0.0004

(D) 5-Fluorouracil
Growth inhibition (% of control)

(E) Na iodoacetate
Growth inhibition (% of control)

(F) Acifluorfen
Growth inhibition (% of control)

(G) Untreated

(H) Sodium iodoacetate 30μM

(I) Acifluorfen 1200μM
Figure 5

A

DLD1 cells

B

HCT116 cells

C

Relative loss or gain of animal weight (%)

Time post cell injection (days)

Tumor size (mm³)

Time post cell injection (days)
HIGHLY EXPRESSED GENES IN RAPIDLY PROLIFERATING TUMOR CELLS AS NEW TARGETS FOR COLORECTAL CANCER TREATMENT

Sarah Bazzocco, Elena Andretta, Paulo Rodrigues, et al.

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