Head and neck cancer (HNC) is the eighth most common malignancy worldwide, comprising a diverse group of cancers affecting the head and neck region. Despite advances in therapeutic options over the last few decades, treatment toxicities and overall clinical outcomes have remained disappointing, thereby underscoring a need to develop novel therapeutic approaches in HNC treatment. Uroporphyrinogen decarboxylase (UROD), a key regulator of heme biosynthesis, was identified from an RNA interference–based high-throughput screen as a tumor-selective radiosensitizing target for HNC. UROD knockdown plus radiation induced caspase-mediated apoptosis and cell cycle arrest in HNC cells in vitro and suppressed the in vivo tumor-forming capacity of HNC cells, as well as delayed the growth of established tumor xenografts in mice. This radiosensitization appeared to be mediated by alterations in iron homeostasis and increased production of reactive oxygen species, resulting in enhanced tumor oxidative stress. Moreover, UROD was significantly overexpressed in HNC patient biopsies. Lower preradiation UROD mRNA expression correlated with improved disease-free survival, suggesting that UROD could potentially be used to predict radiation response. UROD down-regulation also radiosensitized several different models of human cancer, as well as sensitized tumors to chemotherapeutic agents, including 5-fluorouracil, cisplatin, and paclitaxel. Thus, our study has revealed UROD as a potent tumor-selective sensitizer for both radiation and chemotherapy, with potential relevance to many human malignancies.

INTRODUCTION

Ionizing radiation therapy (RT) plays a critical role in cancer patient management; curability however, could be limited by tolerance of normal surrounding tissues. Thus, the development of therapeutic strategies to enhance the therapeutic ratio is of great importance. Unfortunately, many of the currently used radiosensitizers are neither selective nor tumor-specific. This is a particular concern in the management of head and neck cancer (HNC), whereby RT is the primary curative modality. In HNC, tumors are located in close proximity to critical organs, which when damaged compromise patients’ long-term quality of life and lead to mortality.

HNC is the eighth most common malignancy worldwide, comprising a diverse group of cancers affecting the sinuses, nasal and oral cavities, pharynx, larynx, and other sites in this region (1, 2). In addition to the anatomic and molecular heterogeneity of HNC, most patients present with locally advanced disease, and/or suffer from other comorbidities, rendering HNC particularly challenging to treat. Despite advances in therapeutic options over the last few decades, treatment toxicities and overall clinical outcomes have remained disappointing (3). Even the most effective RT regimens achieve local control rates of 45 to 55%, with disease-free survival (DFS) rates of only 30 to 40% for patients with locally advanced head and neck squamous cell carcinomas (HNSCC) (4). Furthermore, meta-analyses have documented concurrent RT with chemotherapy to offer an absolute survival advantage of only 6.5% at 5 years (5). These modest results underscore an urgent need to develop novel therapeutic approaches in the treatment of HNC.

Among the new therapies for HNC, molecularly targeted agents have gained momentum (6, 7). However, an effective strategy to select appropriate patients for these therapies does not yet exist, perhaps because of the complexities of radiation response. Ionizing radiation (IR) induces a myriad of physicochemical changes at the cellular and molecular level, most of which have not yet been clearly elucidated. Hence, in the current study, we describe an unbiased RNA interference (RNAi)–based high-throughput screen (HTS) for the large-scale identification of anticancer radiosensitizing molecular targets. Uroporphyrinogen decarboxylase (UROD), a key regulator of heme biosynthesis, was discovered as a potent modulator of tumor radioresponse. Hereafter, we present in vitro and in vivo characterizations of UROD-mediated radiosensitization and discuss its clinical implications in the management of HNC.

RESULTS

RNAi HTS identifies radiosensitizing targets

The preliminary screen of the Human siGENOME Druggable and Protein Kinase siRNA Libraries identified 188 target sequences with potential radiosensitizing effects at 2 Gy (unit of absorbed dose of ionizing radiation) in FaDu cells (human hypopharyngeal squamous cell cancer), a clinically relevant model for the study of HNC (8); the “hit”
threshold was defined as 4 SDs below the mean after B-score normalization (Fig. 1A). The validity of the screen was corroborated by the identification of known radiosensitizing targets, such as ATM (ataxia-telangiectasia mutated), ATR (ATM- and Rad3-related), and aurora kinase A (9, 10). To confirm the initial HTS results, we transfected FaDu cells with the 188 small interfering RNAs (siRNAs) ± IR (Fig. 1B) and eliminated those that decreased the surviving fraction by >30% in the absence of IR, leaving 67 potential hits. Targets that reduced the surviving fraction by >50% at 2 Gy relative to their unirradiated counterparts were selected for further evaluation. Ingenuity Pathways Analysis identified the top-scoring functional biological network among the radiosensitizing targets as involving cell death, cancer, and/or cellular compromise (table S1); the top molecular and cellular functions included cell growth and proliferation (table S2).

**UROD is a potent radiosensitizing target for HNC**

UROD, a key regulator of heme biosynthesis, was identified from the HTS as a potent modulator of tumor response to IR, and was selected for further evaluation because of its novelty in the context of human cancers and also because it is a well-characterized enzyme, thereby increasing its potential “druggability.” Clonogenic survival curves confirmed that UROD down-regulation significantly enhanced radiosensitivity of FaDu cells, a highly aggressive radioreistant HNC cell line, in a dose-dependent manner (Fig. 1C). Radiation enhancement ratios (RERs) of 2.0, 1.7, and 1.6 were observed at 2, 4, and 6 Gy, respectively; a RER of >1 denotes synergistic radiosensitization (11). The Chou-Talalay combination index (CI) (12) further confirmed the synergistic interaction between UROD siRNA (siUROD) and IR, wherein the CI remained significantly below 1 for all tested combinations (Fig. 1D). Corroboration of siRNA-mediated UROD knockdown, which was determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR) and immunoblotting, demonstrated a significant suppression of both mRNA and protein expression by 48 hours after transfection (Fig. 1, E and F). To ensure that this observation was not due to off-target effects, we used a rescue plasmid expressing target mRNA refractory to siRNA due...
Fig. 2. The radiosensitizing effect of UROD knockdown is independent of porphyrin accumulation. (A) Heme biosynthetic pathway. CPOX, coproporphyrinogen oxidase; PPOX, protoporphyrinogen oxidase; Fe, iron. (B) Porphyrin synthesis in mock-, siCTRL-, or siUROD1-transfected FaDu cells was artificially induced with ALA (500 µM, 4 hours) before porphyrin extraction at 24 hours after transfection. Porphyrins were quantified spectrophotometrically and normalized to total cell number. Representative spectral scans (575 to 750 nm) are shown. **P < 0.01, siUROD1 + ALA compared with siCTRL or untreated + ALA. au, arbitrary units. (C) Fluorescence microscopy images of transfected cells ± ALA (500 µM, 1 hour). Mitochondria and nuclei were stained with MitoTracker Green and Hoechst 33342, respectively. Porphyrins excited with a wavelength of ~400 nm emit red fluorescence at a peak of ~635 nm. Scale bar, 10 µm. (D) ALA-treated (250 to 1000 µM, 4 hours) and siCTRL- or siUROD1-transfected (48-hour transfection) FaDu cells were irradiated (4 Gy), and then cell viability was assessed 96 hours later by MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt] assay. **P < 0.01, siCTRL ± IR compared with siUROD1 ± IR. Data are means ± SEM (n = 3).

to silent mutations. Cotransfection of FaDu cells with siUROD and the rescue plasmid completely abrogated any siUROD-mediated effects, with or without IR (Fig. 1G), confirming a siUROD-specific process.

To further verify that the observed results were not a consequence of off-target effects, we repeated the above experiments using a second distinct siRNA targeting UROD, as well as in another HNC cell line, CAL33, a well-established model for squamous cell carcinoma of the oral tongue. Similar results were obtained with clonogenic survival curves, confirming both siRNA sequences to significantly enhance radiosensitivity of FaDu and CAL33 cells in a dose-dependent manner with RERs of >1 at all IR doses (fig. S1, A and B); UROD knockdown was confirmed by qRT-PCR and immunoblotting (fig. S1, C to F).
siUROD-mediated radiosensitization differs from photodynamic therapy

UROD is the fifth enzyme in the heme biosynthetic pathway (Fig. 2A) and catalyzes the decarboxylation of uroporphyrinogen to coproporphyrinogen (13). Because porphyrinogens are unstable and readily oxidized to fluorescent porphyrin molecules, UROD down-regulation was functionally validated by indirectly measuring uroporphyrinogen accumulation from overall changes in amounts of oxidized porphyrin (uroporphyrin and other highly carboxylated porphyrins). Spectrofluorometrically, porphyrin accumulation with siUROD alone was negligible (~1.3-fold increase versus untreated control; Fig. 2B); thus, FaDu cells were pretreated with δ-aminolevulinic acid (ALA) to artificially induce porphyrin synthesis. ALA plus siUROD significantly increased amounts of intracellular porphyrin relative to ALA alone or siCTRL-treated cells (~18.1-fold versus ~9.9- and ~10.1-fold increase). Similar observations were obtained by fluorescence microscopy (Fig. 2C), wherein cells treated with ALA plus siUROD exhibited enhanced porphyrin accumulation, reflecting the disruption of heme biosynthesis by siUROD.

Because most currently used photosensitizers in photodynamic therapy (PDT) are porphyrin-based (14), it was important to compare the radiosensitizing effects of siUROD to commonly used photosensitizers. ALA-based PDT is a well-established anticancer therapy that uses the heme precursor ALA to induce accumulation of protoporphyrin IX (PPIX) in neoplastic cells (15). When exposed to visible light, PPIX becomes excited and induces reactive oxygen species (ROS) formation, leading to oxidative stress–mediated cell death. Here, siUROD plus IR was markedly more cytotoxic compared to the negligible effects of ALA plus IR (Fig. 2D), indicating that the effects of siUROD were independent of intracellular porphyrin accumulation (Fig. 2, B and C) and thus distinct from PDT.

UROD down-regulation promotes radiation-induced apoptosis

The enhanced tumor radiosensitivity observed with UROD suppression (Fig. 1C) was mediated in part by G2-M cell cycle arrest (Fig. 3A), with a coordinate induction of DNA double-strand breaks (DSBs), reflected by increased overall amounts of γ-H2AX protein and formation of nuclear foci in siUROD plus IR–treated FaDu cells compared with IR alone (Fig. 3, B and C). The significantly prolonged G2-M arrest and concomitant increase in the sub-G1 population (Fig. 3A and fig. S2A) suggested that the DNA damage induced by siUROD plus IR resulted in higher levels of cell death than by IR alone, corroborated by more residual DSBs observed at 24 hours after IR (Fig. 3B).
central role of apoptosis in siUROD plus IR-mediated cytotoxicity in FaDu cells was further demonstrated by the induction of caspase activation (Fig. 3D) and depolarization of the mitochondrial membrane potential ($\Delta \Psi_m$) (Fig. 3E), both classical hallmarks of apoptosis; apoptosis was also elevated in siUROD plus IR–treated CAL33 cells (fig. S2B).

**siUROD-mediated radiosensitization increases cellular oxidative stress**

Heme biosynthesis occurs within the cytoplasm and mitochondrion (fig. 2A), the latter being a major source of intracellular free radicals (16). Thus, to investigate whether siUROD mediated its radiosensitizing effects by perturbing ROS homeostasis, amounts of intracellular oxidants were measured. Mitochondrial superoxide anion radicals were more prevalent in siUROD plus IR–treated compared with IR– or siUROD–treated FaDu (Fig. 4A and fig. S3A) and CAL33 (fig. S3B) cells. Similarly, global ROS production, as measured by CM-H$_2$DCFDA, which detects many other ROS (hydrogen peroxide, hydroxyl radical, peroxyl radical, and peroxynitrite anion), was highest in siUROD plus IR–treated FaDu (Fig. 4B and fig. S3C) and CAL33 (fig. S3D) cells.

Many tumors produce reduced amounts of antioxidants compared to their normal counterparts (17, 18). Hence, ROS production in normal oropharyngeal (NOP) and oral epithelial (NOE) cells was assayed after exposure to siUROD ± IR (Fig. 4, C and D), with equivalent degrees of UROD knockdown as verified by qRT-PCR (fig. S3E). At 72 hours after IR, both normal cell lines demonstrated significantly less ROS accumulation compared to FaDu or CAL33 cells, particularly in combination with UROD down-regulation [dihydroethidium (DE): 1.4-fold/1.3-fold compared with 1.8-fold/4.3-fold increase in NOP/NOE and FaDu/CAL33, respectively; CM-H$_2$DCFDA: 1.5-fold/2.0-fold compared with 2.7-fold/4.0-fold increase in NOP/NOE and FaDu/CAL33, respectively]. The differential degrees of ROS translated into higher survival rates for normal compared with FaDu or CAL33 cells after siUROD ± IR (Fig. 4E and fig. S3F; NOP or NOE compared with FaDu or CAL33 RERs, $P < 0.01$), exposing a therapeutic window to exploit the differential antioxidant capacity between normal and tumor cells to achieve tumor-selective siUROD radiosensitization.

**Given that ROS production is regulated by oxygen tension, and hypoxia diminishes radiosensitivity, the effects of O$_2$ on siUROD radiosensitization were also examined.** siUROD alone retained activity under
hypoxia comparable to that under normoxic conditions, and displayed only a partial reduction in radiosensitization (Fig. 4F). To further understand the mechanisms of siUROD plus IR–mediated cytotoxicity, we examined relative mRNA expression of a panel of genes involved in oxidative stress response. As expected, antioxidants involved in maintaining cellular redox homeostasis, including superoxide dismutases (SOD1 and SOD2), glutathione peroxidase (GPX1), and mitochondrial ferritin (FTMT), were all up-regulated in FaDu cells in response to siUROD plus IR (Fig. 4G and fig. S3G).

**siUROD knockdown perturbs cellular iron homeostasis**
The induction of mitochondrial ferritin, a nuclear-encoded iron-sequestering protein, in FaDu cells transfected with siUROD ± IR prompted further investigations into the role of iron homeostasis in siUROD-mediated effects. Mitochondria are intimately involved in iron trafficking for heme biosynthesis and the formation of iron-sulfur clusters (19). These organelles, which are also the major source of ROS, have developed efficient mechanisms to segregate free iron from ROS, thereby preventing the production of harmful hydroxyl radicals (‘OH) through Fenton-type reactions (20). Accordingly, up-regulation of the FTMT antioxidant in siUROD ± IR–treated FaDu cells (Fig. 4G) was associated with markedly elevated intracellular ferrous (Fe²⁺) and ferric (Fe³⁺) iron, visible as diffuse deep purple staining within the cells (Fig. 5A). The relative changes in iron species (Fig. 5B), with Fe²⁺ reduction compared with Fe³⁺ increase after IR, are likely related to the Fenton reaction, whereby IR can induce hydrogen peroxide (H₂O₂) formation, which consumes Fe²⁺, and in the process generates ‘OH, converting Fe²⁺ to Fe³⁺. Correspondingly, the overall cellular labile iron pool (LIP), detected with calcein AM, was considerably higher in siUROD ± IR–treated FaDu (fig. S4A) and CAL33 cells, which also demonstrated a significant up-regulation of FTMT (fig. S4, B and C).

To corroborate the central role of excess cellular iron in mediating siUROD radiosensitization, we introduced the iron chelator deferoxamine before IR in FaDu cells. Significant suppression (~50%) of siUROD plus IR–induced apoptosis was observed (Fig. 5C), underscoring the critical role of iron in mediating this radiosensitization process.

**siUROD radiosensitizes HNC models in vivo**
To evaluate the radiosensitizing efficacy of UROD knockdown in vivo, we injected transfected FaDu cells into the gastrocnemius muscle of severe combined immunodeficient (SCID) mice, followed by local tumor RT. Mice implanted with siUROD- or siCTRL-transfected cells started to form tumors at ~23 and ~9 days, respectively, delaying the time to reach a tumor plus leg diameter (TLD) of 14 mm by ~14 days (P < 0.001; Fig. 6A). When combined with RT, siUROD appeared to synergistically suppress the tumor-forming capacity of FaDu cells, wherein tumors developed at ~37 and ~12 days in the siUROD plus RT and siCTRL plus RT groups, respectively, extending the mean time to reach 14 mm by ~27 days (P < 0.001; Fig. 6A). Similarly, tumor growth delays were observed in mice implanted with FaDu cells transfected with a second distinct UROD siRNA ± RT followed for ~40 days (Fig. 6B).

The therapeutic efficacy of siUROD plus RT in treating established FaDu tumors was also evaluated. Tumor-bearing mice were systemically treated with siRNA complexed to a cationic polymer polyethylenimine ± local tumor RT, trying to recapitulate the clinical scenario. Although no difference in tumor growth was observed between the siUROD versus siCTRL groups, siUROD plus RT caused a significant reduction in tumor size compared to the siCTRL plus RT arm with both UROD siRNA sequences (P < 0.001; Fig. 6, C and D). The extent of tumor growth delay was reflected by the degree of in vivo UROD knockdown, verified by both immunohistochemistry and immunoblotting (Fig. 6, E to H). Although...
this treatment regimen was not optimized for absorption, distribution, metabolism, or excretion, a therapeutic benefit was nonetheless observed. These data suggest that improving the pharmacokinetics and bioavailability of siUROD may render this therapeutic approach highly effective, based on the significant suppression of tumor-forming capacity when FaDu cells were fully exposed to siRNA-mediated UROD knockdown (Fig. 6, A and B). This therapeutic regimen was well tolerated based on the minimal differences in mouse body weights between the treatment groups (fig. S5, A and B).

UROD knockdown modulates radiosensitivity of several cancer models

To assess the applicability of siUROD-induced radiosensitization to other human cancers, we evaluated additional HNC, lung, cervix, prostate, and breast cancer cell lines. All cell lines demonstrated radiosensitization after equivalent degrees of UROD knockdown, verified by qRT-PCR (Fig. 7A and fig. S6A). To further verify the role of UROD in modulating tumor radiosensitivity, we introduced exogenous UROD protein into the most radiosensitive HNC cell line, UTSCC-42a (fig. S6, B and C), to determine whether this phenotype could be reversed. Indeed, overexpression of UROD before IR protected the UTSCC-42a cells against radiation-induced apoptosis (~53% reduction compared with empty vector control; Fig. 7B) and clonogenic cell death (Fig. 7C), substan-tiating the critical role of UROD in modulating radiosensitivity. UROD overexpression itself increased the clonogenic potential of UTSCC-42a cells in the absence of IR (Fig. 7C).

UROD has clinical implications in the management of HNC

The clinical importance of our findings was determined with the analysis of pretreatment tumor biopsies from patients with stage III or IV nonmetastatic HNSCC, who were all participants in an RT clinical trial (21). Notably, UROD mRNA expression was significantly higher (~11-fold) compared to that of normal laryngeal and tonsillar epithelial tissues (P < 0.05; Fig. 7D). Furthermore, patients with expression of UROD in the lowest quartile experienced a superior DFS compared with those with the highest UROD expression (P = 0.06; Fig. 7E), consistent with the notion that elevated UROD conferred radiosensitivity (Fig. 7, B and C), and supporting the strategy of reducing UROD to increase radiocurability.

UROD deficiency is responsible for the clinical syndrome of porphyria cutanea tarda (PCT), a rare nonfatal metabolic disorder characterized by elevated cellular porphyrin and iron (13). Thus, we examined whether a naturally occurring state of UROD deficiency could recapitulate our findings. Untransformed fibroblasts from familial PCT patients demonstrated minimal cytotoxicity comparable to UROD functional primary normal human fibroblasts (Fig. 7F), corroborating our previous data that siUROD-mediated radiosensitization is tumor-selective (Fig. 4E).
The potential applications of the siUROD sensitization strategy were further broadened when nontoxic doses of cisplatin, 5-fluorouracil, or paclitaxel were significantly sensitized in FaDu and CAL33 cells by UROD inhibition in a dose-dependent manner (Fig. 7G and fig. S6, D and E). These drugs are commonly used in HNC management; hence, inhibiting UROD could play a potentially important role in enhancing the outcome for both radiotherapy and chemotherapy in HNC patients.

**DISCUSSION**

Intrinsic oxidative stress in cancer cells, due in part to oncogenic transformation with resultant increased metabolic activity and mitochondrial dysfunction, has long been recognized to promote genetic instability, cell growth, and proliferation (22). Recently, this distinct biochemical feature has been exploited for selective anticancer thera-

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**Fig. 7.** UROD has clinical implications in the management of human cancers. (A) Cell viability assessment of siCTRL- or siUROD1-transfected cancer cells at 96 hours after IR (2 Gy) by MTS assay. Human HNC (red), lung (black), cervix (blue), prostate (orange), and breast (green) cancer cell lines. **P < 0.01, siCTRL ± IR compared with siUROD1 ± IR. (B) UTSCC-42a cells transfected with pUROD or pVector for 48 hours were irradiated (2 Gy). Apoptotic fractions were assessed by flow cytometry 72 hours after IR. Representative histogram of cell cycle distribution is shown. ***P < 0.001, pUROD + IR versus pVector + IR. (C) Clonogenic survival of UTSCC-42a cells transfected with pUROD or pVector for 48 hours, and then irradiated (0 to 4 Gy). Colonies were counted 14 days after IR. *P < 0.05; **P < 0.01, pUROD compared with pVector for each IR dose. (D) Total RNA was extracted from 38 HNSCC patient tumor biopsies and 5 normal laryngeal and tonsillar epithelial tissues and then assessed for relative UROD mRNA expression. Relative change was determined by normalizing to β-actin expression and comparing to the average from normal tissues. Solid line, mean fold change. *P < 0.05, tumor versus normal tissues. (E) Kaplan-Meier plot of disease-free survival (DFS) for the HNSCC patients from (D), tri-chotomized based on interquartile range (low, medium, or high UROD mRNA expression). DFS was defined as the absence of relapse or death, calculated from the time of diagnosis. Median follow-up time was 6.9 years (range, 2.3 to 10.8 years). (F) Cell viability assessment of irradiated (2 Gy) primary normal human fibroblasts (MRC5 and GM05757) and untransformed fibroblasts from PCT patients (GM01482, GM00977, GM00961, and GM01041) 96 hours after IR by MTS assay. *P < 0.05, MRC5 compared with PCT fibroblasts. (G) siCTRL- or siUROD1-transfected FaDu cells were treated with increasing doses of cisplatin (0.01 to 0.25 μM), 5-fluorouracil (5-FU) (1 to 2.5 μM), or paclitaxel (0.0001 to 0.001 μM) for 24 hours, and then assessed for cell viability 96 hours later. **P < 0.01; ***P < 0.001, siCTRL ± drug compared with siUROD1 ± drug. Data are means ± SEM (n = 3).
pies (23, 24), wherein elevated basal ROS-mediated signaling rendered neoplastic cells more vulnerable to manipulations that enhanced oxidative stress. Thus, the addition of exogenous ROS-inducing agents would increase ROS to toxic levels, triggering death in cancer cells with dysregulated antioxidant defense mechanisms (17, 18). In contrast, normal cells have a greater capacity to contend with oxidative insults by virtue of their lower basal ROS output, along with an intact antioxidant response system. Consistently, in this current study, ROS were significantly augmented in tumor cells after siUROD plus IR (Fig. 4, A and B, and fig. S3, A to D), with the induction of cell cycle arrest and apoptosis (Fig. 3A and fig. S2). The compensatory antioxidant response (Fig. 4G and fig. S3G) was inadequate compared to the capacity of normal cells, which demonstrated only a modest elevation in ROS, with minimal consequences on viability (Fig. 4, C to E).

The uniqueness of our UROD discovery relates to the opportunity to perturb iron homeostasis as the initiator of oxidative stress in tumor cells. Several lines of evidence support an iron-mediated mechanism of radiosensitization for UROD down-regulation, although we cannot preclude other iron-independent mechanisms. First, mitochondrial ferritin was significantly up-regulated by siUROD (Fig. 4G and figs. S3G and S4C), which was associated with a concomitant increase in intracellular iron (Fig. 5, A and B, and fig. S4, A and B). Second, siUROD plus IR-induced apoptosis was significantly suppressed by the iron chelator deferoxamine (Fig. 5C). Although iron is required for many critical biological processes, including respiration, DNA synthesis, and O2 transport, it can also be a powerful catalyst for ROS formation (19). In the presence of H2O2, Fe2+ can be oxidized to Fe3+ through the Fenton reaction, producing highly toxic hydroxyl radicals. When heme synthesis is disrupted, large quantities of iron, which would normally be incorporated into PPIX to form heme, continue to be imported into the mitochondria, causing an elevation in FTMT to sequester the excess Fe2+, thereby minimizing oxidative damage (25). In cancers, the accretion of cellular iron is further exacerbated by the overexpression of transferrin receptor-1, a major mechanism of iron uptake, to sustain the high requirements of cellular and protein turnover, plus DNA synthesis (26). Our data demonstrate the elevated iron and consequential ROS formation due to siUROD alone to be nonlethal; significant cytotoxicity was only observed when combined with IR, which is clinically advantageous because RT is anatomically targeted. Presumably, with siUROD alone, cancer cells can still cope with the excess free Fe2+ and resultant increase in the ambient concentration of free radicals. However, the additional ROS insults induced by IR may overwhelm the cancer cells’ antioxidant capacity, thereby resulting in the observed enhanced cell death.

Excess Fe2+ might also increase the effective range of radicals produced by γ-radiation. Upon IR, superoxide and hydroxyl radicals are formed (27), both of which can react with themselves to form H2O2, initiating the Fenton reaction and, ultimately, oxidative damage. Thus, the same phenomenon (that is, iron overload) that cancer cells rely on for rapid proliferation and DNA synthesis could be exploited for the liberation of detrimental radicals with γ-radiation, exposing the double-edged sword of iron in cancer cells.

Similar to PDT, our siUROD radiosensitizing strategy exploits the heme pathway to harness its anticancer effects; however, siUROD is distinct and may be superior for several reasons. Tumor hypoxia severely hampers PDT efficacy, because molecular O2 is a prerequisite for the production of photo-induced singlet oxygen molecules (28). However, siUROD plus IR retained radiosensitizing efficacy even under hypoxia (Fig. 4F), likely because of its reliance on the Fe2+-catalyzed Fenton reaction to yield highly cytotoxic radicals. H2O2 can be generated by recombination of free radicals formed from water radiolysis (27); hence, there is less reliance on the presence of O2. The applicability of PDT is further limited because the light source used to excite porphyrins and its derivatives occupies the visible spectrum, which cannot penetrate tissues >0.8 cm, restricting PDT application to superficial lesions (29). Moreover, porphyrins cannot be excited by the high-energy photons of x-rays or γ-rays (30), thereby accounting for the modest radiosensitizing effects of porphyrins (29, 31). The possibility of using siUROD as an adjunct to photosensitizers also warrants additional examination, further broadening its potential clinical application.

There is a paucity of literature surrounding UROD and cancer, with only two previous studies reporting increased UROD activity in breast tumors compared with normal tissues (32, 33). The current study demonstrates UROD to be markedly overexpressed in primary HNSCC biopsies compared with corresponding normal tissues (Fig. 7D). A potential predictive value for UROD was also revealed, wherein lower pretreatment UROD mRNA expression appeared to correlate with improved DFS in HNSCC patients treated with RT (Fig. 7E). The power of this association may be underestimated because of the skewed outcome, in that there were only 8 nonrelapsed compared with 30 relapsed patients. Thus, additional evaluation of more balanced HNC cohorts is definitely warranted. The possible role of UROD overexpression in predicting radioresistance was strongly supported by the reversal of the radiosensitive phenotype of UTSCC-42a cells (Fig. 7, B and C), thereby facilitating the selection of cancer patients who would be amenable to UROD-mediated radiosensitization.

Thus, down-regulating UROD has important implications in the management of human cancers. Its potential therapeutic application is broad and effective in the tumor-selective enhancement of radia-

MATERIALS AND METHODS

BrdU-based siRNA HTS

The Human siGENOME Druggable and Protein Kinase siRNA Libraries (Dharmacon) were provided by the Toronto Samuel Lunenfeld Research Institute (SLRI) HTS Robotics Facility. Automation of the 96-well siRNA transfection and bromoephoxuridine (BrdU) cell proliferation assay (Exalpha Biologicals) were performed with the BioMek FX (Beckman Coulter), SpectraMax Plus384 microplate reader (Molecular Devices), and SLRI robotics platform.

Working stock solutions of siRNA were prepared in Opti-MEM I reduced serum media (Invitrogen). Reverse transfections (final concentration of 40 nM siRNA) were performed with Lipofectamine 2000
Local tumor RT (4 Gy) was delivered on days 5 and 13 after intraperitoneal injection for up to 2 weeks. siRNAs were mixed with in vivo jetPEI formulation and injected intraperitoneally with 600 pmol per injection. The average size of the tumors was ~8 mm, and mice were injected intraperitoneally with 600 pmol of siRNA.

The right-tailed Fisher's exact test was used to calculate P values and scores (P score = −log10 P value), indicating the likelihood of genes being observed together in a network because of random chance.

### Supplementary Material

www.sciencetranslationalmedicine.org/cgi/content/full/3/67/67ra7/DC1

**Materials and Methods**

Fig. S1. UROD down-regulation radiosensitizes HNC cell lines.

Fig. S2. UROD knockdown plus irradiation induces apoptosis in FaDu and CAL33 cells.

Fig. S3. siUROD-mediated radiosensitization enhances oxidative stress in FaDu and CAL33 cells.

Fig. S4. UROD knockdown elevates the intracellular labile iron pool.

Fig. S5. Systemic UROD siRNA plus local RT is tolerated in tumor-bearing mice.

Fig. S6. UROD demonstrates clinical significance in human cancers.

**Table S1.** Top-scoring molecular and cellular functions.

**Table S2.** Top-scoring molecular and cellular functions.

**Table S3.** Primer sequences for mRNA expression analyses.

**References**

1. D. M. Parkin, F. Bray, J. Ferlay, P. Pisani, Global cancer statistics, 2002. CA Cancer J. Clin. 55, 74–108 (2005).

2. S. I. Pai, W. H. Westra, Molecular pathology of head and neck cancer: Implications for diagnosis, prognosis, and treatment. Annu. Rev. Pathol. 4, 49–70 (2009).

3. A. L. Carvalho, I. N. Nishimoto, J. A. Califano, L. P. Kowalski, Trends in incidence and prognostic factors for head and neck cancer in the United States: A site-specific analysis of the SEER database. Int. J. Cancer 114, 806–816 (2005).

4. J. Bourhis, J. Overgaard, H. Audy, K. K. Ang, M. Saunders, J. Bernier, J. C. Horiot, A. Le Maître, T. F. Paiak, M. G. Poulsen, B. O’ Sullivan, W. Dobrovolsky, A. Hiliakas, K. Skladkowski, J. H. Hay, L. H. Pintco, C. Falla, K. K. Fu, R. Sylvester, J. P. Pignon; Meta-Analysis of Radiotherapy in Carcinomas of Head and neck (MARCH) Collaborative Group. Hyperfractionated or fractionated accelerated radiotherapy in head and neck cancer: A meta-analysis. Lancet 368, 843–854 (2006).

5. J. P. Pignon, A. Le Maître, E. Maillard, J. Bourhis; MACH-NC Collaborative Group. Meta-analysis of chemotheraphy in head and neck cancer (MACH-NC): An update on 93 randomised trials and 17,346 patients. Radiother. Oncol. 92, 4–14 (2009).

6. J. A. Bonner, P. M. Harari, J. Giralt, N. Azarnia, D. M. Shin, R. B. Cohen, C. U. Jones, R. Sur, D. Raben, J. Jassem, R. Ove, M. S. Kies, J. Baselga, H. Youssoufian, N. Amellal, E. K. Rowinsky, K. K. Ang, Radiotherapy plus cetuximab for squamous-cell carcinoma of the head and neck. N. Engl. J. Med. 354, 567–578 (2006).

7. A. Bozec, P. Formento, S. Lassalle, C. Lippens, P. Hofman, G. Milano, Dual inhibition of EGFR and VEGFR pathways in combination with irradiation: Antitumour supra-additive effects of radiation response by inhibition of Aurora-A kinase using siRNA or a selective Aurora kinase inhibitor PHA680632 in p53-deficient cancer cells. Br. J. Cancer 97, 65–72 (2007).

8. C. Petersen, D. Zips, M. Krause, W. Volkel, H. D. Thames, M. Baumann, Recovery from sublethal damage during fractionated irradiation of human FaDu SCC. Radiother. Oncol. 74, 331–336 (2005).

9. A. Choudhury, A. Cuddihy, R. G. Bristow, Radiation and new molecular agents part I: Targeting ATM-ATR checkpoints, DNA repair, and the proteasome. Semin. Radiat. Oncol. 16, 51–58 (2006).

10. Y. Tao, P. Zang, V. Frascogna, Y. Le克莱斯, A. Auperin, J. Bouris, E. Deutsch, Enhancement of radiation response by inhibition of Aurora-A kinase using siRNA or a selective Aurora kinase inhibitor PHA680632 in p53-deficient cancer cells. Br. J. Cancer 97, 1664–1672 (2007).

11. B. Fertil, H. Dertinger, A. Courdi, E. P. Malaise, Mean inactivation dose: A useful concept for intercomparison of human cell survival curves. Radiat. Res. 99, 73–84 (1984).

12. T. C. Chou, P. Talalay, Quantitative analysis of dose-effect relationships: The combined effects of multiple drugs or enzyme inhibitors. Adv. Enzyme Regul. 22, 27–55 (1984).

13. R. W. Lambrecht, M. Thapar, H. L. Bonkovsky, Genetic aspects of porphyria cutanea tarda. Semin. Liver Dis. 27, 99–108 (2007).

14. K. Berg, P. K. Selbo, A. Weyergang, A. Dietze, L. Prasmickaite, A. Bonsted, B. Ø. Engesaeter, E. Angell-Petersen, T. Warloe, N. Frandsen, A. Høgset, Porphyrin-related photosensitizers for cancer imaging and therapeutic applications. J. Microsc. 218, 133–147 (2005).

15. J. C. Kennedy, R. H. Pottier, Endogenous protoporphyrin IX, a clinically useful photosensitizer for photodynamic therapy. J. Photochem. Photobiol. B 14, 275–292 (1992).

16. M. Valko, D. Leibfritz, J. Moncol, M. T. C. Cronin, M. Mazur, J. Telser, Free radicals and antioxidants in normal physiological functions and human disease. Int. J. Biochem. Cell Biol. 39, 44–84 (2007).

17. T. D. Oberley, L. W. Oberley, Antioxidant enzyme levels in cancer. Histol. Histopathol. 12, 525–533 (1997).

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Statistical analyses

In all cases, statistical significance between treatment groups was determined with Student’s t test and one-way analysis of variance (ANOVA). The Ingenuity Pathways Analysis software (Ingenuity Systems) was used to identify functional biological networks from the HTS data. The right-tailed Fisher’s exact test was used to calculate P values and scores (P score = −log10 P value), indicating the likelihood of genes being observed together in a network because of random chance.

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**In vivo tumor model**

All animal experiments used 6- to 8-week-old SCID BALB/c female mice in accordance with the guidelines of the Animal Care Committee, Ontario Cancer Institute, University Health Network. TLDs and body weights were recorded thrice weekly; mice were euthanized by CO2 once TLDs reached ~14 mm.

**Tumor formation assay**

Cells transfected with siCTRL or siUROD (siUROD1 or siUROD2) for 48 hours were harvested and implanted into the left gastrocnemius muscle of SCID mice (2.5 × 10^5 viable cells in 100 µl of growth medium per mouse), followed immediately by administration of local tumor RT (4 Gy). Mice were immobilized in a Lucite box, and the tumor-bearing leg was exposed to 225 kV (13 mA) at a dose rate of 3.37 Gy/min (X-RAD 225Cx Biological X-Ray Irradiator; MDS Nordion) at a dose rate of 0.84 Gy/min. Cells were incubated for an additional 72 hours, at which time BrdU (Exalpha Biologicals) was added to each well. After 24 hours, cells were monitored for BrdU incorporation on a SpectraMax Plus 384 microplate reader according to the manufacturer’s specifications.

**Transfections**

siRNAs targeting UROD [hs_UROD_2 (siUROD1) and hs_UROD_8 (siUROD8)] HP GenomeWide siRNAs and a scrambled control (AllStars Negative Control siRNA) were purchased from Qiagen; an additional nonoverlapping siRNA against UROD (siGENOME UROD siRNA; siUROD2) was obtained from Dharmacon. A plasmid vector containing the protein-coding sequence of UROD (hs_UROD_IM_1 QIAgene Expression Construct) and an empty vector control (pQE-TriSystem Vector) were also purchased from Qiagen. All transfections were performed in complete media without antibiotics with Lipofectamine 2000 and 40 nM siRNA and/or 1 µg of plasmid DNA.

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**Therapeutic tumor growth assay**

Cells were implanted into the left gastrocnemius muscle of SCID mice (2.5 × 10^5 viable cells in 100 µl). Once the TLDs reached an average of ~8 mm, mice were injected intraperitoneally with 600 pmol of siRNA complexed to in vivo jetPEI (Polyplus-Transfection), thrice a week for up to 2 weeks. siRNAs were mixed with in vivo jetPEI following the manufacturer’s specifications (nitrogen/phosphate ratio: 8). Local tumor RT (4 Gy) was delivered on days 5 and 13 after intraperitoneal injections.
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18. J. Yang, E. W. Lam, H. M. Hammad, T. D. Oberley, L. W. Oberley, Antioxidant enzyme levels in oral squamous cell carcinoma and normal human oral epithelium. J. Oral Pathol. Med. 31, 71–77 (2002).

19. V. Hower, P. Mendes, F. M. Torti, R. Laubenbacher, S. Akrman, V. Shulava, S. V. Torti, A general map of iron metabolism and tissue-specific subnetworks. Mol. Biosyst. 5, 422–443 (2009).

20. H. J. H. Fenton, Oxidation of tartaric acid in presence of iron. J. Chem. Soc. 65, 899–910 (1894).

21. B. Cummings, T. Keane, M. Pintilie, M. Payne, D. E. Payne, F. F. Liu, R. Bissett, M. McLean, P. Galllin, B. O’Sullivan, Five year results of a randomized trial comparing hyperfractionated to conventional radiotherapy over four weeks in locally advanced head and neck cancer. Radiat. Oncol. 85, 7–16 (2007).

22. T. P. Szatrowski, C. F. Nathan, Production of large amounts of hydrogen peroxide by human tumor cells. Cancer Res. 51, 794–798 (1991).

23. X. J. Wu, X. Hua, Targeting ROS: Selective killing of cancer cells by a cruciferous vegetable derived pro-oxidant compound. Cancer Biol. Ther. 6, 646–647 (2007).

24. P. T. Schumacker, Reactive oxygen species in cancer cells: Live by the sword, die by the sword. J. Biol. Chem. 276, 24437–24440 (2001).

25. D. R. Richardson, D. S. Kalinowski, S. Lau, P. J. Jansson, D. B. Lovejoy, Cancer cell iron metabolism and the development of potent iron chelators as anti-tumour agents. Biochim. Biophys. Acta 1790, 702–717 (2009).

26. I. F. Tannock, S. A. Hill, R. G. Bristow, L. Harrington, J. Wrana, F.-F. Liu, Uroporphyrinogen decarboxylase is a radiosensitizing target for head and neck cancer. Proc. Natl. Acad. Sci. U.S.A. 104, 5079–5084 (2007).

27. J. B. Mitchell, S. McPherson, W. DeGraff, J. Gamson, A. Zabell, A. Russo, Oxygen dependence of hematoporphyrin derivative-induced photoinactivation of Chinese hamster cells. Cancer Res. 45, 2008–2011 (1985).

28. J. D. Phillips, H. A. Bergonia, C. A. Reilly, M. R. Franklin, J. P. Kushner, A porphomethene inhibitor of uroporphyrinogen decarboxylase causes porphyria cutanea tarda. Proc. Natl. Acad. Sci. U.S.A. 104, 5079–5084 (2007).

29. F. G. Whitby, J. D. Phillips, J. P. Kushner, C. P. Hill, Crystal structure of human uroporphyrinogen decarboxylase. EMBO J. 17, 2463–2471 (1998).

30. N. M. Navone, C. F. Polo, A. L. Frisardi, N. E. Andrade, A. M. Battle, Heme biosynthesis in human breast cancer—mimetic “in vitro” studies and some heme enzymic activity levels. Int. J. Biochem. 22, 1407–1411 (1990).

31. N. M. Navone, A. L. Frisardi, E. R. Resnik, A. M. Battle, C. F. Polo, Porphyrin biosynthesis in human breast cancer. Preliminary mimetic in vitro studies. Med. Sci. Res. 16, 61–62 (1988).

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