Published in final edited form as:

Gynecol Oncol. 2010 January ; 116(1): 21–27. doi:10.1016/j.ygyno.2009.10.040.

Expression of metabolically targeted biomarkers in endometrial carcinoma

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Abstract

Objectives—The differential metabolic phenotype observed between malignant and non-transformed cells may constitute a biochemical basis for therapeutic intervention. Increased glucose uptake is one of the major metabolic changes found in malignant tumors, a process that is mediated by glucose transporters such as Glut1. Cellular growth can be regulated by mTOR in response to the nutrient milieu. In this study, we sought to determine if endometrial carcinoma cells express Glut1 and mTOR, and if inhibition of these factors is cytotoxic to endometrial carcinoma cells *in vitro*.

Methods—Expression of Glut1, pAkt, and pmTOR was assessed in tissue microarrays constructed from 42 type I and 34 type II endometrial tumors by immunohistochemistry, and in a panel of endometrial carcinoma cell lines. Representative endometrial carcinoma cells with wild type or mutant endogenous PTEN were treated with the glucose analog 2-deoxyglucose (2-DG) and rapamycin, an mTOR inhibitor or cisplatin. Inhibition of cell growth and mechanism of cell death was determined.

Results—Glut1, pAkt, and pmTOR were expressed strongly in both types I and II endometrial carcinoma. 2-DG and rapamycin induced apoptotic cell death in type I endometrial carcinoma cells, and profound growth inhibition and cytostasis in type II endometrial carcinoma cells.

Conclusions—Glut1, pAkt, and pmTOR are overexpressed in endometrial carcinomas. Distinct alterations in the phosphatidylinositol 3'-kinase (PI3K) pathway upstream of mTOR, such as pAkt, may identify endometrial carcinoma patients who may benefit from adjuvant treatment with mTOR inhibitors and/or glucose analogs.

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Conflict of interest statement

None of the authors have any conflicts of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ygyno.2009.10.040.

Keywords

Endometrial carcinoma; Glut1; pAkt; pmTOR

Introduction

Endometrial carcinoma is the most common gynecologic malignancy in the United States [1]. The majority of patients with endometrial carcinoma are diagnosed with stage I or II disease, and the 5 year survival for patients with early stage disease is approximately 60–75%. In contrast, the 5 year survival for patients with advanced, stage III or IV disease, is 10–29% [1]. Women with recurrent or metastatic endometrial carcinoma represent a heterogeneous group. Depending on prior therapy and the nature of the recurrence, these patients are often left with palliative options alone.

Tumor cells exhibit altered metabolism, characterized by increased glucose uptake and elevated glycolysis [2]. Cancer cells frequently display high rates of aerobic glycolysis in comparison to non-transformed cells, although the molecular basis of this phenomenon remains poorly understood. The first regulatory step in glucose metabolism is glucose uptake. Glucose uptake is controlled via a family of facilitative glucose transporters. In several tumor types, cells rely on the Glut1 glucose transporter as a primary source for intracellular glucose [3,4]. Others have shown that activated Akt may increase Glut1 protein synthesis and promote accumulation of Glut1 on the surface of lymphoid cells [5–7]. The glucose analog 2-deoxyglucose (2-DG), which can be taken up via the Glut1 receptor, but cannot be metabolized, is currently in clinical trials in several tumor types [8,9].

Tumor glycolysis has also been linked to activation of the Akt/mTOR pathway. In an experimental leukemia model, Akt activation stimulated glucose consumption without affecting the rate of oxidative phosphorylation suggesting that activation of the Akt oncogene is sufficient to stimulate the switch to aerobic glycolysis characteristic of cancer cells [10]. Expression of glycolytic enzymes can be regulated by mTOR, a serine/threonine protein kinase downstream of Akt, that regulates cell growth, proliferation, and survival in response to growth factors and nutrients. Treatment with rapamycin suppresses the rate of glycolysis [11]; to date, several mTOR inhibitors have been utilized clinical trials with modest success [12,13]. The identification of biomarkers to predict tumor sensitivity to these agents would markedly facilitate patient selection.

We hypothesized that the differential metabolic phenotype observed between malignant and non-transformed cells may constitute a biochemical basis for therapeutic intervention. Our goal in undertaking this study was to determine if targeting metabolic signaling pathways in endometrial carcinoma cells would have antineoplastic activity. Aberrant expression of the facilitative glucose transporter Glut1 as well as phosphorylation of Akt and mTOR has been described in endometrial carcinoma [14–17], yet it is not entirely clear whether all endometrial carcinoma cells are susceptible to inhibition of these factors. We hypothesized that endometrial carcinoma cells would display increased glucose uptake, and that this phenotype could be exploited for therapeutic intervention by using the glucose analog 2-DG. Furthermore, we reasoned that inhibition of mTOR dependent signaling with rapamycin

should mimic nutrient and growth factor deprivation, resulting in growth inhibition and cell death.

Materials and methods

Reagents and cell lines

Rapamycin was purchased from LA Laboratories. 2-Deoxy-D-glucose (2DG), Grade III was purchased from Sigma-Aldrich Inc. Cisplatin was obtained from Bedford Laboratories. Representative endometrial carcinoma cell lines with wild type PTEN (Hec-1B, KLE) and mutant PTEN (Ishikawa and RL95-2) [18] were generously provided by Dr. K. Cho (University of Michigan, Ann Arbor, MI).

In vitro growth inhibition assay

The sulforhodamine B assay was used according to the method of Skehan et al. [19]. Cells were treated as indicated, and at indicated time points, cells were fixed with 50% trichloroacetic acid at 4 °C and allowed to sit for an additional 24 h. Cells were stained with 0.4% sulforhodamine B. Protein-bound stain was solubilized with 150 µl of 10mM unbuffered Tris base, and the cell density was determined using a fluorescence plated reader (wavelength 570). All samples were run in at least triplicate.

Immunoblotting

For preparation of S-100 cytosolic lysates, cells were suspended in Buffer A isotonic lysis buffer and were homogenized using a 28.5-gauge needle. For preparation of whole cell extracts, cells were harvested and lysed with RIPA buffer. Immunoblotting was performed with indicated primary antibodies. After incubation with secondary antibody, the reactions were developed by enhanced chemiluminescence using the Luminol Reagent (Santa Cruz). The monoclonal antibody for caspase-9 was obtained from Stressgen. Polyclonal antibodies for total and phosphorylated Akt and mTOR were obtained from cell signaling. Polyclonal antibodies for Glut-1 were obtained from Abcam.

Flow cytometry analysis

The percentage of apoptotic cells was determined at indicated time points by nuclear propidium iodide staining. In this assay, the nuclei of apoptotic cells exhibit a sub-G₀ profile characteristic of DNA fragmentation [20]. Analysis was performed using Lysis II software of a FACScan flow cytometer (Beckton Dickinson, Mountain View, CA). Values were expressed as the mean $\pm$ SD from duplicate cultures.

TMA analysis

All H&E stained slides of the tumors were reviewed, and areas of tumor were identified. Two to three tissue cores (1.0 mm in diameter) per tumor were taken from spatially separate areas in a single donor block from each case using a tissue microarrayer (Chemicon Advanced Tissue Arrayer). Cores were arrayed into a recipient block at predetermined coordinates. The H&E stained sections from donor and recipient paraffin blocks were used to confirm the area of tumor from which cores were retrieved.

Tissue microarrays (TMAs) were constructed using 75 cores from 42 patients with type I endometrial cancer, 91 cores from 33 patients with type II endometrial cancer, and 20 cores from benign endometrial samples. Tumors were histopathologically classified according to the International Federation of Gynecology and Obstetrics (FIGO) criteria. Clinicopathologic and demographic data were collected from medical records under an IRB-approved protocol.

For immunohistochemistry, 5 μ M thick sections were cut from the respective arrays, deparaffinized, and dehydrated. Immunohistochemical staining for p-Akt, p-mTOR, and Glut1 was performed using a streptavidin peroxidase procedure. The antibodies were obtained from Cell Signaling and Chemicon International, respectively. Antigen bound primary antibody was detected using standard avidinbiotin immunoperoxidase complex (Dako).

Expression scores for membrane staining of Glut1, and cytoplasmic expression of pAkt and pmTOR were based on the percentage of cells that were reactive with antibodies to Glut-1 (Chemicon International, 1:800), pAkt (Cell Signaling 1:100), or pmTOR (Cell Signaling, 1:50) as follows: negative (score = 1, no staining), weak (score = 2, <25% of cells staining, any intensity), moderate (score = 3, 25–75% of cells staining, any intensity), and strong (score = 4, >75% of cells staining, any intensity). All scoring was performed by a single author (MPK).

Statistical analysis

Expression scores (1 to 4) per patient for primary tumor were calculated using the median of the core-specific scores. In cases where the median was the midpoint between score values, the score was rounded. The potential association between patient level expression of pmTOR, Glut1, and pAkt was explored using contingency tables, with significant associations determined using the Fisher's exact test statistic. *P*-values at or below 5% indicated meaningful associations. Differences between type I and type II endometrial tumors, and benign endometrium was the primary objective.

Results

Glut1, pAkt, and pmTOR expression in endometrial carcinoma

Membranous Glut1 was strongly expressed in both type I and type II endometrial carcinomas (53.8% and 46%, respectively). In contrast, benign endometrium did not have any significant Glut1 expression (Figs. 1A–C, $p < 0.001$). Because there is some evidence that activated Akt may increase Glut1 protein synthesis, and promote accumulation of Glut1 on the surface of tumor cells [5–7], we next analyzed expression of phosphorylated Akt in endometrial tumors using an antibody against phosphorylated Ser473 of Akt. Immunostaining of our endometrial carcinoma tissue microarrays revealed that a greater proportion of type I endometrial tumors stained positively for p-Akt as compared to type II endometrial tumors (83.3% and 61.3%, respectively, $p < .05$). In comparison with endometrial carcinoma samples, a significantly smaller proportion of benign endometrium expressed pAkt (74.0% versus 46.2%, $p = 0.02$, Figs. 1D–F). The downstream effector of Akt, mTOR,

was of particular interest, as mTOR can regulate transcription and translation in response to the nutrient milieu. Immunohistochemical analysis of mTOR activation was performed using an antibody directed at phosphorylated mTOR Ser2448, which recognizes the active form of the mTOR kinase (Figs. 1G–I). Cytoplasmic pmTOR was expressed in both types I and II endometrial carcinoma (82.9% and 76.7%, respectively), as well as in benign endometrium.

To confirm that Glut1, pAkt, and pmTOR are expressed in cultured endometrial carcinoma cells, expression levels of these proteins were determined by immunoblotting. Glut1 expression was seen in all cell lines tested (Fig. 2A). Next, expression of pAkt was determined. As shown in Fig. 2B, HEC1B did not express significant levels of pAkt in complete or serum free media, while RL95-2 displayed constitutive activation of pAkt. mTOR activation was analyzed using an antibody directed at phosphorylated mTOR Ser2448, which recognizes the active form of the mTOR kinase. HEC1B and Ishikawa cell lines had higher expression levels of pmTOR as compared to the KLE and RL95-2 cell lines (Fig. 2C).

Growth inhibitory effect of Rapamycin and 2-deoxyglucose

As both the HEC1B and RL95-2 cell lines appeared to express activated mTOR, we next sought to determine whether inhibition of mTOR could inhibit cell growth. First, to confirm that rapamycin treatment resulted in inhibition of mTOR, we determined that rapamycin treatment resulted in inhibition of the mTOR substrate phosphorylated-pS6K1 in representative HEC1B and RL95-2 cells (Fig. 2D). Rapamycin treatment of HEC1B, KLE, Ishikawa, and RL95-2 cells resulted in a modest dose and time dependent reduction of viable cells (Supplementary Fig. 1).

Next, the same panel of cell lines was treated with 2-deoxy-D-glucose (2-DG). 2-deoxy-D-glucose (2-DG) is a glucose analog, which can be taken up through the Glut1 receptor, yet cannot undergo further glycolysis. 2DG treatment of HEC1B, KLE, Ishikawa, and RL95-2 endometrial carcinoma cell lines resulted in a time and dose dependent growth inhibition (Supplementary Fig. 2).

2DG and rapamycin both appeared to enhance the growth inhibitory effect of cisplatin (Fig. 3). To confirm that 2DG and rapamycin were inducing cell death rather than growth inhibition, two representative cell lines (HEC1B and RL95-2) were treated with 2DG, rapamycin, cisplatin alone, or in combination, and viability was determined using the trypan blue assay. In the RL95-2 cell line, 2DG, rapamycin, and cisplatin induced cell death, as treatment was associated with a decrease in total number of cells, and, in parallel, a decrease in the proportion of viable cells (Figs. 3E, F). In contrast, in the HEC1B cell line, although treatment resulted in a dramatic decrease in total number of cells, the proportion of viable cells did not change, indicating that, in this cell line, these agents appeared to induce profound growth inhibition (Figs. 3G, H).

2DG and rapamycin enhance cisplatin induced apoptosis

To further characterize the mechanism of cell death induced by cisplatin, 2DG, rapamycin, or combinations as indicated, treated cells were stained with propidium iodide and the fraction of cells with a less than G₀/G₁ DNA content, indicative of apoptosis, was analyzed

by flow cytometry (Figs. 4A, B). Both the RL95-2 and HEC1B cell lines are relatively resistant to cisplatin induced apoptosis, as only 10–20% of cells were apoptotic following 24 h of treatment. 2DG or rapamycin treatment alone did not result in apoptotic cell death in either cell line. In contrast, in the RL95-2 cell line, after 48–72 h of incubation with cisplatin-2DG or cisplatin-rapamycin, approximately 40–50% of cells underwent apoptosis (Fig. 4A). In the HEC1B cell line, the addition of 2DG or rapamycin to cisplatin increased apoptotic response to from 10% to 20% by 72 h of treatment (Fig. 4B).

To determine whether the intrinsic apoptotic pathway was activated in response to treatment with cisplatin, 2DG, rapamycin, or combinations as indicated, immunoblot analysis of treated cells was performed to determine activation of caspase-9 (Figs. 4C, D). In the RL95-2 cell line, caspase-9 activation (indicated by the appearance of the proteolytic fragments of caspase-9, p37/35) was not seen in cells treated with vehicle control, cisplatin, 2DG, or rapamycin alone at 24 h of incubation (Fig. 4C, lanes 1–4). The combination of cisplatin and 2DG or cisplatin and rapamycin at 24 h resulted in activation of the intrinsic apoptotic pathway as caspase-9 activation was seen (Fig. 4C lanes 5, 6). By 48 h of incubation, treatment with cisplatin alone, or cisplatin with 2DG or rapamycin all resulted in robust caspase-9 activation (Fig. 4C, lanes 8, 11, 12). In the HEC1B cell line, the combination of cisplatin and rapamycin appeared to induce modest caspase-9 activation after 48 h of treatment (Fig. 4D), consistent with data presented in Fig. 4B.

Rapamycin can promote or inhibit apoptosis

There is some evidence that in selected cell types, rapamycin can inhibit Akt, and NF κ B activation, and suppresses Bcl-x_L expression [21]. Because rapamycin/cisplatin induced apoptosis in RL95-2 cells, but only growth inhibition in HEC1B cells, we next investigated the effect of rapamycin on expression of the anti-apoptotic proteins Bcl-2, Bcl-x_L, and pAkt. In HEC1B cells, treatment with cisplatin, rapamycin, 2DG, or combinations of cisplatin with rapamycin or 2DG did not affect Bcl-2 or Bcl-x_L expression levels (Fig. 5A). In contrast, in the RL95-2 cells, treatment with cisplatin and rapamycin resulted in a reduction in both Bcl-2 and Bcl-x_L expression levels (Fig. 5B). In HEC1B cells, treatment with rapamycin alone or rapamycin with cisplatin resulted in activation of Akt, suggesting that, in this cell line, mTORC1 was inhibited (Fig. 5C). In RL95-2 cells, treatment with rapamycin with cisplatin resulted in a modest reduction in pAkt, suggesting that mTORC2 may be inhibited (Fig. 5D).

Discussion

Tumor cells are metabolically distinct from normal, untransformed cells. Tumor cells exhibit increased glucose uptake and elevated glycolysis, a phenomenon first described by Otto Warburg [2]. Loss of p53 function or activation of Akt can induce the metabolic changes seen in the Warburg effect [22]. To date, exploitation of the Warburg effect has been utilized primarily for diagnostic purposes, using positron emission tomography (PET) imaging. Drugs that alter cancer cell metabolism, specifically at the level of glycolysis, may have significant therapeutic activity in cancer cells.

Recent studies indicate that components of the Akt/mTOR signaling pathway are aberrantly expressed in several tumor types, and that these factors may represent targets for therapy. PTEN is a tumor suppressor gene that is frequently mutated in endometrial cancers, which leads to constitutive activation of Akt and upregulation of mTOR [23], suggesting that mTOR inhibitors may be useful in the treatment of this tumor type. Recent data suggest that uterine serous carcinomas may harbor alterations in the PI3K-Akt-mTOR pathway [24], and that rapamycin and cisplatin may exert a synergistic effect on inhibition of cell growth and induction of apoptosis in several endometrial carcinoma cell lines [17]. Several phase II clinical trials utilizing the rapamycin analogues CCI-799 (tensirolimus) and AP-23573 in patients with advanced or recurrent endometrial cancer have demonstrated modest response rates [25]. The ability to predict which patients would derive the most clinical benefit from treatment with mTOR inhibitors would undoubtedly enhance treatment outcome. Our data indicate that the mTOR inhibitor rapamycin induces apoptotic cell death in one cell type (RL95-2), and profound growth inhibition and cytostasis in another cell type (HEC1B). Although both of these cell lines appeared to harbor constitutive activation of mTOR and its downstream target p70S6K (Figs. 2C, D), distinct alterations in the phosphatidylinositol 3'-kinase (PI3K) pathway upstream of mTOR resulted in different results following rapamycin treatment. mTOR is an essential component of two distinct multi-protein complexes centered around mTOR, mTORC1 and mTORC2, and inhibition of these two complexes results in divergent effect on Akt activation [26]. Our data support the hypothesis that, in cells harboring constitutive activation of Akt, rapamycin will inhibit mTORC2 and induce apoptosis, whereas in cells that do not express pAkt, rapamycin treatment inhibits mTORC1, resulting in Akt activation and cytostasis.

2DG is a glucose analog that competitively inhibits cellular uptake and utilization of glucose. Upon entering the cell, 2-DG is phosphorylated by hexokinase. However, unlike glucose, 2-DG cannot be further metabolized, resulting in inhibition of glucose metabolism [27]. Others have investigated the use of 2-DG, for cancer therapy, based on the hypothesis that intracellular limitation of glucose availability can inhibit the carcinogenic process. Pilot studies have shown that 2-DG can enhance the growth inhibitory effect of chemotherapy in xenograft models [28]. In fact, the use of 2-DG for cancer treatment has been patented, and there are several ongoing clinical trials utilizing this agent for the treatment of cancer [29]. The disparate effects of 2DG in RL95-2 and HEC1B cells (apoptosis vs. cytostasis) are not entirely clear at this time. However, recent evidence indicates that 2DG inhibition of cell growth is not entirely due to inhibition of glucose catabolism, and that the effects of 2DG as a cancer therapeutic may involve multiple regulatory pathways [30]. Reassessment of the mechanisms through which 2DG treatment blocks tumor cell growth will likely lead to the identification of novel targets.

Differences in the metabolic phenotype observed between malignant and non-transformed cells may constitute a biochemical basis for therapeutic intervention. Our studies indicate that endometrial carcinoma cells express Glut1, Akt, and mTOR, all of which contribute toward the regulation of cellular metabolism. The use of the mTOR inhibitor rapamycin, and the glycolytic inhibitor 2DG profoundly inhibits endometrial carcinoma cell growth *in vitro*. Activation of specific PI3K pathway components may be predictive of the ultimate therapeutic success of mTOR inhibitors.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Glut1, pAkt, and pmTOR are overexpressed in ovarian carcinoma. Tissue microarrays (TMAs) were constructed using 75 cores from 42 patients with type I endometrial cancer, 91 cores from 33 patients with type II endometrial cancer, and 20 cores from benign endometrial samples. Expression of Glut1 was absent in benign endometrium, whereas 53.8% and 46% of type I and type II endometrial carcinomas expressed Glut 1 (A–C, $p < .05$). Expression of pAkt was significantly higher in both type I and type II endometrial carcinomas as compared to benign endometrium (74% and 46.2%, respectively, $p < .05$, D–F). Expression of pmTOR was present in the majority of endometrial carcinomas as well as in benign endometrium (G–I).

Fig. 2. Glut1, pAkt, and pmTOR are expressed in endometrial carcinoma cell lines. (A) Glut1 expression was analyzed by immunoblotting in endometrial carcinoma cell lines HEC1B, Ishikawa, KLE, and RL95-2. (B) Representative HEC1B and RL95-2 cells expressed minimal and high levels of pAkt respectively in complete media and serum free conditions. (C) pmTOR expression was analyzed by immunoblotting in endometrial carcinoma cell lines HEC1B, Ishikawa, KLE, and RL95-2. (D) Treatment with rapamycin resulted in diminished expression of the mTOR substrate p70S6K in HEC1B and RL95-2 cells.

Fig. 3. 2DG and Rapamycin induce cell death in RL95-2 and cytostasis in HEC1B cells. (A) HEC1B, (B) Ishikawa, (C) KLE, and (D) RL92-2 endometrial carcinoma cells were incubated in media containing cisplatin, 2DG, rapamycin alone, and in combination for 24, 48, and 72 h. Growth was assessed at each timepoint using the sulforhodamine B assay. Data are presented as means $\pm$ S.D. Representative RL95-2 and HEC1B cells were incubated with cisplatin, 2DG, and rapamycin alone and in combination. At 24, 48, and 72 h time points,

cells were harvested and stained with trypan blue. Total cell number (E, G) and viability (F, H) were assessed. Data are presented as means $\pm$ S.D.

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Fig. 4.

2DG and rapamycin enhance cisplatin induced apoptosis. (A) RL95-2 and (B) HEC1B cells were treated with cisplatin, 2DG, and rapamycin alone and in combination. At 24, 48, and 72 h time points, cells were harvested and stained with propidium iodide. The proportion of cells in the subG0 fraction reflecting apoptotic DNA fragmentation was determined by flow cytometry. Data are presented as means $\pm$ S.D.

Fig. 5. 2DG and rapamycin enhance cisplatin induced activation of Caspase-9. (A) RL95-2 and (B) HEC1B endometrial carcinoma cells were incubated in media containing cisplatin, 2DG, and rapamycin alone and in combination for 24 h. Cytosolic lysates were analyzed by immunoblotting for expression of the pro- and cleaved forms of caspase-9.