GlcNAcylation Plays an Essential Role in Breast Cancer Metastasis

Yuchao Gu, Wenyi Mi, Yuqing Ge, Haiyan Liu, Qiong Fan, Cuifang Han, Jing Yang, Feng Han, Xinzhi Lu, and Wengong Yu

Abstract

GlcNAcylation, a dynamic posttranslational modification, is involved in a wide range of biological processes and some human diseases. Although there is emerging evidence that some tumor-associated proteins are modified by GlcNAcylation, the role of GlcNAcylation in tumor progression remains unclear. Here, we show that GlcNAcylation enhances the migration/invasion of breast cancer cells in vitro and lung metastasis in vivo. The decrease of cell surface E-cadherin is the molecular mechanism underlying GlcNAcylation-induced breast cancer metastasis. p120 and β-catenin, but not E-cadherin, are GlcNAcylated; the GlcNAcylation of p120 and β-catenin might play roles in the decrease of cell surface E-cadherin. Moreover, immunohistochemistry analysis indicated that the global GlcNAcylation level in breast tumor tissues is elevated significantly as compared with that in the corresponding adjacent tissues; further, GlcNAcylation was significantly enhanced in metastatic lymph nodes compared with their corresponding primary tumor tissues. This is the first report to clearly elucidate the roles and mechanisms whereby GlcNAcylation influences the malignant properties of breast cancer cells. These results also suggest that GlcNAcylation might be a potential target for the diagnosis and therapy of breast cancer. Cancer Res; 70(15); OF1–8. ©2010 AACR.

Introduction

GlcNAcylation is an O-linked β-N-acetylglucosamine (O-GlcNaC) moiety linked to the side chain hydroxyl of a serine or threonine residue (1). GlcNAcylation is found on numerous cytoplasmic and nuclear proteins. The addition of O-GlcNAc to proteins is catalyzed by O-GlcNAc transferase (OGT) and its removal is catalyzed by O-GlcNAc-selective N-acetyl-β-d-glucosaminidase (O-GlcNAcase, OGA). This dynamic and reversible posttranslational modification, analogous to phosphorylation, is emerging as a key regulator of protein function by regulating protein activity, protein-protein interaction, localization, or protein degradation (2). GlcNAcylation is involved in a wide range of biological processes, such as signal transduction, transcription, cell cycle progression, and metabolism (3).

GlcNAcylation not only has a role in normal biological processes, but its faulted regulation is also involved in some human diseases such as diabetes and neurologic disorders. Some of the proteins that have a role in the pathology of human diseases have been shown to be GlcNAcylated. In diabetes, multiple proteins in the phosphoinositide-3-kinase/AKT signaling cascade, such as RS-1, PI3K, and AKT, are GlcNAcylated and the modification induces phosphoinositide-3-kinase/AKT signaling suppression and insulin resistance (4–6). Global GlcNAcylation is reduced in the brain tissue of patients with Alzheimer’s disease, and the GlcNAcylation of human tau is reduced. The reduction of GlcNAcylation of tau results in its hyperphosphorylation, which, in turn, causes it to aggregate into the paired helical filaments that constitute the visible neurofibrillary tangles characteristic of the disease (7). These results showed that the deregulation of GlcNAcylation in some key proteins plays etiologic roles in some human diseases.

Several tumor-associated proteins have also been identified as GlcNAcylated proteins. For instance, c-Myc is GlcNAcylated mainly on Thr58, which is a known hotspot for mutation in lymphoma and is a major GSK3 phosphorylation site (8). The GlcNAcylation of c-Myc at Thr58 could competitively inhibit phosphorylation and thus suppress the proteasome-mediated degradation of c-Myc. The p53 protein is a transcription factor essential for the prevention of cancer formation (9). GlcNAcylation at Ser149 stabilizes p53 by blocking ubiquitin-dependent proteolysis (10). Although the effects of GlcNAcylation on some tumor-associated proteins have been elucidated, the roles of GlcNAcylation in cancer progression have not been investigated.

In this study, GlcNAcylation levels were examined in human breast tumor tissues and matched adjacent breast tissues. The global GlcNAcylation level was altered through OG T silencing or OGA inhibition in breast cancer cells, and the effects of GlcNAcylation on the malignant properties of...
breast tumors and the molecular mechanisms underlying GlcNAcylation-mediated breast cancer migration and metastasis were determined.

Materials and Methods

Cell cultures
The 4T1 cell line (kindly provided by Dr. Fred R. Miller at the Barbara Ann Karmanos Cancer Institute, Detroit, MI) is derived from a tumor that arose spontaneously in a wild-type BALB/c mouse (11). Cells were cultured in DMEM supplemented with 10% FCS (DMEM-10), 1 mmol/L of mixed nonessential amino acids, and 2 mmol/L of L-glutamine. HBL100 and MCF7 cell lines were purchased from the Cell Bank of Type Culture Collection of the Chinese Academy of Sciences, and cultured in DMEM and α-MEM supplemented with 10% FCS, respectively. To elevate GlcNAcylation levels, cells were treated with 50 μmol/L of PUGNac (Toronto Research Chemicals) or 100 μmol/L of NButGT (synthesized as described previously; ref. 12) for 48 hours or the indicated time periods.

Plasmid construction
The shRNA-expressing lentiviral vectors of mouse OGT and E-cadherin was constructed as described in the Supplementary Information.

Immunoprecipitation and immunoblotting
For immunoprecipitation, cells were lysed in radioimmunoprecipitation assay buffer [25 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 1% NP40, 0.1% SDS, 1 mmol/L EDTA, 1 mmol/L Na3VO4, 10 mmol/L NaF, containing a protease inhibitor cocktail]. For coimmunoprecipitation, the cell lysis was diluted 2-fold with PBS. For reimmunoprecipitation, the pellet was solubilized in 1% SDS [10 mmol/L Tris (pH 7.5), 100 mmol/L NaCl, 1% SDS, and 1% NP40] and diluted 10-fold with 1% NP40 lysis buffer before immunoprecipitation. Precipitation with succinylated wheat germ agglutinin (sWGA)–agarose beads (Vector Laboratories) was performed as previously described (13). Pulldown assays were performed as described previously (14). Cytoskeleton-binding proteins were separated as previously described (13). Immunoblotting was performed according to established protocols and developed by enhanced chemiluminescence–detecting reagents (GE Healthcare).

O-GlcNAc metabolic labeling and detection of p120
O-GlcNAc metabolic labeling was performed using Click-iT GlcNacZ metabolic glycoprotein-labeling reagent (tetra-acetylated N-azidoacetylgalactosamine) from Invitrogen. In brief, cells were cultured in complete medium and when 70% confluency was obtained, replaced with fresh medium containing either DMSO (1:1,000) or 50 μmol/L of Click-iT GlcNacZ. Cells were labeled for 24 hours and lysed in radioimmunoprecipitation assay buffer. p120 was immunoprecipitated from the cell lysate and subsequently analyzed with Click-iT Protein Analysis Detection Kit (Invitrogen) according to the protocols of the manufacturer.

Immunohistochemical studies
A breast cancer tissue microarray (TMA; OD-CT-RpBre03-002; Shanghai Outdo Biotech, Co.) was constructed with 31 formalin-fixed, paraffin-embedded breast cancer tissues and their corresponding adjacent breast tissues. Breast cancer with corresponding metastatic lymph nodes TMA (BR1005; Biomax, Inc.) was constructed with 50 formalin-fixed, paraffin-embedded breast cancer tissues and their corresponding metastatic lymph nodes.

Immunohistochemistry was performed on the TMAs by using DAKO Liquid DAB Substrate Chromogen System (Dako Cytomation) and a monoclonal mouse antibody against O-GlcNAc, RL2 (1:200). Fromowitz’s standard was used to semiquantitatively assess the staining of O-GlcNAcylation (15, 16). A detailed description is provided in the Supplementary Information.

Cell migration and invasion assay
These procedures were performed as previously described (17). Cell migration was assayed using Transwell chambers (6.5 mm; Corning) with 8 μm pore membranes. The lower chamber was filled with 600 μL of NIH-3T3 conditioned medium with or without OGA inhibitors. Cells (5 × 104) were suspended with 100 μL of medium (DMEM with 1% FCS) and plated into the upper chamber with or without OGA inhibitors. After 20 hours, the number of cells appearing by crystal violet staining on the undersurface of the polycarbonate membranes was scored visually in five random fields at ×100 magnification using a light microscope.

For invasion assays, the upper face of the membrane was covered with 70 μL of Matrigel (1 mg/mL; BD Biosciences). Invasion assay was performed using the same procedure as the migration assay, except that the incubation time of the experiment was prolonged to 24 hours.

Primary tumor growth and lung metastasis
These procedures were performed as previously described (17, 18). Female BALB/c mice, 8 to 10 weeks of age, were used for the experiment. In brief, mice (six to eight mice per group) were anesthetized with sodium pentobarbital (50 mg/kg body weight), and tumor cells (5 × 105) in 10 μL of DME-10 were injected into the mammary gland. The weight of the primary tumors and the number of metastatic nodules on the lung surface were evaluated 30 days after tumor cell injection. The animals were housed and cared for according to the guidelines set up by the National Science Council of the Republic of China.

Cell aggregation assay
Cell aggregation assays were performed as described previously (19). In brief, cells were incubated for 30 minutes at 37°C in Hepes-buffered saline containing 0.1% trypsin and 1 mmol/L of CaCl2. After the addition of soybean trypsin inhibitor (Sigma), the cells were washed, resuspended, and incubated for 30 minutes at 37°C with constant rotation at 70 rpm.

Statistical analysis
Data were analyzed with Student’s t test using the SPSS 11.0 software program (SPSS, Inc.). P < 0.05 was considered statistically significant. Data are presented as mean ± SEM.
Results

GlcNAcylation level is associated with breast cancer formation and metastasis

To learn the pathophysiologic significance of GlcNAcylation in breast cancer, we determined the GlcNAcylation level in breast tumor tissues and the corresponding tumor adjacent tissues by immunohistochemistry analysis. A breast cancer TMA was used in this study, which was constructed with 31 formalin-fixed, paraffin-embedded breast cancer tissues and their corresponding adjacent breast tissues. The immunohistochemistry results were analyzed by Fromowitz’s standard. The results indicated that the intensity of GlcNAcylation immunostaining in breast cancer tissues was markedly enhanced as compared with that in the adjacent tissues (Fig. 1A and B). Furthermore, the GlcNAcylation of 50 breast cancer tissues and their corresponding metastatic lymph nodes was analyzed. The results showed that GlcNAcylation was significantly elevated in metastatic lymph nodes (Fig. 2A and B). These results strongly suggested that GlcNAcylation might play important roles in breast cancer formation and metastasis.

GlcNAcylation promotes breast cancer malignancy

To determine whether GlcNAcylation is involved in breast cancer progression, the GlcNAcylation level in metastatic 4T1 cells was suppressed by lentiviral-mediated OGT silencing and was elevated with OGA-specific inhibitors NButGT and PUGNAc. The expression of shOGT1 and shOGT2 dramatically reduced the expression of OGT and the global GlcNAcylation level in 4T1 cells (Supplementary Information, Fig. S1A); NButGT and PUGNAc treatment obviously elevated global GlcNAcylation levels in 4T1 cells (Supplementary Information, Fig. S1B). To investigate whether GlcNAcylation affects cell proliferation and anchorage-independent growth, growth curve assays and soft agar colony assays were performed. Surprisingly, partial OGT silencing and OGA inhibition did not affect the proliferation and colony formation ability of 4T1 cells (Supplementary Information, Fig. S1D and E).

To test whether the roles of GlcNAcylation in breast cancer malignancy was a special case in 4T1 cells, HBL100 and MCF7 cells were examined. The results indicated that NButGT treatment enhanced the invasion ability of these cell lines, but did not markedly affect the proliferation and colony formation ability of these cells (Supplementary Information, Fig. S3 and 4).

Based on the roles of GlcNAcylation on the malignancy of breast cancer cells as described above, we next examined the effects of GlcNAcylation on the tumor formation and metastasis of breast cancer cells in vivo. Consistent with the results in vitro, OGT silencing in 4T1 cells did not have a significant effect on primary tumor weight (Fig. 3A), but dramatically reduced the number of visible metastatic nodules on the lung surface of tumor-bearing mice (Fig. 3B and C). Additionally, we detected OGT in the primary tumors by real-time reverse transcription-PCR and immunoblotting. The results indicated that the expression of OGT was effectively suppressed in shOGT tumor tissues (Supplementary Information, Fig. S5).
GlcNAcylation promotes breast cancer lung metastasis through decreasing cell surface E-cadherin

The above results and a previous report (20) have shown that GlcNAcylation could influence intercellular adhesion. Taking into account the important roles of E-cadherin in epithelial cell-cell adhesion and cancer malignance, the expression and distribution of the components of E-cadherin/catenin complex were examined. The effects of GlcNAcylation on the expression of E-cadherin, β-catenin, and p120 were first examined by immunoblotting. The results indicated that E-cadherin and β-catenin were slightly elevated by OGT silencing and inhibited by OGA inhibitors, but p120 expression was not changed (Fig. 4A). Then, a coimmunoprecipitation assay was used to detect the effects of GlcNAcylation on the formation of the E-cadherin/catenin complex. As shown in Fig. 4B, the association of E-cadherin, β-catenin, and p120 increased in the OGT-silenced cells and decreased in the OGA-inhibited cells. Previous reports have shown that the binding of E-cadherin/catenin complex to the cytoskeleton is essential for strong cell-cell adhesion (21), so we
investigated the effect of GlcNAcylation on the formation of the E-cadherin/catenin/cytoskeleton complex. Triton X-100–insoluble and -soluble proteins were extracted and E-cadherin, β-catenin, and p120 were detected by immunoblotting. The results indicated that the cytoskeleton-associated (Triton X-100–insoluble) E-cadherin, β-catenin, and p120 were markedly elevated in the OGT-silenced cells and reduced in the OGA-inhibited cells; E-cadherin and β-catenin in the Triton X-100–soluble portions were not significantly affected by GlcNAcylation, but the soluble p120 were reduced in OGT-silenced cells and elevated in the OGA-inhibited cells (Fig. 4C). Immunofluorescence assays were also used to determine the distribution and colocalization of E-cadherin, β-catenin, and p120. The results showed that E-cadherin, β-catenin and p120 were significantly increased on the cell surface, especially at cell-cell contact sites in the OGT-silenced cells, and obviously decreased in OGA-inhibited cells (Supplementary Information, Fig. S6), which was consistent with the above results. Then, cadherin-mediated intercellular adhesion was examined, we found that OGT silencing enhanced cadherin-mediated intercellular adhesion and OGA inhibition reduced cadherin-mediated intercellular adhesion (Fig. 4D). Altogether, these findings showed that GlcNAcylation decreased the cell surface E-cadherin and thus reduced intercellular adhesion.

To determine whether GlcNAcylation altered breast cancer cell migration and metastasis via E-cadherin, E-cadherin was silenced in 4T1 cells. The lentiviral-mediated shEcad2 and shEcad4 vectors markedly silenced E-cadherin expression, and shEcad1 and shEcad3 vectors only slightly silenced E-cadherin expression (Supplementary Information, Fig. S7A and B). Cell migration assays showed that the migration ability was negatively correlated with the expression of E-cadherin in 4T1 cells (Supplementary Information, Fig. S7C). Afterward, 4T1-shCtrlhygro and 4T1-shEcad4 cells were treated by PUGNAc (Supplementary Information, Fig. S8A), and the migration assay indicated that PUGNAc markedly enhanced the migration of 4T1-shCtrlhygro but only slightly enhanced the migration of 4T1-shEcad4 cells (Fig. 5A). Furthermore, OGT/E-cadherin double-knockdown cells were generated by coinfection with lentiviral shOGT1 and shEcad4 vectors; immunoblotting analysis showed that both GlcNAcylation level and E-cadherin were efficiently silenced (Supplementary Information, Fig. S8B). Cell migration assays indicated that OGT knockdown in E-cadherin–silencing 4T1 cells inhibited cell migration less efficiently than that in the control 4T1 cells (Fig. 5B). We then investigated the role of E-cadherin in the suppression of lung metastasis induced by OGT silencing in 4T1 cells. The results indicated that silencing of E-cadherin in OGT knockdown cells did not affect the primary tumor weights (Fig. 5C) but significantly compensated for the inhibition of lung metastasis induced by OGT knockdown (Fig. 5D). The expression of OGT and E-cadherin were also suppressed in the OGT and E-cadherin silencing primary
tumor tissues (Supplementary Information, Fig. S9). Therefore, we can conclude that the decrease of cell surface levels of E-cadherin was one of the main mechanisms underlying the GlcNAcylation-induced breast cancer cell migration and metastasis.

**The GlcNAcylation of p120 and β-catenin**

To clarify the mechanisms underlying the decrease of cell surface E-cadherin induced by GlcNAcylation, we first detected the GlcNAcylation of E-cadherin, β-catenin, and p120 by using GlcNAcylation-specific antibodies (RL2 and CTD110.6, each known to bind to some but not all GlcNAcylated proteins) and sWGA-agarose (sWGA recognizes terminal β-GlcNAc residue). GlcNAcylation of E-cadherin could not be detected by using these methods (data not shown). p120 could be stained by RL2 but not by CTD110.6, and the staining was partly eliminated by OGT silencing and completely inhibited by 100 mmol/L of GlcNAc (Fig. 6A and B). Additionally, p120 could be precipitated by sWGA-agarose and the precipitation was diminished by 500 mmol/L of GlcNAc (Fig. 6C). As previously reported (13), GlcNAcylation of β-catenin was detectable by RL2, CTD110.6, and sWGA in 4T1 cells (Supplementary Information, Fig. S10). Furthermore, an O-GlcNAc metabolic labeling assay was used to confirm the GlcNAcylation of p120 (Fig. 6D). These results showed that both β-catenin and p120 were GlcNAcylated in 4T1 cells, which might play important roles in their binding to E-cadherin, and thus, the cell surface localization of E-cadherin.

**Discussion**

It has been shown that GlcNAcylation plays important roles in some human diseases such as diabetes, neurologic disorders, and cardiovascular disease (3). In this study, we found out that global GlcNAcylation levels are associated with breast cancer formation and metastasis. These results strongly suggested that GlcNAcylation might play important roles in breast cancer progression.

Several studies have shown that GlcNAcylation acts as a cellular regulator of growth and division. The deletion of OGT in embryonic stem cells is lethal (22), and OGT tissue-specific mutation results in the loss of GlcNAcylation in specific tissues and causes T-cell apoptosis and fibroblast growth arrest (23). It was also reported that OGT inhibition prevented G2-M transition in *Xenopus laevis* oocytes (24). However, the study of Haltiwanger and colleagues indicates that PUGNAc had no effect on the growth rate of any of the cell lines...
examined (25). Our findings indicated that OGT knockdown and OGA inhibition do not affect breast cancer cell proliferation and anchorage-independent growth in vitro and tumor growth in vivo. Proliferation of some of the other cells examined (including MCF7, HBL100, 67NR, HeLa, NIH3T3, and HT29) was not significantly affected by chronic NButGT treatment. In this study, although GlcNAcylation was suppressed or elevated, the cycling of GlcNAcylation was not absolutely eliminated. These results suggested that the fluctuation of GlcNAcylation, to a certain extent, could not affect the growth of most cell types.

In this study, we showed that GlcNAcylation enhanced cell migration and metastasis mainly through the decrease of cell surface E-cadherin in breast cancer cells. However, the results also indicated that the migration of 4T1-shOGT1/shEcad4 was still weaker than that of 4T1-shCtl/shEcad4 cells and E-cadherin silencing only partly compensated for the inhibition of lung metastasis induced by the OGT knockdown. These results suggested that GlcNAcylation might also enhance breast cancer metastasis through the regulation of other proteins or signaling pathways. Human cancer is widely recognized as an intricate multistep process that involves malfunction in proto-oncogenes, tumor suppressor genes, and other key cellular genes implicated in cell proliferation, differentiation, survival, and genome integrity (26, 27). Several different combinations of gene activation and/or inactivation can be found in the genome of morphologically similar human cancers (27). As a type of posttranslational protein modification, certain functions of GlcNAcylation depend on the changes in function of the proteins as a result of this modification. These results implied that the roles and mechanisms of GlcNAcylation in cancer metastasis are context-dependent and might be influenced by cell type, microenvironment, and oncogenic events acquired during the course of tumor evolution.

E-cadherin, which contributes to epithelial cell-cell adhesion, is the prototype of the cadherin class. The extracellular domain of E-cadherin mediates homophilic cell-cell contact, and the cytoplasmic tail of E-cadherin is highly conserved and binds directly to p120 and β-catenin (21). These cytoplasmic interactions regulate the adhesive function of E-cadherin. The binding of β-catenin is essential for the transportation of E-cadherin to the membrane and for the association of p120 (29–31). Zhu and colleagues showed that the induction of apoptosis by agents that cause endoplasmic reticulum stress results in the GlcNAcylation of newly synthesized E-cadherin; however, the GlcNAcylation of E-cadherin was undetectable in untreated cells (13). GlcNAcylation of E-cadherin blocks cell surface transport, resulting in reduced intercellular adhesion in some apoptotic processes (13). In this study, our results revealed that GlcNAcylation decreased cell surface E-cadherin and suppressed cell-cell adhesion. We found that β-catenin and p120, but not E-cadherin, were GlcNAcylated in 4T1 cells, which might be a novel mechanism underlying the regulation of E-cadherin–mediated intercellular adhesion in normal tissue morphogenesis and maintenance, as well as in human disease states. To clarify this mechanism, the GlcNAcylation sites of β-catenin and p120 need to be determined and the role of site-specific GlcNAcylation needs to be investigated in future studies.

In a previous study, GlcNAcylation levels were measured in 12 matched primary breast tumors and adjacent breast tissue samples (32). The authors claimed that GlcNAcylation was decreased in tumor tissues, especially in proteins in the molecular mass range of 45 to 65 kDa. However, they also pointed out that the GlcNAcylation patterns found in the tumor and adjacent tissue were complex, and that there were more labeled bands in most tumor tissues in some molecular mass range of proteins. In this study, a breast cancer TMA was used to analyze GlcNAcylation in 31 breast cancer tissues and their corresponding adjacent breast tissues by immunohistochemistry. Our findings indicated that the global GlcNAcylation levels were significantly elevated in tumor tissues; moreover, GlcNAcylation was significantly enhanced in the metastatic lymph nodes compared with their corresponding primary tumor tissues. As a dynamic posttranslational modification, GlcNAcylation was very easily removed from the tissue samples during storage and handling. In our study, all the paired breast cancer tissues and the adjacent breast tissues were treated simultaneously, which could prevent the false results from the differential removal of GlcNAcylation. Taken together, the results suggested that GlcNAcylation could be important in promoting malignant transformation in breast cancer and might be a potential marker for the diagnosis of breast cancer.

The "Warburg effect" was described more than 80 years ago as a peculiar condition of cancer cells, which consists of an increase in glycolysis that is maintained in conditions of high oxygen tension and gives rise to enhanced lactate production (33). In addition, cancer cells enhance glucose uptake and use elevated amounts of glucose as a carbon source for anabolic glycolysis. The glucose entering the cell (2–5%) is used to produce UDP-N-acetylglucosamine (UDP-GlcNAc) through the hexosamine biosynthetic pathway (34). Furthermore, OGT catalyzes GlcNAcylation of protein using UDP-GlcNAc as the donor substrate, and its activity is tightly dependent on the concentration of UDP-GlcNAc in the cell. Therefore, the Warburg effect supports our results in theory, the enhanced glucose uptake and metabolism might lead to elevated intracellular GlcNAcylation in cancer tissues. The elevation of UDP-GlcNAc and subsequent GlcNAcylation might be one of the reasons why the Warburg effect constitutes an advantage for tumor progression. Reciprocally, GlcNAcylation, which has been proposed as a nutrient sensor (35), might be involved in the mechanisms leading to the Warburg effect. Consistent with our proposal, a recent report showed that p53 knockdown triggers the Warburg effect and elevates the GlcNAcylation of a number of proteins (including IKKβ); on the other hand, the elevated GlcNAcylation of IKKβ enhances its activity and upregulates aerobic glycolysis (36). Based on these results, we proposed that elevated GlcNAcylation might be universally involved in various types of cancer.
Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Fred R. Miller for providing 4T1 cells, Gerald W. Hart for providing anti-OGT antibody (clone AL28), and William C. Hahn and Kai Simons for providing pLkO.1 vectors.

Grant Support

National Basic Research Program of China (973 Program; 2003CB716402) and National High-tech R&D Program (2007AA09Z418 and 2007A091506).

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Received 05/28/2009; revised 04/04/2010; accepted 05/27/2010; published OnlineFirst 07/06/2010.

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Cancer Res  Published OnlineFirst July 7, 2010.