The Metastasis-Associated Gene CD24 Is Regulated by Ral GTPase and Is a Mediator of Cell Proliferation and Survival in Human Cancer

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Abstract

Ral GTPases are important mediators of transformation, tumorigenesis, and cancer progression. We recently identified the metastasis-associated protein CD24, a glycosyl phosphatidyl inositol–linked surface protein, as a downstream target of Ral signaling by profiling the expression of RalA/B–depleted bladder carcinoma cells. Because CD24 is highly expressed in bladder and many other tumor types, we sought to determine whether CD24 plays an essential role in maintaining the malignant phenotype. Here, we show that loss of CD24 function in cell lines derived from common tumor types is associated with decreased rates of cell proliferation, clonogenicity in anchorage-independent growth, and anchorage-independent proliferation, and survival. We further evaluated CD24 protein expression in a bladder cancer tissue microarray and found that CD24 is an independent prognostic factor for disease-free survival in patients. Taken together, these results are the first to report that the prognostic marker CD24 is an important mediator of tumor growth and survival.

Introduction

Recent reports reveal a compelling role for Ral GTPase signaling in carcinogenesis and tumor progression (reviewed in ref. 1). The Ral family of GTPases, comprising the 85% identical paralogues RalA and RalB, constitutes a distinct group of Ras-related small GTPases that are activated in a Ras-dependent manner via several RalGEFs and signal through several effectors (2). Activation of the Ral pathway is among the most basic requirements for transformation of human cells, and Ras-mediated transformation depends on activation of RalA (3). Extending our prior findings of Ral involvement in cancer cell motility (4), we recently reported antagonistic roles for these two GTPases in cancer cell motility and a cooperative role for both in cancer cell growth (5). An analysis of gene expression in Ral-depleted cells identified CD24 as a putative transcriptional target.6 CD24 is a mucin-like cell surface protein consisting of a short, heavily glycosylated protein core linked to plasma membrane rafts domains via glycosyl-phosphatidylinositol.

Historically, CD24 was developed as a cluster of differentiation (CD) marker of hematopoietic lineages that has been found to be overexpressed in many common malignancies (6) and has been associated with the metastatic phenotype (7). Reported functions for CD24 include leukocyte signal transduction (8), regulation of B-cell apoptosis (9), and leukocyte adhesion (10). Despite the multitude of human tissue studies correlating CD24 expression with malignancy, to date, no report has definitively evaluated whether CD24 is important in maintaining this phenotype or merely a transformation-associated epiphenomenon. Here, we report that CD24 is central to maintaining cancer cell growth, anchorage-independent proliferation, and survival. We further evaluated CD24 protein expression in a bladder cancer tissue microarray and found that CD24 is an independent prognostic factor for disease-free survival in patients. Taken together, these results are the first to report that the prognostic marker CD24 is an important mediator of tumor growth and survival.

Materials and Methods

Cell culture, proliferation and cell cycle assays, and microscopy. All cell lines used are derived from human cancer and maintained as described (American Type Culture Collection, Manassas, VA). Cells were transfected with small interfering RNA (siRNA) for GL2, RalA, RalB, RalA/B, or CD24 as described (5), then quantified daily using Alamar Blue (Biosource, Camarillo, CA). Clonogenic assays for anchorage-independent growth employed cells 24 hours after transfection and evaluated as described (11). For cell cycle analysis, cells were transfected with siRNA, harvested, fixed in ethanol, and stained with propidium iodide. Cells (10,000) were analyzed on a FACS Calibur cytometer using CellQuest Software (Becton Dickinson, San Diego, CA). Actin cytoskeletal visualization and microscopy were as described (11). Our immunoblotting and detection results are the first to report that the prognostic marker CD24 is an important mediator of tumor growth and survival.

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1 American Association for Cancer Research, doi:10.1158/0008-5472.CAN-05-3855

6 G. Oxford et al., in preparation.
from the Lenberg et al. oligonucleotide microarray series of normal kidney and renal cell carcinomas (12) and the Chen et al. cDNA array study of normal liver and hepatocellular carcinoma (13) are publicly available. The human bladder cancer tissue microarray and our immunohistochemistry methods have been described (14). Anti-CD24 (monoclonal; clone 24C02; 1:100 dilution; Lab Vision, Fremont, CA) was applied to tissue microarray sections for 1 hour at room temperature. CD24 expression scoring was 0, 1+ (<10% positive cells), 2+ (10-50% positive cells), and 3+ (>50% positive cells). This was carried out by one pathologist (H.F.F.) blinded to patient outcome.

**Statistical methods.** In vitro and human oligonucleotide microarray studies were analyzed using a two-tailed Student's t test. In microarray studies of CD24 down-regulation with Ral knockdown, we employed a one-tailed t test. In CD24 immunohistochemistry analyses, stage and grade categories with low frequencies were combined with the next higher category of stage or grade. Associations with stage and grade were tested with Pearson's χ² test. The log-rank test was used to compare disease-free survival distributions among levels of expression. Cox proportional hazards models were used to estimate the effect of CD24 on survival time, adjusting for stage and grade, treating CD24 levels as categorical. Plots and analysis were carried out with the SAS software (SAS Institute, Inc., Cary, NC).

**Results and Discussion**

**Ral GTPases regulate CD24 expression.** To identify genes regulated by Ral signaling, we did a transcriptional analysis, using high-density oligonucleotide microarrays, of Ral-depleted UM-UC-3 urothelial carcinoma cells 72 hours after siRNA transfection (Fig. 1A). We observed decreased expression of CD24, minimally in RalA-depleted (80.1% of control; P = 0.33) and RalB-depleted (50.2% of control; P = 0.13) cells but substantially (17.9% of control; P = 0.05) following double knockdown of both RalA and RalB (RalA/B) compared with a GL2 luciferase control non-targeting duplex (Fig. 1B). We confirmed this regulation by real-time RT-PCR in an independent experiment in UM-UC-3 cells (Fig. 1C) and extended it to HeLa cells (Fig. 1C), a commonly studied cancer cell line. RalA/B knockdown led to decreased CD24 on the level of protein as well, as assayed by immunoblotting in both UM-UC-3 and HeLa (Fig. 1D). CD24 appears as a wide band of varying molecular weights (~30-60 kDa) due to the presence of multiple glycoforms, as reported before (6). Although the specific transcriptional mechanism of Ral regulation of CD24 remains unknown, Ral has been shown to signal to transcription through signal transducers and activators of transcription, Jun, nuclear factor-κB, AFX, and TCF pathways, (reviewed in ref. 15). Our data suggest that transcriptional targets of Ral signaling, such as CD24, should not be overlooked in the search for “drugable” or otherwise therapeutically viable targets for cancer treatment.

**CD24 plays a role in cell proliferation in vitro.** To develop siRNA for CD24, we initially used a pool of four siRNAs to evaluate effects of CD24 depletion. We then tested four individual duplexes and identified two nonoverlapping siRNAs that consistently reduced growth. We finally selected one of these duplexes exhibiting effective CD24 depletion for further experiments and verified that this duplex did not substantially induce protein kinase R, a surrogate for the induction of an IFN response (ref. 16; data not shown).

We observed substantial knockdown of CD24 protein in a panel of tumor cell lines from common epithelial human cancers, including UM-UC-3 urothelial carcinoma cells, DU145 prostate carcinoma cells, HeLa cervical adenocarcinoma cells, MCF-7 breast adenocarcinoma, and SAOS-2 osteosarcoma cells (Fig. 2A-E, insets). Depletion of CD24 by siRNA ranged from nearly complete in UM-UC-3 and DU145, and HeLa to partial in the cells with the highest CD24 expression, MCF-7, and SAOS-2. Following siRNA-mediated depletion of CD24 in these five cell lines, we observed significant...
decreases in cell number. Specifically, over a 4-day period, the
decreases in number compared with GL2 control ranged from 26%
to 68% and in all cases were significant (Fig. 2A-E). This
observation of reduced in vitro proliferation in five cell lines
derived from diverse cancer types underscores an important and
fundamental role for CD24 in cellular proliferation of cancers
derived from both epithelial and mesenchymal origins.

**CD24 knockdown modulates clonogenicity of cancer cells in soft agar.** As anchorage-independent growth is one hallmark of
tumorigenicity and metastatic competence, we used siRNA for CD24 to determine if UM-UC-3 and DU145 cells are dependent on
CD24 expression for their clonogenicity in agar. In UM-UC-3, we
observed a ~75% reduction (P < 0.01) in colony formation at 15
days, whereas in DU145, we observed a ~73% reduction (P < 0.001)
in colony formation at 25 days compared with the same cells
transfected with GL2 (Fig. 2F).

**Knockdown of CD24 is associated with significant effects on the actin cytoskeleton and induction of apoptosis.** We recently
reported the observation that depletion of RalA/B in UM-UC-3 cells
was associated with a significant decrease in stress fibers in
adherent cells (5). Interestingly, in keeping with a role for CD24 as a
Ral transcriptional target, depletion of CD24 was also associated
with a significant change in cell morphology and the actin
cytoskeleton. In phase contrast, we observed control-transfected
cells growing to confluence (Fig. 3A) and displaying epithelial
morphology identical to untreated cells at 72 hours (data not shown). At 48 hours (Fig. 3B) and 72 hours (Fig. 3C), CD24 siRNA-
treated cells seemed more flat and rounded and exhibited unusual
phase-dark radial protrusions. By 72 hours, CD24 siRNA-treated
cells began to round up and lose adhesion to the substratum.
Floating cells were evident in culture (data not shown). We stained
for actin stress fibers in CD24 knockdown cells to ask whether
CD24 depletion also resulted in modulation of the actin
cytoskeleton. By fluorescence microscopy, at 72 hours, the GL2
control siRNA-treated cells displayed prominent stress fibers
(Fig. 3D), as described before (5). However, in CD24 siRNA-treated
cells at 48 hours (Fig. 3E) or 72 hours (Fig. 3F), cells showed
increasingly disordered morphology, loss of stress fibers, rounding,
and nuclear condensation evident in Hoechst 33342 nuclear
staining at 72 hours, suggestive of apoptotic induction.

Having observed nuclear condensation microscopically, we did
cell cycle analysis using propidium iodide staining and flow
cytometry on UM-UC-3 and DU145 cells 96 hours after transfection
to assess the proportion of cells undergoing apoptosis as a result
of CD24 depletion and subsequent cytoskeletal rearrangements.
Apoptosis, as defined by a sub-G0-G1 hypodiploid population in
CD24 siRNA-treated samples of these cell lines (Fig. 3G and H),
occurred in 12.2% and 13.2% of UM-UC-3 and DU145 cells,
respectively, compared with GL2-treated controls (in both cell lines <1%). Taken together with the effects on agar growth, these results suggest that the effects of CD24 on the metastatic competence are likely mediated in part by its effects on survival.

**CD24 protein expression in human cancer tissue is a prognostic factor for survival.** CD24 overexpression has been reported in many tumor types (6). As we observed a striking *in vitro* phenotype following CD24 depletion in cell lines derived from breast and prostate cancer, in two common malignancies, where CD24 immunohistochemistry has been proven to be of prognostic value (6), we investigated a potential association of CD24 expression with bladder cancer.

Using previously generated microarray data (11), we found an ~4.4 fold overexpression of CD24 in bladder cancer compared with normal mucosa (P = 0.01; Fig. 4A). We also analyzed other publicly available microarray data sets to ascertain if CD24 is a biomarker for tumor types not yet reported. Using the Lenberg et al. oligonucleotide microarray series of renal cell carcinomas and normal kidney tissue (12), we note that CD24 is ~3.3-fold overexpressed in renal cell carcinoma (P < 0.01). In the Chen et al. cDNA microarray series of hepatocellular carcinomas and human liver tissue samples that are publicly available (13), we find that CD24 is overexpressed in hepatocellular carcinoma compared with normal liver (P < 0.01).

Based on these results, we carried out immunohistochemistry for CD24 on a tissue microarray of human bladder cancer patients (14). Although we found no statistically significant associations between clinical stage or grade with CD24 expression, adjustment for differences in stage and grade through multivariate analyses showed that patients with higher levels of CD24 (CD24, 3+) tended to have shorter disease-free survival times than those with lower levels (CD24, 1+), approaching significance even in our small cohort (P = 0.07). Figure 4B shows representative histologic sections for levels of CD24 staining, and Fig. 4C shows Kaplan-Meier estimation of disease-free survival time by CD24 expression. This observation that CD24 is an independent prognostic factor for bladder cancer disease-free survival is striking. It further extends the breadth of the prognostic use of CD24 immunohistochemistry to bladder cancer, in addition to breast, prostate, lung (6), and colon (17) cancers, which together comprise a vast majority of solid malignancies.
malignancies diagnosed in the United States (18). Furthermore, as most patients with bladder cancer die of metastatic disease, this supports the notion that CD24 is an important contributor to metastatic competence.

A model for CD24 function. To date, the functions ascribed to CD24 include induction of apoptosis (9), localization with lipid rafts (8, 9), and activation of tyrosine kinase signaling (8), assayed via inactivating approaches using extracellular antibody-mediated crosslinking. However, as study authors have noted (9), the physiologic correlate or specificity to CD24 function of these methods is unknown. Overexpression experiments are also limited, as invasive cancer cell lines may either not change their phenotypes due to limiting numbers of signaling intermediates or alter them in a potentially artifactual way. Here, we overcome these limitations by using a specific, reverse genetic approach to deplete CD24 expression and characterize effects in cell lines from a diverse set of cancer types.

Friederichs et al. recently reported that CD24 could function as a cell adhesion molecule in a murine tail vein model of metastasis (7). In this study, CD24, as a ligand for P-selectin, mediated pulmonary arrest of adenocarcinoma cells when stably cotransfected with an appropriate fucosyltransferase for sialylLe\(^\text{a}\) modification. A priori, this metastatic adhesive function alone could potentially explain the clinical prognostic associations of CD24. However, our findings of a CD24 role in proliferation and survival of cancer cells as well as the observations here and elsewhere (6) of overexpression of CD24 at many primary tumor sites support a role in transformation and tumorigenesis. Interestingly, CD24 has been shown to modulate integrin function, including LFA-1, or \(\alpha_\text{L} \beta_2\) (19) and VLA-4, or \(\alpha_4 \beta_1\) (20), in leukocyte adhesion studies. Cell death in response to loss of integrin-mediated cell-matrix adhesion has been termed “anoikis,” and recent reports find that “anoikis resistance” may be an essential property of metastasis-competent cells (reviewed in ref. 21). Given our actin cytoskeletal findings in CD24-depleted cells, we are currently investigating whether CD24 functions through regulating integrin-mediated survival signals in cancer cells. Taken together, these data support a dual-functional model for CD24 in cancer: both in proliferation and survival at the tumor origin and metastases as well as adhesive function during hematogenous dissemination of cancer cells. Clearly, in vivo systems employing cells stably expressing RNA interference for CD24 are necessary to address the relative contribution of CD24 to tumorigenesis versus metastasis.

In summary, our results suggest a novel and important role for CD24 in the regulation of proliferation and survival of cancer cells. We have shown that CD24 is regulated by Ral GTPases, which

Figure 4. CD24 in human bladder cancer. A, Affymetrix analysis expression analysis of CD24 mRNA shows fold overexpression in bladder cancer compared with normal bladder mucosa. Columns, fold change; bars, SE. B, tissue microarray staining for CD24. Representative scoring of 1+, 2+, and 3+ levels. C, Kaplan-Meier estimation of disease-free survival as a function of CD24 immunohistochemical expression staining.
suggests that CD24, and potentially other transcriptional targets of Ral signaling, may mediate important functional aspects of this GTPase subfamily that is emerging as a central player in cancer. This report of a causal role for CD24 in growth and survival of many cancer cells of various histologic types, as well as the apparent generality of CD24 overexpression in different tumors, highlights the need to strongly consider this molecule as a therapeutic target.

References

Acknowledgments
Grant support: NIH grant CA075115 (D. Theodorescu) and Medical Scientist Training Program training grant T32GM007267 (S.C. Smith).

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We thank Drs. Mikael Herlevsen, Martin Schwartz, Yongde Bao, Charles Owens, and Joanne Lannigan for discussions and assistance and Dr. Peter Altevogt for the SWA11 monoclonal antibody.
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