Published in final edited form as:

Int J Gynecol Cancer. 2013 May ; 23(4): 615–621. doi:10.1097/IGC.0b013e31828b4eb5.

Ribonucleotide Reductase Expression in Cervix Cancer: a Radiation Therapy Oncology Group Translational Science Analysis

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Abstract

Objective—To evaluate pretherapy ribonucleotide reductase (RNR) expression and its effect upon radiochemotherapeutic outcome in women with cervical cancer.

Methods/Materials—Pretherapy RNR M1, M2, M2b immunohistochemistry was done on cervical cancer specimens retrieved from women treated on Radiation Therapy Oncology Group (RTOG) 0116 and 0128 clinical trials. RTOG 0116 enrollees (node-positive stage I_A-IV_A) received weekly cisplatin (40mg/m²) with amifostine (500mg) and extended-field radiation then brachytherapy (85Gy). RTOG 0128 enrollees (node-positive or bulky 5cm stage I_B-II_A, or stage II_B-IV_A) received day 1, 23, 43 cisplatin (75mg/m²), 5-FU (4-day 1gm/m²) during pelvic radiation then brachytherapy (85Gy), plus celecoxib (400mg twice daily, day 1 through 1 year). Disease-free survival (DFS) was estimated univariately by the Kaplan-Meier method. Cox proportional hazards models evaluated the impact of RNR immunoreactivity on DFS.

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CONFLICT OF INTEREST: There are no potential conflicts of interest among the authors and this manuscript. This manuscript has been seen, read, and agreed upon in its content by all designated authors. This manuscript has not been submitted or published elsewhere.

Results—51 tissue samples were analyzed; 13 from RTOG 0116 and 38 from RTOG 0128. M1, M2, and M2b overexpression (3+) frequencies were 2%, 80%, and 47%, respectively. Low-level (0–1+, n=44/51) expression of the regulatory subunit M1 did not associate with DFS (p=0.38). High (3+) M2 expression occurred in most (n=41/51), but without impact alone upon DFS (hazard ratio (HR): 0.54, 95% confidence interval [CI]: 0.2–1.4; p=0.20). After adjusting for M2b status, pelvic node-positive women had increased hazard for relapse or death (HR: 5.5, 95% CI: 2.2–13.8; p=0.0003).

Conclusions—These results suggest that RNR subunit expression may discriminate cervical cancer phenotype and radiochemotherapy outcome. Future RNR biomarker studies are warranted.

Keywords

ribonucleotide reductase; radiosensitivity; cervical cancer

Introduction

Cervical cancer — which is commonly positive for human papillomavirus (HPV) and abnormal p53 signaling — is an aggressive malignancy marked by higher rates of incomplete radiochemotherapeutic response and poorer disease-specific survival if ribonucleotide reductase (RNR) is overactive.^{1–6} Women whose cervical cancers respond poorly to radiochemotherapy have a median survival of 2 years.^{7, 8} Cancer biomarkers able to predict response to standard-of-care cisplatin radiochemotherapy are greatly needed.

As an important supplier of deoxyribonucleotide diphosphates (dNDPs) used as building blocks for DNA, RNR has emerged as a therapeutic target in cervical cancer.⁹ Human cells have three non-identical RNR subunits — one homodimeric subunit α (M1) holding catalytic sites and two small homodimeric subunit β alternatives, M2 or p53-inducible M2b (a.k.a., p53R2), harboring tyrosyl free radicals vital to catalysis. RNR in its M1-M2 form supplies dNDPs for S-phase-specific DNA replication. RNR in its M1-M2b form supports cell-cycle independent dNDP demands and DNA damage responses. Regulation of RNR catalytic activity in resting cells and in cycling cells is limited by differential expression and degradation of M1, M2, and M2b.¹⁰

Although the full implications of overactive RNR are still under investigation, preclinical models show cervical cancer cells have an increased capacity to produce dNDPs through RNR, to repair radiochemotherapy-induced damaged DNA, and to survive radiochemotherapy treatment.^{1–4} Pharmacologic inhibition of RNR lowers catalytic activity, protracts the repair of damage done by radiochemotherapy, and provokes cell death.^{1–4} The cytotoxic effect of RNR inhibition after DNA damage likely exploits a time critical, unmet high dNDP demand with blocked DNA precursor supply.⁹ In prior clinical trials, inhibitors of RNR (*i.e.*, hydroxyurea, 5-fluoruracil, and gemcitabine) have improved radiochemotherapy response.^{11–15} More potent inhibitors of RNR (*e.g.*, 3-aminopyridine-2-carboxaldehyde thiosemicarbazone) further improve radiochemotherapeutic responses.^{5, 6}

One unanswered question is how RNR subunits influence radiochemotherapeutic outcome in women with cervical cancer. One possible answer might be that the subunits facilitate fixing of damaged DNA and suppress the death-provoking effects of radiochemotherapy. This retrospective study was designed to evaluate whether pretherapy RNR M1, M2, and M2b immunohistochemical overexpression was associated with shortened disease-free and overall survival in two clinical trials conducted by the Radiation Therapy Oncology Group (RTOG).

Materials and Methods

Cervical cancer tissue samples

Formalin-fixed, paraffin embedded (FFPE) sections of human uterine cervix cancer tissue were obtained from the RTOG Biospecimen Resource bank (University of California San Francisco, San Francisco, CA) from 13 women with node-positive stage IA-IVA cervical cancer enrolled in RTOG 0116¹⁶ and from 38 women with node-positive or bulky 5cm stage IB-IIA, or stage IIB-IVA from RTOG 0128.¹⁷ Pathologists confirmed diagnoses for cervical cancer and provided quality assurance of the tissue slides and microarrays (FAK, DD).

Patient population

RTOG 0116 enrollees received once weekly cisplatin (40 mg/m²) and extended-field radiation (45 Gy), followed by brachytherapy (40 Gy). Amifostine (500 mg) was given daily on cisplatin and non-cisplatin days, dosing in two equally-divided subcutaneous administrations. A four-field or two-field opposed anteroposterior (AP/PA) extended-field radiation technique was applied.¹⁶ AP/PA portals encompassed T11/T12 interface superiorly and the obturator foramen or 3 cm below disease extension in the vagina inferiorly. Fields included 2 cm wider than the true pelvis with custom blocking to shield bowel, while maintaining at least 1 cm margin on pelvic and para-aortic nodal tissue. The anterior border of lateral fields was 1 cm anterior to the common iliac nodes. The posterior border of lateral fields split T12 to sacral vertebral bodies, maintaining a 3 to 4 cm margin on cervical cancer disease. Brachytherapy prescribed to point A was delivered by low-dose-rate (LDR) or high-dose-rate (HDR) intracavitary systems, with tandem and ovoid applicators preferred. Interstitial brachytherapy was allowed to treat vaginal disease.

RTOG 0128 enrollees received day 1, 23, 43 cisplatin (75 mg/m²), 5-FU (4-day 1 gm/m²) during pelvic radiation (45 Gy), followed by brachytherapy (40 Gy). Celecoxib (400 mg twice daily) was given day one through one year. A four-field pelvic radiation technique was done.^{17, 18} AP/PA portals set the superior border at the L4/L5 interface and the lower border at a transverse line below the obturator foramen. The lateral border was 2 cm wider than the widest true pelvis diameter. On lateral portals, the anterior border was positioned in front of the symphysis pubis at least 1 cm anterior to the common iliac nodes. The posterior border included the entire sacrum ensuring 3 cm margin posterior to the greatest extent of cervical disease. LDR or HDR intracavitary brachytherapy prescribed to point A was done using tandem and ovoid applicators, with allowance of interstitial brachytherapy for treatment of vaginal disease.

Immunohistochemistry

Immunohistochemistry was done as previously described^{5, 19} using commercial, high-relative specificity antibodies targeting RNR M1, M2, and M2b proteins.^{20, 21} Cell nuclei were stained blue by hematoxylin (Biocare Medical, Concord, CA). RNR M1 (rabbit polyclonal [ab81085], Abcam, Cambridge, MA) and RNR M2 (mouse monoclonal [ab57653], Abcam) were both used at 1 µg (1:200) in 199 µL volume of 0.25% casein in phosphate-buffered saline (PBS, Dako North America, Inc., Carpinteria, CA). RNR M2b (rabbit polyclonal [ab8105], Novus Biologicals, Littleton, CO) was used at 1 µg (1:250) in 249 µL volume of 0.25% casein in PBS. A biotin-free probe (MACH 4), followed by a horseradish peroxidase that binds to the MACH 4 probe, allowed mouse and rabbit antibody detection by producing a brown precipitate. Stained slides were viewed on an inverted microscope (Olympus, Center Valley, PA) at 2.5× and 20× magnifications. Two independent pathologists (DD, FAK), blinded to treatment and outcome, scored the brown color staining intensity of M1, M2, and M2b on a 0 (< 5% of cells), 1 (5% to < 25%), 2

(25% to < 75%), and 3 (> 75%) scale.⁵ Discrepancies were reviewed and resolved to a single score. Because M1 and M2b are expressed in cells at low levels throughout the cell cycle,¹⁰ brown color intensities of 2+ and 3+ arbitrarily identified M1 and M2b subunit overexpression. M2 is typically restricted to S-phase of the cell cycle and preclinical *in vitro* cervical cancer cell observations suggest that it is commonly overexpressed,¹ and so, a brown color intensity of 3+ indicated M2 overexpression in this study.

Statistics

A secondary analysis of RTOG 0116 and RTOG 0128 was done to evaluate the potential associations of RNR expression with overall (OS) and disease-free survival (DFS). An OS event was death due to any cause and was measured from the date of randomization to date of death or last follow-up for censored patients. A DFS event was defined as local, regional, para-aortic, or distant disease relapse as well as death due to any cause. DFS was measured from the date of randomization to date of first relapse or last follow-up in censored patients. Univariate product-limit estimates with 95% confidence intervals (CI) were tabulated by the method of Kaplan and Meier²² and RNR staining intensity subgroups were compared using the log-rank test. Cox proportional hazards models were used to adjust for variables potentially associated with outcome.²³ Due to the small number of OS and DFS events, only two-variable multivariate Cox proportional hazards models were run. Variables included in the hazards model included age (continuous), International Federation of Gynecology and Obstetrics (FIGO) stage (I_A-II_B v. III_A-IV_A), tumor stage (T₃ or T₄ v. other), pelvic or iliac lymph node positive disease (yes v. no), para-aortic lymph node positive disease (yes v. no), Zubrod performance status (0 v. 1 or 2) and separate variables for RNR M1 (0-1+ v. 2-3+), M2 (0-2+ v. 3+), and M2b (0-1+ v. 2-3+) staining intensity.

Results

Patients

RTOG 0116 accrued 45 patients between August, 2001, and March, 2007. Of these 45 patients, 13 were eligible and had cervical cancer tissue banked for immunoreactivity studies. RTOG 0128 enrolled 84 patients between August, 2001 and March 2004. Of these 84 patients, 38 patients were eligible and had tissue banked for immunohistochemical analysis. Two pathologists agreed that a total of 51 histopathological specimens had suitable quality and sufficient quantity of malignant cervical cancer available to be included in this analysis (Fig. 1). All analyzed cervical cancer tissue was obtained prior to radiochemotherapy. Patient demographic and efficacy were not statistically significantly different between the patients with and without suitable cervical cancer tissue. Patient demographic and tumor variables were not statistically significantly different between the RNR M1 (0-1+ v. 2-3+), M2 (0-2+ v. 3+), and M2b (0-1+ v. 2-3+) staining intensity subgroups. Patient demographic and tumor variables for the 51 patients included in the analysis are listed in Table 1.

Median follow-up for all patients is 24 months (minimum-maximum, 2 to 44 months). Thirteen (25%) local and/or regional disease relapses have been reported. Fifteen deaths have been recorded. Eight (16%) patients included in this analysis did not receive all of the indicated clinical trial therapy. Two patients on RTOG 0116 did not complete amifostine (patients refused); 3 patients on RTOG 0128 did not complete radiation or chemotherapy (1 due to progression and 2 patients refused) and did not receive brachytherapy; and 3 additional patients on RTOG 0128 did not complete chemotherapy (due to toxicities), but received radiation and brachytherapy.

RNR M1 expression

Human cells need a balanced supply of dNDPs for nuclear and mitochondrial DNA replication and repair.¹⁰ RNR M1 has a long half-life, allowing it to be detected in all phases of the cell cycle.^{24, 25} To test whether undamaged cervical cancer cells expressed RNR M1 at low levels, RNR M1 was assessed immunohistochemically (Fig. 2). The pretherapy expression of RNR M1 in the cytosol was low (0–1+) in the majority of cervical cancers (44 [86%] of 51, Table 2). RNR M1 overexpression (2–3+) was not associated with an increased incidence of pelvic ($p = 0.17$) or para-aortic ($p = 0.33$) lymph node metastases at cervical cancer diagnosis (Fig. 3). There were no deaths, and two (29%) disease-related events in seven patients with RNR M1 overexpression. In this dataset (Table 3), high (2–3+) levels of RNR M1 were not associated with shorter DFS (log-rank $p = 0.38$) or OS (log-rank $p = 0.11$). RNR M1 expression was not associated with DFS in any of the two-variable Cox models.

RNR M2 expression

RNR M2 expression is tightly regulated and typically restricted to the S-phase of the cell cycle.¹⁰ Cells enlist an anaphase promoting complex-Cdh1-mediated degradation of the RNR M2 protein in late mitosis.^{26, 27} To answer the question of whether cervical cancer cells would have elevated RNR M2 levels pretherapy, RNR M2 immunoreactivity was investigated. Viral or mutational silencing of p53 in cervical cancers eliminates the G1/S cell cycle checkpoint, resulting in a compensatory rise in RNR M2.¹ For the purposes of this immunohistochemical analysis and based on these preclinical *in vitro* cervical cancer cell observations, RNR M2 3+ immunoreactivity was arbitrarily deemed as the clinically meaningful level of overexpression. A high 3+ level of RNR M2 expression seen in the cytosol occurred in most (41 [80%] of 51) cervical cancers (Fig. 2, Table 2). Pelvic ($p = 0.43$) or para-aortic ($p = 0.35$) lymph node metastases at diagnosis were not statistically significantly more common when RNR M2 expression was high (3+, Fig. 3). Of those patients that had relapse or death, more patients had RNR M2 overexpression (3+) (16/22=73%) than had RNR M2 (0/1/2+) immunoreactivity (6/22=27%). Of those patients that died, more patients had RNR M2 overexpression (3+) (10/15=67%) than had RNR M2 (0/1/2+) immunoreactivity (5/15=33%). Despite these findings, RNR M2 overexpression (3+) was not statistically associated with lower estimate of DFS ($p = 0.19$) or OS ($p = 0.07$, Table 3). After adjusting for M2 status, pelvic node-positive women were 4.7 (95% CI: 1.9–11.4) times more likely to relapse or die ($p = 0.0006$).

RNR M2b expression

Low levels of RNR M2b can be detected throughout the cell cycle, and induced expression occurs logarithmically after DNA damage in p53/p73-dependent and independent manners.^{2, 10, 28} In some ways, RNR M2b can be considered a DNA damage response element.⁹ Constitutively expressed RNR M2b (*a.k.a.*, p53R2) is held in check by a protein-protein bond with p53.²⁹ It has been suggested that phosphorylated p53 or mutated p53 cannot interact with M2b, allowing the possibility of immediate supply of dNTPs when nuclear or mitochondrial DNA is damaged.^{10, 30} This raises the possibility that cervical cancers with high expression (2–3+) of RNR M2b could facilitate immediate DNA damage repair after radiochemotherapy exposure. To evaluate the association of overexpression of M2b and cancer outcome, immunohistochemical analyses of cervical cancers were conducted. The high (2–3+) level of RNR M2b in the cytosol was seen in more than half (30 [59%] of 51) of cervical cancers (Fig. 2, Table 2). Pelvic ($p = 0.26$) or para-aortic ($p = 0.28$) lymph nodes were not more common when a high (2–3+) level of RNR M2b was detected (Fig. 3). Of the 22 patients with relapses or deaths, high (2–3+) levels of RNR M2b were more common ($n = 14$, 64%). This did not translate into a statistically significantly shortened DFS in univariate ($p = 0.69$) analysis (Table 3). OS was also not associated with

RNR M2b overexpression in univariate analysis ($p = 0.16$). After adjusting for M2b status, pelvic node-positive women had an increased hazard for relapse or death (HR: 4.9, 95% CI: 2.0–12.0; $p=0.0005$).

Discussion

These data indicate that pretherapy expression of RNR M1, M2, and M2b is informative. Expression of the M1 regulatory subunit appears low in the majority of women with cervical cancer (Fig. 2), and even after its overexpression (2–3+), the radiochemotherapeutic control of such cervical cancers was not dampened. These data agree with an earlier contention that most cervical cancers universally have high (3+) levels of the normally S-phase restricted M2 subunit (Fig. 2), perhaps indicating either a high cell growth fraction or enhanced capacity to repair DNA damage.⁹ Even the naturally p53-regulated M2b subunit is elevated (2–3+) in many cervical cancers (Fig. 2), presumably due to viral or mutational modification of a p53 protein-protein interaction with M2b.

What function does the supply and demand economics of dNDPs have in cells? It must be essential, because fluctuating dNDP pools (and by association, deoxynucleoside triphosphate [dNTP]) are harbingers of genotoxic stress and cell death.³¹ HPVs, as DNA viruses, hijack cellular machinery to increase dNDP supply for replication.³² Thus, it is no surprise to find that M2 and M2b levels are high in cervical cancer cells, which commonly show HPV DNA integration. Because no alternative RNR M1 protein has been detected in cells, an abundance of M2 and M2b proteins would likely saturate M1 proteins to form functional RNR. In this state, dNDP overproduction could occur outside of the S-phase of the cell cycle. Since radiochemotherapy damaged cells demand dNDPs to fix DNA (2) and cervical cancers show RNR M2 and M2b overexpression (Fig. 2), it is reasonable to suggest that a proportion of patients with cervical cancer would have a less than optimal radiochemotherapy response. Indirect evidence for this has been shown among women with stage I_{B2} cervical cancer randomly allocated to radiation plus hysterectomy or to radiochemotherapy plus hysterectomy.⁷ In this trial, women whose tumors exhibited a poor pathological response (> 10% viable cells in hysterectomy specimens) had a higher rate of extrapelvic recurrence and hazard for disease progression or death. Whether the women whose cervical cancers poorly responded to radiochemotherapy or to radiation occurred as a consequence of RNR subunit overexpression is not known.

To fill in this particular knowledge gap, pretherapy cervical cancer tissues obtained in RTOG 0116 and RTOG 0128 patient cohorts were studied because immunohistochemical analyses could be reliably conducted and treated patients had a relatively high risk for posttherapy cancer-related events with which to associate RNR immunoreactivity. Overexpression of RNR M1 (2–3+), M2 (3+), and M2b (2–3+) did not correlate with shortened OS or DFS (Table 3). However, multivariate analyses showed that after adjusting for high M2 or M2b immunoreactivity, node-positive women were at an increased hazard for cervical cancer recurrence or death (Fig. 3). In previous studies of RTOG 0128 tissues, dynamic changes in M2b expression occurred in parallel with alteration in expression patterns of cell cycle or DNA replication and repair genes.^{33, 34} More data are needed to sort out the pretherapy molecular biomarkers reassuringly predicting patients who may responder to radiochemotherapy. This is especially true in an era where pharmacological inhibitors of RNR have shown outstanding anticancer potential.^{5, 6}

This analysis would have been strengthened by study of a uniformly treated patient cohort. In this analysis, women enrolled on RTOG 0116 received once weekly cisplatin (40 mg/m²) and extended-field radiation (45 Gy) then brachytherapy (40 Gy), while women enrolled on RTOG 0128 received day 1, 23, 43 cisplatin (75 mg/m²), 5-FU (4-day 1 gm/m²) during

pelvic radiation (45 Gy) then brachytherapy (40 Gy). Since the impact of pretherapy RNR subunit expression on subsequent cancer outcome was of interest, it was necessary to consider the two investigated treatment regimens to be similar in order to incorporate both studies in our analysis. Efficacy of these two approaches has not been compared head-to-head, and so, similar treatment effect was assumed. Lastly, celecoxib was given from day one through one year to patients enrolled on RTOG 0128. Celecoxib exerts its off-target anticancer effect perhaps through inhibition of anti-apoptosis molecules such as PDK1 and its downstream effector AKT1.³⁵ Unknown molecular adaptations to prolonged celecoxib exposure may bias the interpretation of RNR's impact upon DFS and OS.

In conclusion and despite its limitations, this study provides a much needed proof-of-concept that pretherapy cervical cancer RNR M1, M2, and M2b expression is informative. On the basis of these results, prospective collections of uterine cervix tissue adequately powered to study RNR immunoreactivity, DFS, and OS are being considered.

Acknowledgments

SOURCES OF SUPPORT: This project was supported by RTOG grant U10 CA21661, CCOP grant U10 CA37422, and RTOG Biospecimen Resource grant U24 CA114734 from the National Cancer Institute (NCI) and 2010 Pennsylvania Department of Health Formula Grant 4100054841. This manuscript's contents are solely the responsibility of the authors and do not necessarily represent the official views of the National Cancer Institute.

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Figure 1. STROBE diagram for progress through stages of analysis. *STROBE*: Strengthening the Reporting of Observational Studies in Epidemiology.

Figure 2.
Ribonucleotide reductase M1, M2, and M2b immunoreactivity in uterine cervix cancers.
Depicted are examples of brown color staining intensity for M1 (A 1+, B 3+); M2 (C 1+, D 3+); and M2b (E 1+, F 3+). Magnification is indicated.

Figure 3.

RNR M1, M2, and M2b immunoreactivity in cancer tissues and incidence of metastases to pelvic lymph nodes (A) and to para-aortic lymph nodes (B) at diagnosis.

Table 1

Characteristics of patients included in this study from RTOG 0116/0128 (n = 51)

Characteristic		
Age (years)		
	Median	47
	Minimum — Maximum	30 — 69
FIGO stage		
	I _A -II _B	40 (78%)
	III _A -IV _A	11 (22%)
Tumor stage		
	Other	40 (78%)
	T ₃ or T ₄	11 (22%)
Pelvic / Iliac lymph node positive		
	Yes	14 (28%)
	No	37 (73%)
Para-aortic lymph node positive		
	Yes	9 (18%)
	No	42 (82%)
Zubrod performance status		
	0	32 (63%)
	1 or 2	19 (37%)

* Percentages may not add to 100% due to rounding.

Table 2

Ribonucleotide reductase M1, M2, and M2b expression

Group	0+ No. (%)	1+ No. (%)	2+ No. (%)	3+ No. (%)
M1	30 (59)	14 (27)	6 (12)	1 (2)
M2	3 (6)	2 (4)	5 (10)	41 (80)
M2b	6 (12)	15 (29)	6 (12)	24 (47)

No. = number

Table 3

Disease-free and overall survival by ribonucleotide reductase expression

Group	3-year disease-free survival*	3-year overall survival*
M1		
0-1+	54% (CI: 38% — 67%)	60% (CI: 40% — 75%)
2-3+	57% (CI: 08% — 89%)	100% (CI: N/A)
P value	0.38	0.11
M2		
0-2+	38% (CI: 10% — 66%)	47% (CI: 15% — 74%)
3+	57% (CI: 38% — 72%)	66% (CI: 40% — 83%)
P value	0.19	0.07
M2b		
0-1+	62% (CI: 38% — 79%)	61% (CI: 36% — 78%)
2-3+	49% (CI: 27% — 67%)	62% (CI: 27% — 84%)
P value	0.69	0.16

* CI denotes 95% confidence interval. N/A: not applicable.