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Evaluation of mRNA by Q-RTPCR and Protein Expression by AQUA of the M2 Subunit of Ribonucleotide Reductase (RRM2) in Human Tumors

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Abstract

Purpose—The purpose of this study was to evaluate baseline RRM2 protein and gene expression in tumors of patients receiving 3-AP.

Experimental Design—Tumor blocks from patients enrolled in phase I and II clinical studies using 3-AP, were evaluated for RRM2 gene and protein expression by quantitative real time polymerase chain reaction (Q-RTPCR) and automated quantitative analysis, (AQUA).

Results—Esophageal and gastric cancers overexpressed RRM2 protein when compared to prostate cancer (Z-score, 0.68 ± 0.94 SD, vs 0.41 ± 0.84 SD, respectively,; $p=0.04$). Esophageal and gastric cancers also overexpressed RRM2 mRNA when compared to prostate cancer (relative gene expression 2.56 ± 1.49 SD, vs 0.29 ± 0.20 SD, respectively,; $p=0.02$). Protein and gene expression were moderately associated (Spearman's rank correlation =0.30; $p=0.12$).

Conclusion—RRM2 gene and protein expression varies by tumor type and may predict response to 3-AP.

Keywords

Triapine®; 3-aminopyridine-2-carboxaldehyde thiosemicarbazone; ribonucleotide reductase; automated quantitative immunohistochemistry (AQUA); quantitative real time PCR

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Introduction

Ribonucleotide reductase (RR) is the enzyme that catalyzes the conversion of ribonucleoside 5'-diphosphates into 2'-deoxyribonucleotides (1). RR consists of one regulatory subunit, RRM1, and one catalytic subunit, RRM2. Together, RRM1 and RRM2 form the catalytically active RR enzyme. RRM1 has binding sites for ribonucleotides, and its expression in proliferating cells remains consistent throughout the cell cycle. RRM2 contains a tyrosyl-free radical that is stabilized by a non-heme iron center, which is essential for ribonucleotide reduction and conversion of nucleotides to deoxynucleotides (2). This conversion is a rate-limiting step in the production of 2'-deoxyribonucleoside 5'-triphosphates that are necessary for DNA replication, making RRM2 essential in DNA synthesis.

Overexpression of RRM2 is associated with increased cell proliferation (3) and malignant potential in certain cancers and inhibition of RRM2 reduces cellular proliferation *in vitro* and *in vivo* (4,5). RRM2 interacts with a variety of oncogenes which promotes tumor progression (6,7), enhances the invasiveness of cancer cells (8), reduces radiosensitivity in human solid tumors (9) and increases the drug-resistant properties of cancer cells to various chemotherapeutic reagents, including hydroxyurea and gemcitabine (10-13). Therefore, inhibition of RRM2 is a potential therapeutic target for new anticancer agents. Triapine[®] (3-aminopyridine-2-carboxaldehyde thiosemicarbazone, 3-AP, Vion Pharmaceuticals, Inc., New Haven, CT), is a novel small molecule inhibitor of RRM2 that is being evaluated in Phase I and II clinical trials. In this study, we hypothesized that baseline tumor RRM2 expression varies by tumor and RRM2 expression may be used to identify tumors sensitive to 3-AP.

Materials and Methods

Formalin-fixed paraffin-embedded (FFPE) tissue blocks were obtained from 40 of the 43 patients with locally advanced, unresectable or metastatic solid tumors who participated in three clinical trials at the University of Wisconsin: [a phase I combination of 3-AP and doxorubicin (14), a phase I combination of 3-AP and irinotecan (15) and a phase II single agent study of 3-AP in pancreatic cancer (16)]. There were thirteen tissue blocks from patients with pancreatic cancer (four from primary sites and nine from metastatic sites). Other tissue types included: one primary bladder cancer; one primary cervical cancer; four cholangiocarcinoma (one primary, three metastatic); two primary colon cancers; three primary esophageal cancers, one primary gastric cancer, one metastatic lymphoma, three primary melanomas, two primary mesotheliomas, one primary non-small cell lung cancer (NSCLC), two primary prostate cancers, one primary sarcoma and one primary small cell lung cancer (SCLC). All samples were available for automated quantitative immunohistochemistry (AQUA) analysis. Seven individuals with pancreas cancer, two with breast cancer and one with lymphoma did not have sufficient material for RNA analysis. The Health Sciences Institutional Review Board of the University of Wisconsin approved these trials prior to their implementation, and all patients gave informed written consent.

Laser Capture Microdissection

Sections were prepared from each FFPE tissue blocks, and hematoxylin and eosin (H&E) staining was performed. H&E stained slides were reviewed by a pathologist to determine the location of tumor tissue on each slide. Laser capture microdissection (LCM) with the SL μ Cut Laser Microdissection System (Molecular Machines & Industries, Glattbrugg, Switzerland) was utilized to ensure the isolation of only tumor cells. Briefly, the sections were melted at 60°C for 30 min and deparaffinized in xylene. Sections were then rehydrated in graded ethanols, rinsed with DEPC-treated water, stained with toluidine blue, rinsed in

DEPC-treated water, dehydrated in graded ethanols followed by isopropanol and placed in a desiccator until dry.

RRM2 Gene Expression Analysis by Quantitative Reverse Transcriptase Polymerase Chain Reaction (Q-RT-PCR)

After LCM, RNA extraction was performed using the Paradise Whole Transcript RT Reagent System (Arcturus Bioscience, Sunnyvale, CA) which processes FFPE tissue scrapes. Caps were placed in a microcentrifuge tube-containing proteinase K and incubated at 37°C for 16-20 hours. After centrifugation, the caps were removed and the RNA was isolated and treated with DNase following the manufacture's instructions. The total RNA was resuspended and then treated with DNase. Total RNA was stored at -80°C until analyzed. The RNA was quantified via NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE), and the total RNA extracted from FFPE tumor tissue was reverse transcribed using random primers by standard methods.

TaqMan assays were designed for the genes listed in Table 1 using Integrated DNA Technologies (Coralville, IA), keeping the amplicon length < 100bp. Quantitative real-time PCR was performed using the Bio-Rad iCycler IQ system (Hercules, CA). Due to the limited sample supply, the target gene (RRM2) and the endogenous reference gene (YWHAZ) were amplified in a single well. Each well contained 5 pmol/μL of the probes, 5 pmol/μL of the primers, and 12.5 μL of iQ Multiplex Powermix (Bio-Rad) in a 25 μL final reaction mixture. The Multiplex Powermix was heat-activated for 3 min at 95°C. Each of the 50 PCR cycles consisted of 15 sec of denaturation at 95°C and hybridization of primers and probes for 45 sec at 60°C.

Expression levels in the patient samples were determined by the standard curve method using standard cDNA solutions which were serially diluted 5-fold from HepG2 carcinoma cell line. The standard curve and samples were run in triplicate, and nontemplate controls were included in each run. The data was analyzed with the standard curve line equations generated by iQ5 software (Bio-Rad). The starting mass value for the gene of interest and reference gene were calculated by substituting the threshold cycle (Ct) values generated by the iQ5 software into the standard curve formula. The Ct (threshold cycle) is defined as the fractional cycle number at which the fluorescence passes the fixed threshold. The data was expressed as target gene (RRM2)/endogenous gene (YWHAZ). The correlation coefficient (r) for each standard curve exceeded 0.99, and the coefficient of variation for the Ct values was less than 3.5% for all replicates.

Validation Summary

Linearity was determined with five standard curves, and the mean coefficient of determination (r^2) for RRM2 was 0.990 (range 0.982-0.998) and for YWHAZ was 0.996 (range 0.994-0.998). Triplicate determinations of each standard were run on a single plate on five separate occasions over a two-week period. The intra-day variability in the standard Ct readings for RRM2 averaged 0.65% (range 0.09-2.84%), while for YWHAZ the standard Ct readings averaged 0.61% (range 0.06-1.96%). The mean CV in the Ct values for RRM2 over this time ranged from 0.32% (range 0.09-0.68%) for the 400 ng standard to 1.47% (range 0.66-2.84%) for the 0.128 ng standard. For YWHAZ, the mean CV in the Ct values over the standard curve ranged from 0.54% (range 0.31-1.03%) for the 400 ng standard to 1.11% (0.33-1.96%) for the 0.128 ng standard. Triplicate determinations of Ct readings in five patient samples on a single plate had a mean CV for RRM2 of 1.18% (range 0.25-2.21%) over the mass range of 0.19-114.43 ng (all samples were diluted). In the same five patient samples, the mean CV for YWHAZ was 0.70% (range 0.03-1.14%) over the mass range of

0.13-116.12 ng (all samples were diluted). The variability did not change with concentration.

M2 Protein Analysis by Automated Quantitative Analysis (AQUA)

RRM2 expression was determined using the AQUA system (HistoRx, New Haven, CT) as previously described (17-19). Initially, target compartments were localized using a fluorescently tagged rabbit anti-cytokeratin antibody (or the anti-S100 antibody for melanoma cells). 4,6-Diamidino-2-phenylindole (DAPI) was added to visualize nuclei. RRM2 was visualized with an Alexa Fluor 488 labeled tyramide which, like diaminobenzidine, is activated by horseradish peroxidase and results in the deposition of numerous covalently associated Alexa Fluor 488 dyes immediately adjacent to the horseradish peroxidase-conjugated secondary antibody (Figure 1). Using this approach, classical compartments are defined on the basis of molecular co-localization. The cytokeratin compartment is equivalent to all epithelial cells in the tissue section. The S-100 compartment is equivalent to melanoma cells in tissue section. DAPI is the area defined as the cell nucleus. A matched set of H&E sections were used for locating tumor. RRM2 was tagged and measured within the subcellular compartments by the PLACE algorithm as previously described. Data is represented as a Z-score, which was calculated by subtracting the mean AQUA score from the individual AQUA score and dividing by the standard deviation (18,19). Therefore a negative Z-score indicates the protein expression was less than the average and a positive Z-score means it is greater than the average.

Statistical Methods

RRM2 gene expression and protein expression were summarized in terms of number of observations, means and standard deviations. The data were presented in graphical format using boxplots. The comparisons between groups were performed using a non-parametric Wilcoxon Rank Sum test or the Kruskal-Wallis test. Exact p-values were computed for all comparisons. All statistical tests were two-sided, and $P < .05$ was used to indicate statistical significance. Due to the exploratory nature of this study, no adjustments for multiple comparisons were made. Non-parametric Spearman's rank correlation analysis was used to examine the association between protein and gene expression levels. Statistical analysis was performed using SAS[®] (SAS Institute Inc., Cary, North Carolina) version 9.1 software.

Results

RRM2 Expression by Tumor Type

AQUA demonstrated that RRM2 protein localized to the cytoplasm (Figure 1), where it is produced. Table 2 lists the number and types of samples that were analyzed. Comparisons were made for tumor types where at least three tissue blocks were available. The median Z-scores were as follows: pancreatic cancers (n=13) -0.41 ± 0.84 , for cholangiocarcinoma (n=4) -0.21 ± 0.23 , for esophageal and gastric cancers (n=4) 0.68 ± 0.95 , and for melanoma (n=3) it 0.74 ± 0.57 . The p-value for the comparison across all four tumor types is 0.03 (Kruskal-Wallis test), suggesting that at least one tumor type is different than the others (Figure 2). Pairwise comparisons between the groups with the Wilcoxon Rank Sum test demonstrate cholangiocarcinoma and esophageal cancer are significantly different ($p=0.03$), pancreatic cancer and esophageal/gastric cancer are significantly different ($p=0.04$), and that pancreatic cancer and melanoma show a trend towards difference ($p=0.06$).

The RRM2 gene expression relative to the housekeeping gene YWHAZ for pancreatic cancers (n=6) was 0.29 ± 0.20 , for cholangiocarcinoma (n=4) was 0.54 ± 0.52 , for esophageal/gastric cancers (n=4) 2.56 ± 1.49 , and for melanoma (n=3) it was 0.79 ± 0.22 . The p-value for the comparison across all 4 tumor types is 0.03 (Wilcoxon Rank Sum test),

suggesting that at least one tumor type is different than the others. See Figure 3. Pairwise comparisons between the groups with the Kruskal-Wallis test demonstrate that pancreas cancer and esophageal/gastric cancer ($p=0.02$), and prostate cancer and melanoma ($p=0.05$) are significantly different. Cholangiocarcinoma and esophageal cancers show a trend towards difference ($p=0.06$).

Spearman's rank correlation analysis was performed to examine the association between RRM2 protein expression as evaluated by AQUA and gene expression evaluated by Q-RTPCR in baseline tumor specimens. This analysis demonstrated a moderate correlation (Spearman's rank correlation =0.30, $p=0.12$) between the cancers, and two tumor types (SCLC and sarcoma) had highly discordant results (Table 2).

Patient Response

Two patients (5%) achieved a partial response by Response Evaluation Criteria in Solid Tumors (RECIST) (20). One patient with NSCLC, who received 3-AP at 85 mg/m² on days 1-3 of a 21 day cycle and irinotecan 150 mg/m² on day 1 of a 21 day cycle had a Z-score of 0.78 and a relative gene expression value of 2.43. Another patient with bladder cancer, who received 3-AP at 25 mg/m² Days 1-4 of a 21 day cycle and doxorubicin at 60 mg/m², on day 1 of a 21 day cycle had an unconfirmed PR and a Z-score of 0.57 and relative gene expression value of 2.17. While response rates were too low to perform a statistical analysis comparing responders to non-responders, both patients achieving a partial response had relatively high expression levels compared to other patients without a response (Table 2).

Discussion

In this study, we evaluated differences in RRM2 mRNA and protein expression in baseline tumor samples. Our findings demonstrated that RRM2 levels are higher in esophageal/gastric cancer and melanomas when compared to pancreatic cancer. Additionally, esophageal cancers have more expression when compared to cholangiocarcinomas. These differences in RRM2 were significant despite small sample sizes and suggest that relative RRM2 overexpression may play a role in tumor development of esophageal and gastric cancers and melanoma, although confirmatory studies are required.

As RRM2 is the intracellular target of 3-AP, one potential application would be to evaluate RRM2 expression to predict 3-AP -sensitive tumors. Gandera and colleagues recently reported preliminary findings suggesting inhibition of RRM2 gene expression was associated with clinical response to the antisense agent GTI-2040 targeting RRM2 (21). Two phase II trials of 3-AP have recently been reported (22,23) with disappointing results. In a trial of single agent 3-AP in advanced renal cell cancer, Knox and colleagues demonstrated a 7% (1/19) partial response rate (22). Mackenzie and colleagues studied 3-AP in combination with gemcitabine for advanced pancreatic cancer with no responses observed (23). While RRM2 expression was not evaluated, the clinical results are consistent with our own phase II trial of 3-AP in advanced pancreas cancer where no responses were observed (16). Since RRM2 gene expression and protein expression in pancreas cancer was found to be significantly less than in other solid tumors, low RRM2 expression may be a potential mechanism of 3-AP resistance.

While a statistical analysis could not be performed due to a limited sample size, both patients with a partial response had high RRM2 mRNA and protein levels. Since both patients achieving a partial response received chemotherapy in addition to 3-AP, the observed responses cannot be attributed solely to 3-AP relatively high baseline RRM2 expression. The patient with NSCLC received concurrent 3-AP and irinotecan, and the patient with bladder cancer received 3-AP and doxorubicin. However, given the single agent

response rate of second-line irinotecan in metastatic NSCLC is less than 5% (24) and the single agent response rate for doxorubicin in bladder cancer is 17% (25), our data suggests that increased baseline expression of RRM2 and 3-AP may contribute to the activity of the regimen.

In this analysis, protein expression and mRNA expression were moderately correlated. This association can likely be explained by the SCLC and sarcomas that had highly discordant results. The data suggests that for the majority of tumors, transcription is an important mechanism controlling RRM2 protein expression. In some tumors, however, transcription and expression do not appear to be linked, and unknown mechanisms may control protein expression. This analysis also raises an important question regarding the preferred method for expression analysis, which cannot be answered in the current study. Further studies comparing protein expression to mRNA expression to 3-AP response and mechanistic studies to determine the relationship between mRNA expression and protein expression for RRM2 are necessary.

In conclusion, both RRM2 gene and protein expression vary by tumor type in baseline tumor samples. Given the poor phase II activity of 3-AP in renal cell and pancreatic cancer, one potential application of our findings would be evaluating baseline RRM2 to predict tumors sensitive to 3-AP.

Acknowledgments

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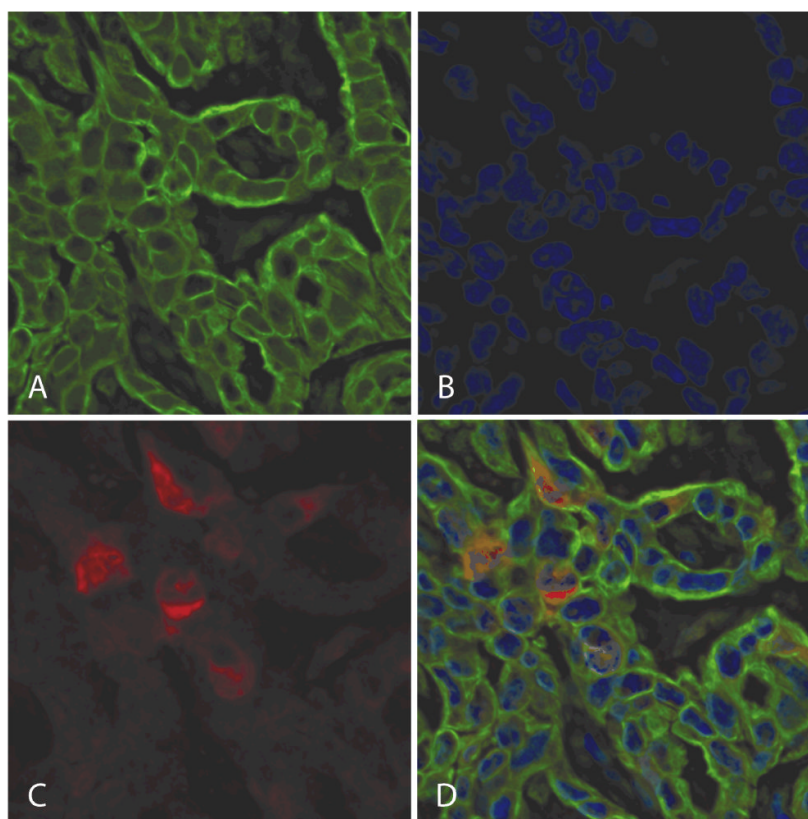


Figure 1.

Protein expression of ribonucleotide reductase M2 (RRM2) was determined using an automated *in situ* quantitative measurement of protein analysis, automated quantitative immunohistochemistry on the basis of immunofluorescence.

- a. Target compartments were localized using a fluorescently tagged Alexa Fluor 555, rabbit anti-cytokeratin antibody (green).
- b. 4,6-Diamidino-2-phenylindole was added to visualize nuclei (blue).
- c. RRM2 was visualized with Alexa Fluor 488-tyramide, human anti-RRM2 (red).
- d. A three color overlay shows localization of RRM2 to the cytoplasm.

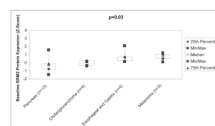


Figure 2.

Boxplot of RRM2 Protein Expression Evaluated by automated quantitative immunohistochemistry. The median Z score for pancreas cancers (n=13) was -0.41 ± 0.84 , cholangiocarcinoma (n=4) was -0.21 ± 0.23 , esophageal and gastric (n=4) was 0.68 ± 0.95 , and melanoma (n=3) was 0.74 ± 0.57 . The p-values for the comparisons across all four tumor types is 0.03 (Kruskal-Wallis test), suggesting that at least one tumor type is different than the others. Abbreviations: Min, minimum; Max, maximum; n, number; RRM2, ribonucleotide reductase M2.

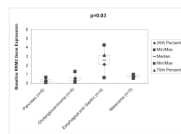


Figure 3.

Boxplot of RRM2 Gene Expression Evaluated by RT- PCR. The relative ribonucleotide reductase M2 gene expression for pancreas cancers (n=6) was 0.29 ± 0.20 , cholangiocarcinoma (n=4) was 0.54 ± 0.52 , esophageal and gastric (n=4) was 2.56 ± 1.49 , and melanoma (n=3) was 0.79 ± 0.22 . The p-values for the comparisons across all 4 tumor types is 0.03 (Kruskal-Wallis test), suggesting that at least one tumor type is different than the others. Abbreviations: RRM2, ribonucleotide reductase M2; n, number; Min, minimum; Max, maximum.

Table 1
Sequences of the primers and probes used in quantitative PCR studies

Gene	Oligonucleotide	Sequence	PCR product size (bp)	GenBank Accession Number
YWHAZ	Forward primer	5'-CCAATGCTTCACAAGCAGAGAGCA-3'	99	NM_003406
	Reverse primer	5'-CTTCTTGTGCATCACCAGCGGCAA-3'		
	Probe	5'-fAGGAGATTACTACCGTTACTTGGCTGAGGq-3'		
RRM2	Forward primer	5'-TTTAGTGAGCTTAGCACAGCGGGA-3'	89	NM_001034
	Reverse primer	5'-AAATCTGCGTTGAAGCAGTGAGGC-3'		
	Probe	5'-rACAGTCCTTTAACCAGCACAGCCAGTq-3'		

f denotes the fluorescein FAM, r denotes the fluorescein TET, q denotes Black Hole Quencher 1

Table 2
Ribonucleotide Reductase M2 Protein and Gene Expression by Tumor Type

Tumor Type (n=Protein/mRNA)	RRM2 Protein Expression (Z-Score) Mean \pm SD	RRM2 Gene Expression Relative to YWHZ Mean \pm SD
Unknown Primary (2)	-7.8 \pm 1.0	0.03 \pm 0.01
Prostate (2)	-0.77 \pm 0.1	0.35 \pm 0.14
Sarcoma (1)	-0.57	3.3
Cervical (1)	-0.56	0.35
Lymphoma (1/0)	-0.52	
Breast (2/0)	-0.42 \pm 0.41	
Pancreas (13/6)	-0.41 \pm 0.84	0.29 \pm 0.20
Cholangiocarcinoma (4)	-0.21 \pm 0.23	0.54 \pm 0.52
Mesothelioma (2)	0.26 \pm 0.23	0.16 \pm 0.14
Colon (2)	0.29 \pm 0.46	0.31 \pm 0.12
Bladder (1)	0.57	2.17
Esophageal/Gastric (4)	0.68 \pm 0.95	2.56 \pm 1.49
Melanoma (3)	0.74 \pm 0.57	0.79 \pm 0.22
NSCLC (1)	0.78	2.43
Small Cell Lung Cancer (1)	3.8	0.03

RRM2: Ribonucleotide Reductase M2

NSCLC: non small cell lung cancer

SD: standard deviation