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Expression of RBM5-Related Factors in Primary Breast Tissue

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Abstract The aim of this study was to examine the expression of the RBM5 tumor suppressor, in relation to RBM6 and RBM10, to obtain a better understanding of the potential role played by these RBM5-related factors in the regulation of RBM5 tumor-suppressor activity. Paired non-tumor and tumor samples were obtained from 73 breast cancer patients. RNA and protein expression were examined by semi-quantitative reverse transcription-polymerase chain reaction and immunoblot, respectively. Data were analyzed using various statistical methods to test for correlations amongst the RBM5-related factors, and between the factors and various pathological parameters. Most notably, RBM5, RBM10v1, and HER2 protein expression levels were elevated in tumor tissue ($P < 0.0001$). RBM5 and RBM10v1 protein expression were significantly positively correlated ($P < 0.001$), as were RBM5 and HER2 protein expression ($P < 0.01$), in both non-tumor and tumor tissue, whereas RBM10v1 and HER2 protein expression were only marginally correlated, in non-tumor tissue ($P < 0.05$). Interestingly, RBM5 and RBM10v1 protein expression were both deregulated in relation to RNA expression in tumor tissue. RBM10v2 and RBM6 RNA were highly significantly positively correlated in relation to various factors relating to poor prognosis ($P < 0.0001$). To our knowledge, this study is the first to examine RBM5 expression at both the RNA and protein level in primary breast tumor tissue, and the first to examine expression of all RBM5-related factors in a comprehensive manner. The results provide a graphic illustration that RBM5-related factors are significantly differentially expressed in breast cancer, and suggest complex inter-related regulatory networks involving alternative splicing, oncogenic expression, and tissue-specific function. J. Cell. Biochem. 100: 1440–1458, 2007.

Key words: cancer; breast; tumor suppressor; RBM5; HER2

Since the first published reference to the RBM5/LUCA-15/H37 gene appeared 7 years ago [Gure et al., 1998], it has been described as a tumor suppressor [Oh et al., 2002, 2006], apoptosis modulator [Sutherland et al., 2000; Mourtada-Maarabouni et al., 2002, 2003; Rintala-Maki and Sutherland, 2004; Rintala-Maki et al., 2004; Oh et al., 2006], cell cycle regulator [Mourtada-Maarabouni and Williams, 2002; Mourtada-Maarabouni et al., 2003; Oh et al., 2006] and RNA binding protein [Drabkin et al., 1999; Edamatsu et al., 2000]. Very little is known, however, about how it functions in any of these capacities. All of the functional studies were conducted using over-expressed, exogenously administered RBM5,

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and thus provide little insight into the normal physiological mechanisms that regulate RBM5 expression and function in the wild-type milieu.

For instance, RBM5 has significant sequence similarity to two other genes, RBM6 and RBM10, both of unknown function [Sutherland et al., 2005]. RBM6 is located at 3p21.3, immediately telomeric to the *RBM5* gene [Gure et al., 1998]. The protein has approximately 30% identity to RBM5 [Timmer et al., 1999]. RBM6 has five known RNA splice variants [Sutherland et al., 2005], at least two of which are likely to encode protein [Gure et al., 1998]. Both putative proteins have a unique amino terminal repeat sequence of unknown function, but differ by the addition of alternative sequence at the same amino terminal site [Gure et al., 1998]. RBM6 was first identified from an autologous antibody screen for human lung cancer antigens, suggesting that its expression is upregulated in lung cancer [Gure et al., 1998].

The *RBM10* gene, located on the X chromosome, has even more sequence homology to RBM5. There are at least three RBM10 RNA transcripts, two of which are identical except for the inclusion of a 77 base-pair (bp) sequence in variant 1 [Sutherland et al., 2005]. RBM10v1 and RBM10v2 have 49% and 54% identity, respectively, with RBM5 [Sutherland et al., 2005]. While there are no functional data concerning RBM10, the high degree of sequence homology with RBM5 suggests either functional similarity, synergy, redundancy, or antagonism. Interestingly, a recent report demonstrates that RBM10 RNA expression is significantly positively correlated with expression of the pro-apoptotic Bax RNA ($P < 0.001$), suggesting that RBM10, like RBM5, is involved in apoptosis modulation [Martinez-Arribas et al., 2006]. The observation that RBM10 is subject to X chromosome inactivation [Coleman et al., 1996; Thiselton et al., 2002] suggests that control of absolute protein levels has functional significance, and thus further suggested to us that the expression of any one RBM5-related product may not be as functionally important as the combined expression, or balance of expression, of the various products.

RBM5 maps to 3p21.3, to a fragile region that is associated not only with the earliest lesion observed in lung cancer [Lerman and Minna, 2000], but also with renal, head and neck, gastrointestinal, breast, and cervical cancers [Kok et al., 1997]. Several reports indicate that RBM5 is, as would be expected of a tumor suppressor, pro-apoptotic molecule and cell cycle inhibitor, downregulated in relation to cancer. For instance, endogenous expression of RBM5 RNA was reportedly downregulated in human breast cancer tissues [Edamatsu et al., 2000], in many lung cancers [Oh et al., 2002], and vestibular schwannomas [Welling et al., 2002]. RBM5 was also one of the genes comprising the 17-gene metastatic signature recently identified in humans by Ramaswamy et al. [2003], and confirmed in mice [Qiu et al., 2004]. In this signature, RBM5 was downregulated in both primary solid tumors and their associated metastases.

In contrast, however, are reports demonstrating upregulation of RBM5 expression in tumors. For instance, endogenous expression of RBM5 RNA was reportedly upregulated in 5-fluorouracil-resistant colorectal and breast cancer cells [Wang et al., 2004], and breast and ovarian cancers [Oh et al., 1999]. In the study reported by Oh et al. [1999], RBM5 RNA expression was positively correlated with expression of the HER2 oncogene.

While upregulation of a candidate tumor suppressor, and pro-apoptotic molecule is not entirely unexpected in a pre-malignant cell that is perhaps still in the process of adjusting to the altered proliferative signals, it is counterintuitive to have a tumor suppressor gene and known apoptosis regulator upregulated in an advanced stage cancer, unless it is either not being translated into protein, the protein is inactive or protein activity is indirectly functionally suppressed. Indirect functional suppression could take the form of interrupted downstream signaling as a result of additional mutations, or altered expression of related factors whose expression contributes to RBM5 function. It was our hypothesis that although upregulated RBM5 is capable of functioning independently to suppress tumor growth and enhance apoptosis, that in the physiological milieu it functions in association with related factors, including variants—generated through alternative splicing—that have been shown to have agonistic apoptogenic modulatory activity.

In the study described herein, we examined the expression of all the RBM5-related factors, in a cancer environment, in order to (a) determine the status of RBM5 protein, and (b)

look for correlations amongst the RBM5-related factors, and between the factors and various tumor parameters, that might help to elucidate the mechanisms regulating RBM5 function. Since RBM5 mRNA had previously been reported to be upregulated in primary breast tumors, particularly in relation to HER2 oncogene expression, we decided to carry out our investigation in primary breast tissue. RBM5-related product expression, including RNA splice variants, was examined in relation to patient age, tumor type, grade and size, grade of ductal carcinoma in situ, HER2 status, nodal involvement, and estrogen receptor (ER) and progesterone receptor (PR) status, as consistently recorded clinical variables.

MATERIALS AND METHODS

Cell Culture

The human breast cancer cell line MDA-MB-231, originally isolated from a pleural effusion of a patient with breast adenocarcinoma, was obtained from the American Type Culture Collection (ATCC # HTB-26). Cells were maintained in D-MEM/F-12 medium containing 15-mM HEPES buffer, L-glutamine, and pyridoxine hydrochloride (Invitrogen, MD), supplemented with 10% fetal bovine serum (Invitrogen), at 37°C in a 5% CO_2-humidified incubator.

Cells were transiently transfected with pcDNA3.1.myc.his (Invitrogen) or pcDNA3.1.myc.his.HER2 (the kind gift of Robert Lafrenie, Sudbury), using Lipofectamine Reagent and Lipofectamine PLUS (Invitrogen), according to the manufacturer's instructions.

Sample Procurement

Paired non-tumor and tumor breast specimens were obtained from patients treated at the Hôpital régional de Sudbury Regional Hospital in Sudbury, Ontario, Canada between 2002 and 2004. All samples were procured with informed consent, according to Institutional Research Ethics Board guidelines. Tissue was obtained from lumpectomies or mastectomies, and in all cases represents invasive carcinoma. Non-tumor tissue was obtained from the periphery of each specimen at the time of surgery, and designated at that time.

Isolation of RNA and Protein From Breast Tissue

Samples were initially embedded in optimal cutting temperature (OCT) compound and frozen in liquid nitrogen, then stored at −80°C. For tissue preparation, each sample was removed from the OCT and 100 mg was homogenized using a #11 scalpel blade in a microfuge tube containing 1 ml of Tri Reagent (Molecular Research Center, Inc.). RNA and protein were extracted from the same piece of tissue, according to the Tri-Reagent manufacturer's instructions. Briefly, phase separation was achieved by the addition of 1-bromo-3-chloro-propane (Sigma) and centrifugation. RNA was precipitated from the aqueous layer using isopropanol. RNA pellets were washed with 75% ethanol, air-dried and suspended in DEPC (Sigma)-treated water. Isolated RNA was subsequently treated with 40 units of RNaseOUT (Invitrogen) and RNA concentrations were determined by optical density at 260 nm. Genomic DNA was removed from the organic phase by ethanol precipitation. Proteins were isolated from the supernatant by isopropanol precipitation and collected by centrifugation. Protein pellets were washed with 0.3 M guanidine hydrochloride (Sigma) in ethanol, air-dried, and suspended in protein resuspension buffer containing 50 mM Tris pH 6.8, 20 mM EDTA, 5% SDS, 5 mM β-glycerophosphate (Sigma). Protein concentrations were determined by Bradford assay using the DC Protein Assay System (Bio-Rad), and analyzed with GraphPad Prism Version 4.0 (GraphPad Software, Inc.).

In addition to the non-tumor and tumor breast samples, RNA and protein was isolated and quantified from MDA-MB-231 breast adenocarcinoma cells. RNA was isolated as described above. Protein was isolated by suspension of cell pellets in lysis buffer containing 50-mM Tris-HCl pH 8, 150 mM NaCl, 0.5% NP-40, 100 mM NaF, 1 mM EDTA pH 8, 1 mM EGTA pH 7.5, and 1% protease inhibitor cocktail (Sigma), followed by centrifugation. Protein concentrations in the supernatants were quantified using the DC Protein Assay (Bio-Rad).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

RNA (1 μg) was treated with 1 unit of DNase I enzyme (amplification grade, Invitrogen), then 500 ng was reverse-transcribed using Superscript II Reverse Transcriptase (Invitrogen) and an oligo-dT primer ($T_{20}VN$). Reverse transcription was achieved by incubation at 42°C for 50 min, followed by heat inactivation at 70°C. For each PCR, 1/20 of the cDNA was used.

The following gene-specific primers were used for PCR: RBM5/RBM5Δ6 (LU15(2) and LU15(3)) [Sutherland et al., 2000], RBM5 + 6/RBM5 + 5 + 6 (LU15(2) and LS5) [Sutherland et al., 2000], RBM10 (RBM10F 5′-TGG CTGGGAAGTGAAACGGA-3′ and RBM10RS 5′-GGATGTTGAGGGAGTGCTGA-3′), RBM6 (RBM6FA 5′-GCGGCGCTGGGTCGGTGGC-3′ and RBM6R 5′-CTGAATGTGGCGTATCCCT GTCCCTA-3′), actin (actinF 5′-CGG GAA ATC GTG CGT GAC ATT AAG-3′ and actinR 5′-TAC TCC TGC TTG CTG ATC CAC ATC-3′) and HER2 (HER2F and HER2R [Aigner et al., 2001]). Platinum Taq Polymerase (Invitrogen) was used to amplify the cDNA using the iCycler thermalcycler (Bio-Rad) and the following programs: 95°C 5 min, followed by 30–40 cycles of 95°C 30 s, annealing for 30 s (HER2 (40 cycles), $T_m = 55$°C: actin (30 cycles), RBM5/RBM5Δ6 (40 cycles), RBM5 + 6/RBM5 + 5 + 6 (40 cycles), $T_m = 58$°C: RBM10 (40 cycles), $T_m = 64$°C: RBM6 (40 cycles), $T_m = 69$°C) and 72°C for 30 s to 1.5 min, followed by an additional extension of 72°C for 10 min. The products of these reactions were electrophoresed through TAE agarose gels (1–2% W/V) containing 0.1 μg/ml ethidium bromide. Gels were visualized using the FluorChem Gel Documentation System (Alpha Innotech) and densitometric analysis was performed using AlphaEaseFC software (Alpha Innotech).

Immunoblotting

Aliquots of each protein (50 μg) were added to sample buffer (10% glycerol, 0.7 M β-ME, 3% SDS, 62 mM Tris-HCl, pH 6.8), boiled for 5 min and loaded onto 7 or 12 % SDS–PAGE gels. Each gel loaded with tissue protein also contained 50 μg of MDA-MB-231 protein, as an internal control, and the BenchMark Prestained Protein ladder (Invitrogen). Following electrophoresis, proteins were electrophoretically transferred to either Hybond-ECL nitrocellulose membrane (Amersham) or PVDF (Pall Life Sciences). Membranes were blocked with 5% non-fat dry milk in TBS-T (20 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 0.05% Tween-20). Seven percent gels containing tissue samples were cut in half and probed with rabbit anti-human neu (C-18) (Santa Cruz, 1:1,000 dilution) or goat anti-human actin (Santa Cruz, 1:1,000 dilution) in 3% non-fat dry milk in TBS-T, overnight at 4°C. Twelve percent gels containing tissue samples were probed with rabbit anti-human LUCA-15-UK [Sutherland et al., 2000] (1:2,500 dilution) or chicken anti-human RBM10v1-92 (generated for us by Gallus Immunotech, Inc., to the unique RBM10v1 sequence DGDYRDQDYRTEQGE, 1:2,500). Secondary antibodies used were goat anti-rabbit IgG-HRP (Santa Cruz, 1:10,000), bovine anti-goat IgG-HRP (Santa Cruz, 1:10,000), and donkey anti-chicken IgY-HRP (Gallus Immunotech, Inc., 1:5,000). Antibody detection utilized an ECL chemiluminescent kit (Amersham) and Hyperfilm ECL (Amersham). Densitometric analysis was performed using AlphaEaseFC software (Alpha Innotech).

Immunohistochemistry

Three micron formalin fixed, paraffin-embedded sections from tumor were placed on charged slides. Slides were labeled with a bar code and placed on an automated Benchmark XT immunohistochemistry (IHC) instrument. Reagents were then automatically applied to the tissue and incubated for a precise time and temperature. Reagents included a pre-treatment solution for deparaffinization and rehydration called EZ prep and an unmasking reagent called CC1. Pre-diluted mouse anti-human HER2/neu clone CB11 primary antibody (Ventana Medical Systems) was incubated at 37°C for 20 min. Protein was detected using a labeled avidin–biotin technique including the chromogen diaminogenzidene (DAB). A hematoxylin counterstain followed by a lithium carbonate bluing agent were also applied. Finally, the slides were dehydrated, cleared, and mounted. All reagents were purchased through Ventana Medical Systems.

Complete strong HER2 membrane positivity was measured on a scale of 0–4. A score of 3+ or greater in 10% or more of the cells was counted as HER2 overexpression. A score of 2+ was considered equivocal and confirmed by FISH analysis (fluorescence in situ hybridization). Blind scoring of all samples was carried out by an independent pathologist.

Pathological Criteria

Tumor grade was classified according to the Nottingham modification of the Scarff-Bloom-Richardson system [Elston and Ellis, 1998]. Tumor size ranges corresponded to those used in the TNM staging system of tumor classification, according to the American Joint Committee on Cancer Staging of Breast Carcinoma (AJCC) criteria, 6th edition [Greene et al.,

2002]. Ductal carcinoma in situ (DCIS) grade was classified as previously described [Silverstein et al., 1995].

HER2 Overexpression Defined

In this study, HER2 protein overexpression was defined by two different methods. First, HER2 overexpression was clinically defined immunohistochemically (IHC) using the CB11 anti-human HER2 antibody on paraffin-embedded tissue sections: as described above, the presence of strong membrane localization (3+ staining intensity on a scale of 0–4) in 10% or more cells indicated "overexpressed" HER2 protein (refer to Fig. 1). Second, HER2 overexpression was defined as any expression level above that found in non-tumor tissue, measured by immunoblotting (IB) using the neu (C-18) HER2 antibody (refer to Fig. 3A,B). Although immunoblotting cannot differentiate membrane localized HER2 from that which is not membrane localized, it has the advantage of assessing HER2 with the same technique, and in the same samples, as used for evaluation of RBM5. In the end, all the data were analyzed using both methods, so as to enable the results to be interpreted with a clinical application in mind, but with basic scientific relevance to total protein expression levels. In addition, because the IHC was carried out on tumor sections that were distinct from the piece of tumor that was analyzed by RT-PCR and immunoblotting, the

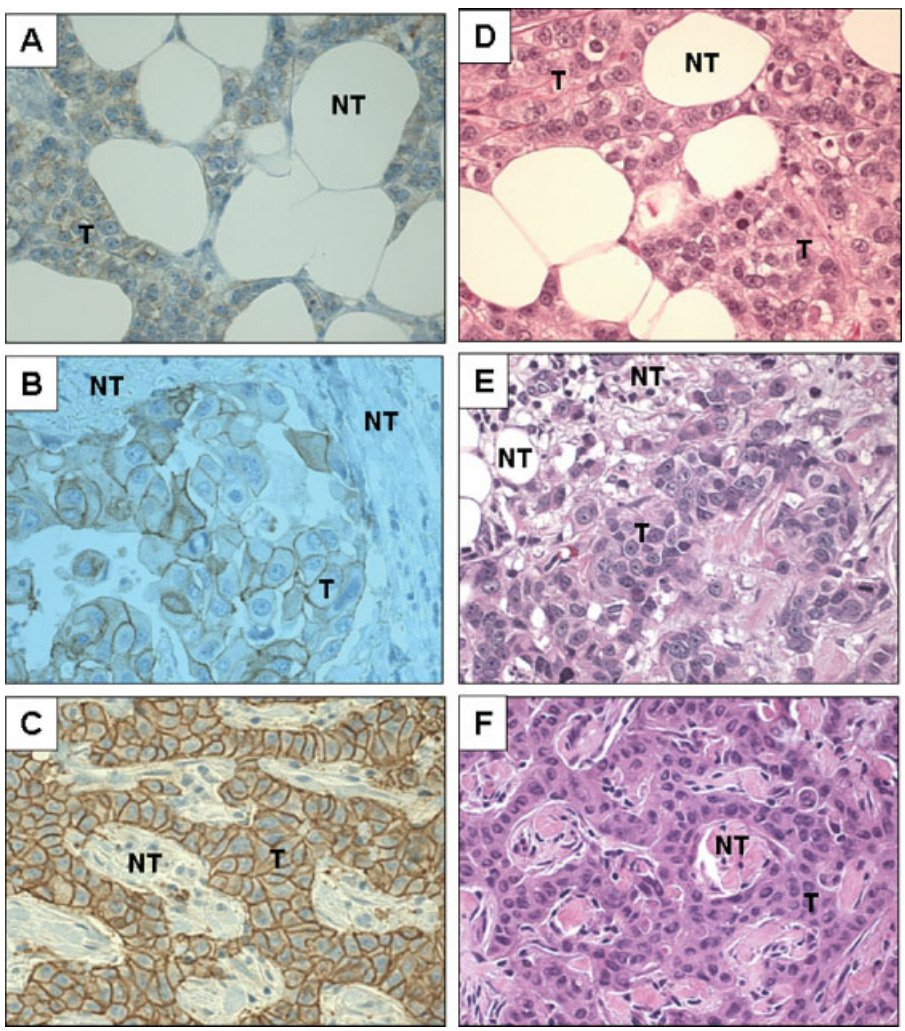

Fig. 1. HER2 expression visualized by IHC. Non-consecutive breast tumor slices, from three different patients, stained with the HER2/neu clone CB11 monoclonal antibody (**A**, **B**, **C**) or hematoxylin-eosin (**D**, **E**, **F**). A: 1+, weak, incomplete staining. B: 2+, complete membrane staining in fewer than 10% of the cells. C: 3+, strong, complete membrane staining in more than 10% of the cells. A and D, Patient #1; B and E, patient #2; C and F, patient #3. NT, non-tumor cells; T, tumor cells (magnification 400×).

dual analysis technique served to confirm the IHC results. According to the IHC-associated definition, 13/61 (21%) of the tumor samples overexpressed HER2, while according to the immunoblotting (IB)-associated definition, 11/61 (18%) overexpressed HER2.

Expression Analyses

A number of control measures were undertaken to ensure data accuracy. For instance, so as to be able to directly correlate RNA and protein expression, all of the RNA and protein from each patient sample was isolated from the identical piece of fresh frozen tissue. For analysis, RNA and protein was electrophoresed through a gel that also contained equal amounts of either RNA or protein from the MDA-MB-231 human breast cancer cell line, to control for reaction, transfer, and band exposure variances. To eliminate sample variability, all MDA-MB-231 RNA and protein used in this study was from the same large, pooled batch of whole cell lysate. Finally, only those samples expressing measurable levels of actin RNA or protein were further examined: this amounted to RNA from 33 non-tumor and 48 tumor samples, and protein from 62 non-tumor and 61 tumor samples (the RNA appeared to be far more labile than the protein). Densitometric data were normalized firstly to the same MDA-MB-231 RNA or protein molecule, and then to the actin RNA or protein. Statistical analyses were performed using these normalized data.

A number of different statistical tests were carried out to examine relationships between the various RBM5-related molecules and pathological parameters. First, distribution of the expression levels around the norm was determined using both the Kolmogorov–Smirnov and the Shapiro–Wilk tests, before and after outlier removal ($>3 \times$ the median): any significance less than $P = 0.05$ signified a non-normal distribution of data. Since the data were found to be non-normally distributed, to examine linear correlations that were not dependent on a parameter, that is, distribution around a norm, Spearman's rho and Kendall's tau b non-parametric distribution tests were used: because the results were very similar, only Spearman's results are reported herein. The Wilcoxon Signed Ranks Distribution Test was used to determine whether any apparent difference in expression levels between two paired samples (e.g., the correlation between RNA and protein expression levels in relation to metastatis status) was significant, whereas the Mann–Whitney *U*-Test was used to determine whether any apparent difference in expression levels between two independent samples (e.g., RBM5 RNA and RBM5 protein) was significant. The Kruskal–Wallis Test was used to determine whether any apparent difference in expression levels between multiple independent variables was significant. Significance was reported as an asymptotic two-tailed *P*-value. Unless otherwise stated, all outliers were included in data sets, so as not to bias the outcome against any valid rare overexpression phenomena. All statistical analyses were performed using SPSS software, version 12.0 (SPSS, Inc.).

RESULTS

RBM6 RNA Expression Was Significantly Elevated in Primary Breast Tumors

All of the mRNA splice variants of RBM5, RBM6, and RBM10 identified to date are depicted in Figure 2A. Gene-specific primers able to amplify regions of each of these variants were used in RT-PCR reactions on total RNA extracted from each of the tissue specimens. Amplicons were visualized electrophoretically, and representative results, from non-paired samples, are presented in Figure 2B.

Each of the RBM5 RNA splice variants that are routinely observed in transformed human cell lines were expressed in both the non-tumor and tumor tissue. Of the three potential RBM10 RNA products, RBM10v1 and RBM10v2 were dramatically variably expressed between patients, while RBM10v3 was rarely detected. Of note, whereas RBM10v2 RNA expression was considerably elevated in relation to either RBM10v1 or RBM10v3 in the control MDA-MB-231 human breast carcinoma cell line, this was never observed in the primary tissue. In addition, the primers used to distinguish between the three known variants of RBM10 detected a number of unexpected amplicons in the primary tissue (not purified for sequence analysis), suggesting the existence of additional, as yet unidentified, RBM10 RNA products. Of the five known RBM6 variants, only three would have been distinguishable with the exon 1 and exon 3 primers used in this study (refer to Fig. 2A). RBM6A and RBM6Δ6 would be indistinguishable from each other, as a single ~900-bp

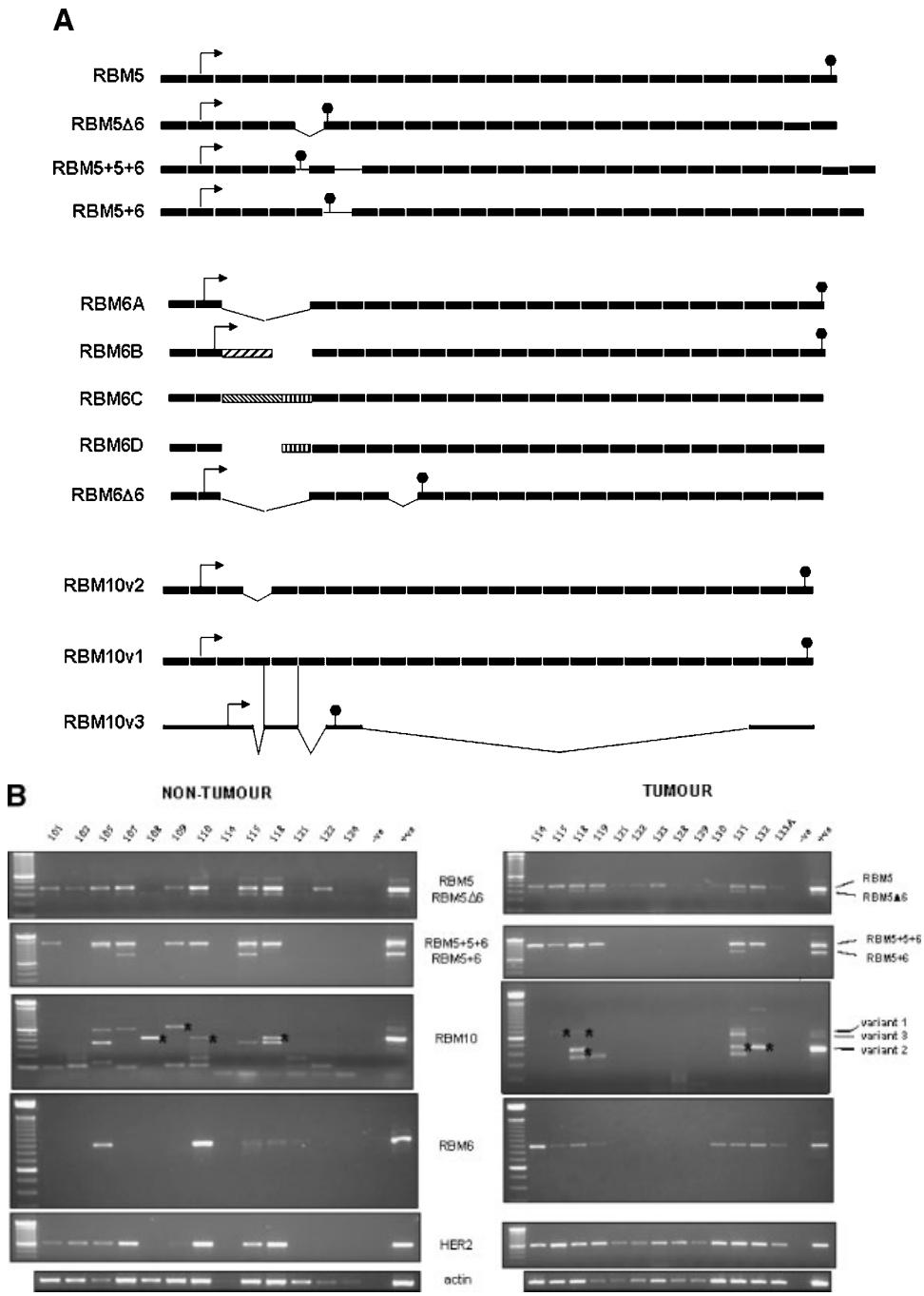

Fig. 2. RNA expression. **A**: Depiction of the various RBM5, RBM6, and RBM10 alternatively spliced RNA transcripts that are currently known to exist [Sutherland et al., 2005]. Arrows represent translation start sites, while stop sign forms represent translation stop sites. Thin horizontal lines represent retained intronic sequence, while thin angled lines represent alternatively deleted sequence. Thick, solid lines represent exons. Thin vertical lines represent the sequence between RBM10v1 and RBM10v3 that is not only identical but also in frame within the protein sequence. Hatched and vertical lines within exons in RBM6 represent alternate exons. **B**: Representative tissue samples, from non-tumor and tumor specimens. RNA was extracted from non-tumor and tumor tissue, and RT-PCR was performed using gene-specific primers to delineate the splice variants of RBM5, RBM10, and RBM6 expressed in primary breast tissue. HER2 expression was also examined. PCR cDNA amplicons were electrophoresed through a 1% agarose gel, followed by staining with 0.1 μg/ml ethidium bromide. *designates amplicons that were identified by the densitometry program to correspond to either RBM10v1 or RBM10v2. Actin was used for semi-quantitative expression analysis and as both a reaction and a loading control. Each lane represents a different tissue sample, either non-tumor or tumor. **C**: Following standardization and normalization, expression data were analyzed using the Wilcoxon Signed Ranks Test to determine which RNA transcripts were significantly different depending on tumor status. The Y-axis on all the box-plots represents the densitometry reading of the normalized, standardized expression data. *P*-values represent the significance of the distribution differences between expression in non-tumor versus tumor tissue.

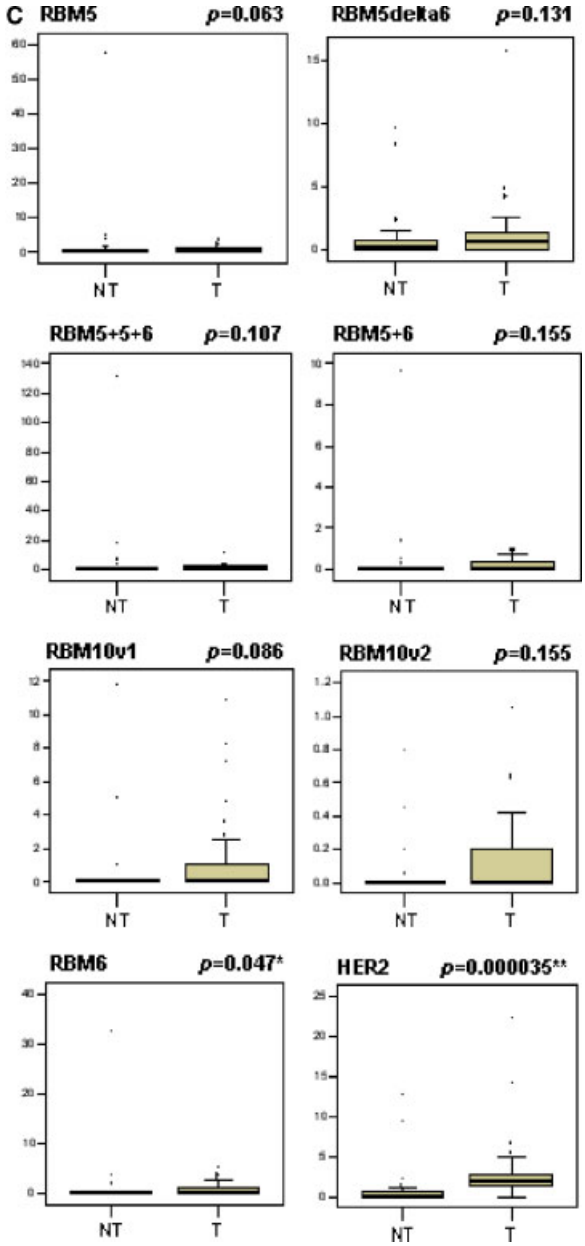

Fig. 2. (*Continued*)

amplicon. The PCR results of the breast tissue demonstrated that only the 900-bp product was amplified in either non-tumor or tumor tissue, indicating that only RBM6A and/or RBM6Δ6 were expressed. Note, as described in Materials and Methods, samples with undetectable levels of actin expression were excluded from the analyses.

Despite the more obvious, patient-specific, differences in RNA expression, it was determined, using the Wilcoxon Signed Ranks Test that the only significant differences in overall RNA expression levels between non-tumor and tumor tissue were observed for the RBM6 amplicon ($P = 0.047$) and HER2 ($P = 0.000035$), expression being significantly elevated in tumor compared to non-tumor tissue. None of the other RBM5-related RNA transcripts was found to be significantly differentially expressed in tumor tissue compared to non-tumor tissue, although expression of both RBM5 and RBM10v1 RNA demonstrated a trend towards significance (Fig. 2C).

Correlations Between RBM6 RNA Expression Levels and Other RBM5-Related Factors Were Dramatically Altered in Relation to Tumor Status and Nodal Involvement

Since many of the RBM5-related factors have no known function, we investigated the presence of expression correlations amongst the factors in an effort to tease out some hypotheses relating to function. The most striking correlations amongst all appeared to involve RBM6 mRNA. For instance, in the non-tumor tissue, the most significant correlation observed amongst all the RBM5-related RNA factors was a positive correlation between RBM6 and the anti-apoptotic factor RBM5Δ6 ($P = 0.00096$, $\rho_s = 0.548$, $n = 33$) (refer to supplementary data Table SI). In the tumor tissue, the most significant correlation was a positive correlation between RBM6 and RBM10v2 ($P = 0.0000018$, $\rho_s = 0.628$, $n = 48$) (supplementary data Table SII). Not only did both of the correlations involve RBM6, but also the altered correlation partners following tumorigenesis suggest that RBM6 expression changes are important to the process.

Of particular interest was the fact that the positive correlation between RBM6 and RBM10v2 RNA expression in tumor tissue was most significant in relation to parameters that are normally predictive of poor prognoses. The prognostic variables considered were tumor grade, tumor size, HER2 protein expression levels, the presence of ductal carcinoma in situ (DCIS), estrogen receptor (ER) status, progesterone receptor (PR) status, the presence of associated lymph node metastases (LNM), and patient age. An outline of the characteristics of our patient cohort is presented in Table I.

For instance, in Grade 1 tumors, there was no correlation between RBM6 and RBM10v2 RNA

TABLE I. Summary of Pathological Parameters for the 61 Tumor Samples

Characteristic	Number of patients	%
Age (years)		
Less than 55	23	38
Greater than or equal to 55	38	62
Tumor type		
Ductal	53	87
Lobular	8	13
Tumor grade		
1	7	11
2	26	43
3	28	46
Tumor size		
2 cm or less	25	41
>2 cm to 5 cm	29	47.5
<5 cm	7	11.5
DCIS grade		
0	17	32
1	1	2
2	13	25
3	22	41
Lymph node metastases		
Positive	27	44
Negative	34	56
HER2 (by IHC)		
Positive	13	21
Negative	48	79
Estrogen receptor		
Positive	48	79
Negative	13	21
Progesterone receptor		
Positive	38	62
Negative	23	38

IHC, immunohistochemistry; DCIS, ductal carcinoma in situ.

expression ($P = 0.628$, $\rho_s = 0.225$, $n = 7$). In Grade 2 tumors, RBM6 and RBM10v2 expression were significantly positively correlated ($P = 0.006$, $\rho_s = 0.595$, $n = 20$). In Grade 3 tumors, the correlation became highly significant ($P = 0.000001$, $\rho_s = 0.873$, $n = 21$). (Note, the n-values may be different from the numbers presented in Table I, since RNA expression was not detectable in every sample). Not only was the correlation between RBM6 and RBM10v2 the most significant correlation between any RBM5-related product and any of the pathological parameters examined, but also the difference in the significance of the correlation between tumor grades was the most dramatic of any parameter.

Tumor size, nodal involvement, and metastatic status combine to define a tumor's "stage," and are universally referred to as the TNM staging classification. In stage 1 tumors, the greatest dimension of the tumor is less than or equal to 2 cm. In stage 2 tumors, the greatest dimension is greater than 2 cm but not more than 5 cm. In stage 3 tumors, the greatest dimension is more than 5 cm. In our breast tumor cohort, 11.5% of the tumors were >5 cm. In the non-overexpressing HER2 tumors (defined by IHC), the correlation between RBM6 and RBM10v2 RNA was noted as highly significant in the middle size category (>2 cm to 5 cm) ($P = 0.000013$, $\rho_s = 0.777$, $n = 23$) compared to the first category (2 cm or less) ($P = 0.048$, $\rho_s = 0.436$, $n = 21$). Limited sample size (n=4) precluded accurate statistical analyses within the largest size category.

In situ, non-invasive, carcinomas are graded on a scale of 0–3. The higher the grade of the in situ carcinoma, the closer the progression to invasiveness. Both ductal and lobular carcinomas have in situ components; however, lobular carcinomas in situ are generally not graded. In our cohort of 61 invasive tumor samples, 53 (87%) were ductal, while only 8 (13%) were lobular. Although some of the lobular carcinomas had an in situ component, because they are not graded they were excluded from our in situ analyses. Within the ductal carcinoma category, 36/53 (68%) had an in situ component: of these, the majority (41%) was Grade 3. Examination of RBM5-related factor expression within these 36 invasive ductal tumors having an in situ component revealed that, at neither the RNA nor the protein level did expression of any of the factors relate to the grade of DCIS. Once again, however, the correlation between RBM6 and RBM10v2 RNA expression was found to be highly significant, particularly in Grade 3 DCIS ($P = 0.000021$, $\rho_s = 0.874$, $n = 15$) compared to either the absence of DCIS ($P = 0.001$, $\rho_s = 0.772$, $n = 14$) or Grade 2 DCIS ($P = 0.236$, $\rho_s = 0.354$, $n = 13$).

ER positive breast tumors are associated with a better prognosis than ER negative tumors [Reiner et al., 1990], and patients with either ER negative or PR negative breast tumors have a better overall survival than patients with both ER and PR negative breast tumors [Reiner et al., 1990], demonstrating the prognostic value of PR status. In our cohort, 48/61 (79%) of the tumors were ER positive, and 23/61 (38%) of tumors were PR negative. When considered individually, none of the RBM5-related factors were found to be significantly related to either ER or PR expression levels, although a trend towards significance was noted for RBM10v1 protein, in the non-overexpressing HER2 samples (refer to Table IV). HER2 protein expression was significantly elevated in the ER positive samples ($P = 0.007$), but demonstrated

no relationship to PR status ($P = 0.264$). On the other hand, the correlation between RBM10v2 and RBM6 RNA expression was significant in both ER negative ($P = 0.021$, $\rho_s = 0.784$, $n = 8$) and ER positive ($P = 0.00025$, $\rho_s = 0.548$, $n = 40$) tumors, although the relationship was not as affected by ER status as by other prognostic variables. The positive correlation between RBM10v2 and RBM6 RNA expression was also observed in both PR-positive ($P = 0.005$, $\rho_s = 0.484$, $n = 32$) and PR-negative ($P = 0.0000043$, $\rho_s = 0.888$, $n = 16$) tumors, having considerably increased significance in the PR-negative tumors.

When the tumor samples were divided into those with associated LNM (+LNM) and those without associated LNM (−LNM), the most significant correlation observed amongst the RBM5-related factors in the (−)LNM primary tumors was between RBM6 and RBM5 + 6 ($P = 0.00017$, $\rho_s = 0.661$, $n = 27$), whereas in the (+)LNM primary tumors, it was between RBM6 and the anti-apoptotic RBM5Δ6 ($P = 0.00026$, $\rho_s = 0.716$, $n = 21$) (see supplementary data Tables SIII and SIV). These results suggest that alterations in RBM6 expression are related to both tumorigenesis and metastasis.

The fact that the significance of the positive correlation between RBM6 and RBM10v2 RNA expression increased in relation to advanced tumor grade, increased tumor size, advanced grade of DCIS, and loss of PR expression suggests that the coordinated expression of specific RBM6 and RBM10 variants is an important aspect of breast tumorigenesis.

RBM5 and RBM10v1 Protein Expression Was Significantly Elevated in Primary Breast Tissue

We next examined expression of the RBM5-related factors at the protein level. Despite the presence of at least four different RBM5 RNA transcripts, only two (RBM5 and RBM5Δ6) have been shown unequivocally to encode protein [Mourtada-Maarabouni et al., 2002, 2003]. Unfortunately, the LUCA-15-UK antibody appears unable to detect endogenous RBM5Δ6 protein, which has only previously been observed as a 17 kDa HA-tagged product [Mourtada-Maarabouni et al., 2003]. Thus, only the protein encoded by full-length RBM5 (~115 kDa) was investigated in this study. Likewise, only RBM10v1 protein was detected in this study, since the antibody was raised against peptide lacking from the RBM10v2 sequence (see Materials and Methods). No antibody for RBM6 was available.

Representative immunoblot protein expression data for RBM5, RBM10v1 and HER2, from four paired non-tumor and tumor samples, is presented in Figure 3A. The protein expression is further detailed, by individual patient, in Figure 3B and the significance of the rank distribution differences is presented in the boxplots depicted in Figure 3C. It was determined that RBM5 ($P < 0.0001$), RBM10v1 ($P < 0.0001$), and HER2 ($P < 0.0001$) protein expression levels were all significantly upregulated in the tumor compared to the non-tumor tissue.

RBM5 and RBM10v1 Protein Expression Levels Were Positively Correlated

We next investigated whether or not a relationship existed between the expression of RBM5 and RBM10v1 protein expression levels. We found that RBM5 and RBM10v1 protein expression levels were significantly positively correlated in both non-tumor and tumor tissue, more significantly in the non-overexpressing HER2 tumors, and independently of nodal involvement (Table II). While expression of RBM5 RNA was marginally reduced ($P = 0.052$) in primary tumors with associated LNM, expression of RBM10v1 RNA was unrelated ($P = 0.294$) to the metastatic status of the primary tumor. Expression of neither RBM5 ($P = 0.663$) nor RBM10v1 ($P = 0.684$) protein, however, was related to LNM status of the primary tumor (Table IV). The fact that RBM5 RNA but not protein expression levels were reduced in primary tumors with nodal involvement suggests that RBM5 protein was stabilized in this subset of tumors. In addition, the consistently strong positive correlation between the expression of both RBM5 and RBM10v1 protein suggests that they are functionally inter-related.

Protein Stability Was Altered in Relation to Tumor Status

As outlined in Table III, not only were RBM5, RBM10v1, and HER2 protein expression levels elevated in tumor compared to non-tumor tissue, but also each protein was expressed more frequently. Of particular interest was the

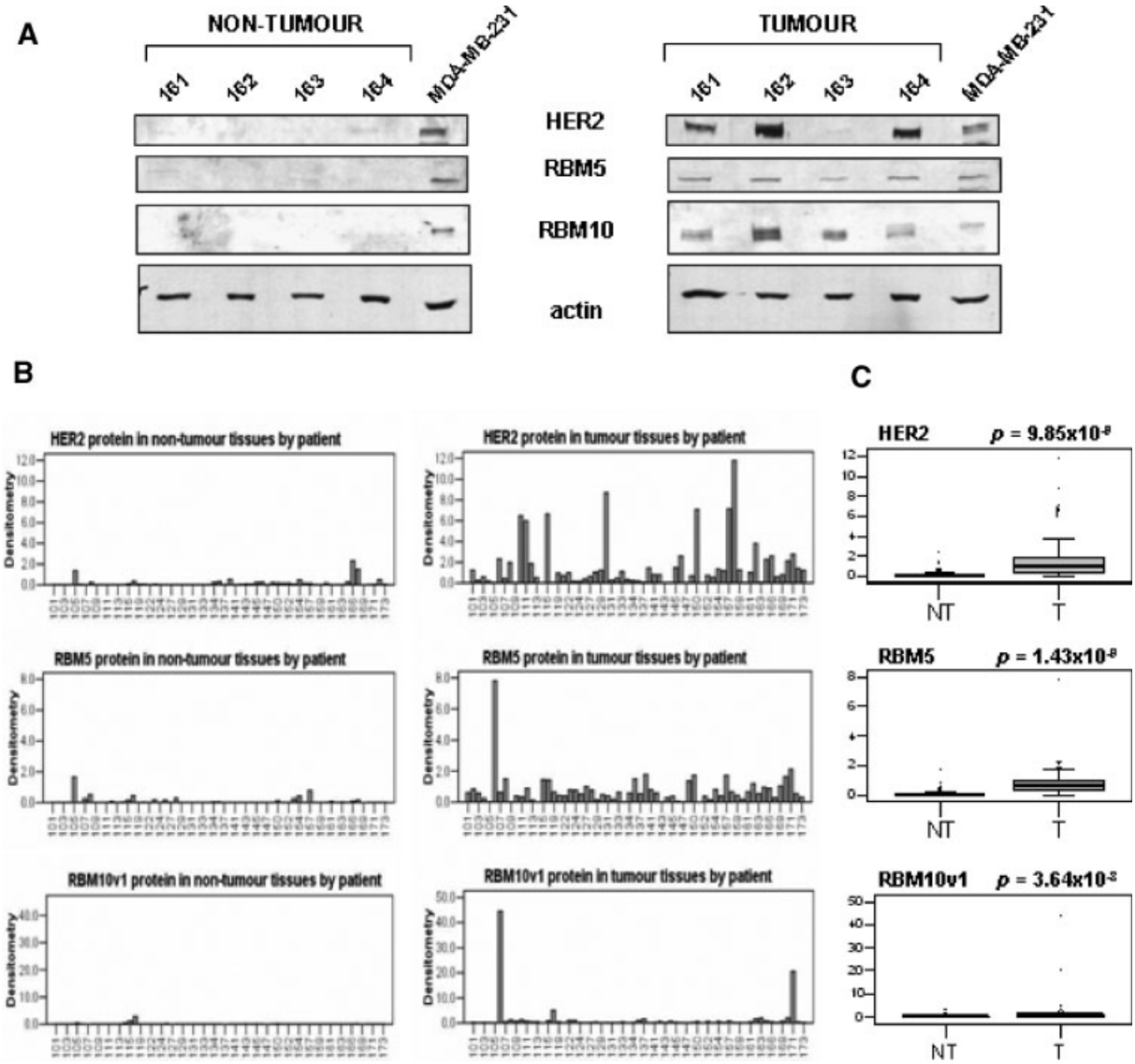

Fig. 3. Protein expression. **A**: RBM5, RBM10v1, and HER2 protein expression was measured by immunoblot. Detailed, are representative results from matched non-tumor and tumor specimens from four different patients. Whole cell lysate from the human breast cancer cell line MDA-MB-231 was loaded onto every gel, for normalization purposes, and to serve as a control for transfer and exposure variabilities between gels. Actin protein was monitored for normalization purposes, and as a loading control. **B**: The distribution of RBM5, RBM10v1, and HER2 protein in non-tumor and tumor samples was plotted in a boxplot, and (**C**) the significance of the data distribution was determined using the Wilcoxon Signed Ranks test. The Y-axis represents the densitometry reading of the normalized, standardized expression data. *P*-values represent the significance of the distribution differences between expression in non-tumor versus tumor tissue.

TABLE II. Protein Correlations Between RBM5 and RBM10v1

	Non-tumor	Tumor	Low HER2[b]	High HER2[b]	(−) LNM	(+) LNM
Correlation coefficient	0.428	0.501	0.456	0.682	0.533	0.516
Sig. (2-tailed)[a]	0.00082***	0.000039****	0.001**	0.010*	0.001**	0.006**
N	58	61	48	13	34	27

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$.
****$P < 0.0001$, as determined from the Spearman's rho test.
[a]*P*-values represent asymptotic two-tailed significance.
[b]as defined by IHC.

TABLE III. Expression Frequencies

	Variable	Number of samples with expression
Non-tumor tissues	RBM5 protein	21/62 (34%)
	RBM5 RNA	27/33 (82%)
	RBM10v1 protein	9/58 (16%)
	RBM10v1 RNA	3/33 (9%)
	HER2 protein	31/62 (50%)
	HER2 RNA	16/33 (48%)
Tumor tissues	RBM5 protein	56/61 (92%)
	RBM5 RNA	47/48 (98%)
	RBM10v1 protein	53/61 (87%)
	RBM10v1 RNA	13/48 (27%)
	HER2 protein	54/61 (89%)
	HER2 RNA	44/48 (92%)

observation that while 82% of the non-tumor specimens expressed RBM5 RNA but only 34% expressed RBM5 protein, approximately equal numbers of tumor specimens (~95%) expressed both. These findings suggest that in non-tumor tissue, there is a level of RBM5 translational control that is absent following tumorigenesis. For RBM10v1, whereas protein expression parallels that of RNA in the non-tumor tissue, in tumor tissue, protein expression was observed in more specimens than was RNA, suggesting increased protein stabilization of RBM10v1 protein in the tumor samples. In contrast to either RBM5 or RBM10v1 protein expression, HER2 RNA and protein expression levels appeared to be closely linked in both non-tumor and tumor samples. These findings suggest that a significant alteration in protein stabilization mechanisms is associated with RBM5-related factors during tumorigenesis.

RBM5 and RBM10v1 Protein Expression in Relation to Indicators of Poor Prognosis

The expression of RBM5 and RBM10v1 protein was examined in relation to the various prognostic indicators outlined in Table I. The results indicated that RBM5 protein expression levels were related to patient age, while RBM10v1 protein expression levels were related to tumor type.

The incidence of breast cancer increases in relation to the increasing age of the patient, plateauing around 75 years of age [Shen et al., 2005]; however, the younger the onset of the disease, the poorer the prognosis, particularly in women under 30 years of age [Xiong et al., 2001]. In our patient cohort, the mean age was 62.98 years, while the median age was 62.50 years. Age range was 51 years, from 40 to 91. Thirty-seven percent of our cohort was under 55 years of age.

Expression of only one RBM5-related RNA product—RBM5—was found to differ in relation to patient age (Table IV). The relationship was only significant, however, once the samples were divided into those not-overexpressing or those overexpressing HER2 (refer to Materials and Methods for HER2 overexpression details). RBM5 protein expression levels were significantly higher in the non-overexpressing HER2 tumors from patients under 55 years of age than in the non-overexpressing HER2 tumors from patients whose age was greater than or equal to 55 years ($P = 0.041$, as defined by IHC: the mean rank distribution in patients less than 55 years = 30.09, whereas the mean rank distribution in patients greater than or equal to 55 years = 21.44). The fact that the relationship between RBM5 expression and age was statistically significant irrespective of the HER2 overexpression determination method ($P = 0.041$, IHC; $P = 0.005$, IB), reinforced the strength of the observations. Average RBM5 RNA levels were unrelated to patient age, reinforcing our previous observation that the regulation of RBM5 protein expression levels occurs post-transcriptionally.

Invasive ductal carcinoma is the most common form of breast cancer, accounting for about 70% of all breast carcinomas, while invasive lobular carcinomas account for roughly 5% [Frykberg, 1999]. As summarized in Table I, 87% of our breast cancer specimens were invasive ductal carcinomas and 13% were invasive lobular carcinomas. At the RNA level, while the expression of the anti-apoptotic RBM5 splice variant RBM5Δ6 was significantly lower in lobular than in ductal carcinomas ($P = 0.047$: mean rank in lobular = 12.90, mean rank in ductal = 25.85), this relationship was not observed when HER2 overexpression was defined by IB analysis. At the protein level, however, the expression of RBM10v1 was significantly lower in lobular carcinomas compared to ductal carcinomas ($P = 0.005$, Table IV). This relationship between RBM10v1 protein expression and tumor type was dependent on HER2 expression status: only in the tumors not overexpressing HER2 was there a significant difference in the average expression level of RBM10v1 between ductal and lobular

TABLE IV. Relationship Between Protein Expression Levels and Various Clinicopathological Parameters

	All cases					(−) HER2					(+) HER2				
Parameters	Total no. of patients	Number expressing RBM5	RBM5	Number expressing RBM10v1	RBM10v1	Number of patients	Number expressing RBM5	RBM5	Number expressing RBM10v1	RBM10v1	Number of patients	Number expressing RBM5	RBM5	Number expressing RBM10v1	RBM10v1
Age (years)															
Less than 55	23	22	0.079	20	0.360	17	16	**0.041***	15	0.305	6	6	0.886	5	0.943
Greater than or equal to 55	38	34		33		31	28		27		7	6		6	
Tumor type															
Ductal	53	49	0.104	48	**0.005****	43	39	0.338	39	**0.001****	10	10	0.287	9	0.469
Lobular	8	7		5		5	5		3		3	2		2	
Tumor grade															
1	7	6	0.371	6	0.306	7	6	0.568	6	0.453	0	0	0.391	0	0.391
2	26	24		22		20	19		18		6	5		4	
3	28	26		25		21	19		18		7	7		7	
Tumor size															
2 cm or less	25	22	0.581	22	0.405	22	19	0.799	20	0.095	3	3	0.673	2	0.719
>2 cm to 5 cm	29	28		27		24	23		22		5	5		5	
>5 cm	7	6		4		2	2		0		5	4		4	
DCIS grade															
0	17	16	0.434	15	0.201	16	15	0.386	15	0.142	1	1	0.426	0	0.214
1	1	1		1		1	1		1		0	0		0	
2	13	12		13		11	10		11		2	2		2	
3	22	20		19		15	13		12		7	7		7	
Lymph node metastases															
Positive	27	25	0.663	23	0.684	20	19	0.992	18	0.967	7	6	0.391	5	0.668
Negative	34	31		30		28	25		24		6	6		6	
HER2 (by IHC)															
Positive	13	12	0.549	11	0.860				n/a					n/a	
Negative	48	44		42											
Estrogen receptor															
Positive	48	44	0.660	41	0.127	37	34	0.941	31	0.071	11	10	0.167	10	0.621
Negative	13	12		12		11	10		11		2	2		1	
Progesterone receptor															
Positive	38	35	0.732	32	0.283	30	28	0.670	25	0.332	8	7	0.770	7	0.714
Negative	23	21		21		18	16		17		5	5		4	
	61	56		53		48	44		42		13	12		11	

(−) HER2, non-overexpressed HER2; (+) HER2, overexpressed HER2, as defined by immunohistochemistry (IHC); DCIS, ductal carcinoma in situ; n/a, not applicable.
Values represent asymptotic two-tailed significance, with asterisks denoting $^{*}P < 0.05$ and $^{**}P < 0.01$, either from the Mann–Whitney U-test (for two-level parameters) or the Kruskal–Wallis H-test (for multi-level parameters).

carcinomas ($P = 0.001$, Table IV). This relationship was statistically significant according to either definition of HER2 overexpression (IHC or IB: $P = 0.009$ by IB), thus reinforcing the strength of the observed relationship.

In summary, expression of neither RBM5 nor RBM10v1 protein was particularly related to factors associated with poor prognosis.

RBM5 and HER2 Protein Expression Levels Were Positively Correlated

Oh et al. [1999] had previously determined that RBM5 RNA expression was positively correlated with HER2 RNA expression in 15 primary breast tumor tissues. While our analyses in 33 primary non-tumor and 48 primary tumor samples did reveal a positive correlation between RBM5 and HER2 RNA expression in the non-tumor tissue ($P = 0.001$, $\rho_s = 0.546$, $n = 33$), it did not reveal any correlation between RBM5 and HER2 RNA expression in the tumor tissue ($P = 0.198$, $\rho_s = 0.189$, $n = 48$) (see supplementary data Tables SI and SII). At the protein level, however, a positive correlation was observed in both non-tumor ($P = 0.007$, $\rho_s = 0.342$, $n = 62$) and tumor tissue ($P = 0.00047$, $\rho_s = 0.434$, $n = 61$) (Table V). We noted that when RBM5 protein expression in the tumor samples was examined in relation to "low" (non-overexpressed) and "high" (overexpressed) HER2 protein expression levels, the average RBM5 protein expression level was not significantly different with regard to HER2 status (determined using a Mann–Whitney U-test, $P = 0.549$) suggesting that RBM5 and HER2 protein expression levels were not positively correlated in HER2 overexpressing tumors. Indeed, further analyses revealed that the significant positive correlation between RBM5 and HER2 protein expression was only significant in the tumors that did not overexpress HER2, regardless of the HER2 overexpression definition method employed (e.g., IHC or IB) (Table V). These results demonstrated that there was no correlation between RBM5 and HER2 expression when HER2 was overexpressed, that is, in aggressive breast cancers, suggesting that the original hypothesis of Oh et al. [1999], that RBM5 contributes to the aggressive phenotype associated with HER2 overexpressed breast tumors, is unlikely to be valid.

Interestingly, despite the strong positive correlation between RBM5 and RBM10v1 protein expression, only a marginal positive correlation was observed between RBM10v1 and HER2, in either non-tumor or tumor tissue (Table V). This result suggests that the relationship between HER2 and RBM5 is more significant than the relationship between HER2 and RBM10v1, and that the correlation between RBM5 and RBM10v1 occurs in a subset of cells that is distinct from the subset in which RBM5 protein expression is strongly positively correlated with HER2 expression.

RBM5 and HER2 Protein Levels Were Most Significantly Positively Correlated in Low HER2 Expressing Primary Tumors With Associated Lymph Node Metastasis

Using a Mann–Whitney U-test, RBM5 RNA expression was shown to be marginally related to the LNM status of the primary tumor ($P = 0.052$); however, no relationship was

TABLE V. Correlations Between RBM5, RBM10v1, and HER2 Protein Expression in Non-(Low) and Over-(High) Expressing HER Primary Breast Tumors

			IHC		IB	
	Non-tumor	Tumor	Low HER2	High HER2	Low HER2	High HER2
RBM5						
Correlation coefficient	0.342	0.434	0.468	0.490	0.444	0.164
Sig. (2-tailed)[a]	0.007**	0.00047***	0.00079***	0.089	0.001**	0.631
N	62	61	48	13	50	11
RBM10v1						
Correlation coefficient	0.272	0.249	0.182	0.278	0.153	−0.173
Sig. (two-tailed)	0.039*	0.053	0.216	0.357	0.289	0.612
N	58	61	48	13	50	11

(−) LNM, lymph node metastasis negative; (+) LNM, lymph node metastasis positive; IHC, immunohistochemistry; IB, immunoblot.
[a]P-values represent asymptotic two-tailed significance, with asterisks denoting $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, from the Spearman's rho test.

observed at the protein level ($P = 0.663$). Neither HER2 RNA nor protein expression was related to the LNM status of the primary tumor ($P = 0.323$ and $P = 0.942$, respectively). However, in an attempt to better understand the positive correlation between RBM5 and HER2 protein in tumor tissue, we examined the relationship between RBM5 protein expression and metastatis more closely, by investigating the relationship between RBM5 and HER2 in primary tumors associated with LNM. Tumor samples were divided into those having associated LNM and those that did not, and the correlation between RBM5 and HER2 protein in these subcategories was examined. Of all the patient samples, 34/61 (56%) were from tumors with no associated LNM, while 27/61 (44%) were from tumors with associated LNM. Using the Spearman's rho rank test, the correlation between RBM5 and HER2 protein was found to be only significant in the primary tumor samples that were associated with LNM ($P = 0.001, \rho_s = 0.607$, $n = 27$) (no associated LNM: $P = 0.065$, $\rho_s = 0.321$, $n = 34$). Further subdivision of the breast tumor samples with and without LNM, into those not overexpressing and those overexpressing HER2, revealed that RBM5 and HER2 were most significantly positively correlated in the non-overexpressing HER2 tumors that had associated LNM ($P = 0.003$, $\rho_s = 0.627$, $n = 20$) (see Supplementary data Table SV). (One must, however, be cognizant of the reduced sample numbers, especially in the HER2 overexpressing category.) The data therefore suggest that when metastatic disease is absent, the correlation between RBM5 and HER2 in the non-aggressive tumors is weakened.

HER2 Overexpression Was Associated With Reduced RBM5 + 5 + 6 RNA Expression

It is interesting to note that although there appears to be no relationship between RBM5 and HER2 RNA expression in HER2 overexpressing tumors, we did observe reduced expression of the potentially pro-apoptotic variant RBM5 + 5 + 6 ($P = 0.016$), the anti-apoptotic variant RBM5Δ6 ($P = 0.021$) and RBM10v2 ($P = 0.023$) in relation to overexpressed HER2 protein; however, since the reduced expression was only observed when HER2 overexpression was defined by IHC, not by IB, the results were not considered highly significant in this data cohort.

Interestingly, in previous functional studies carried out in the lab, we had demonstrated that upregulation of exogenous HER2 in the MDA-MB-231 human breast adenocarcinoma cell line resulted in a dramatic downregulation of RBM5 + 5 + 6. We therefore extended this analysis to include all the RBM5-related RNA transcripts, with particular focus on RBM5Δ6 and RBM10v2. As seen in Figure 4, the only transcript that was found to have significantly altered expression as a result of HER2 overexpression remained RBM5 + 5 + 6.

This mechanistic result validates the breast tissue expression data, which also demonstrated decreased expression of RBM5 + 5 + 6 in HER2 overexpressing primary breast tumors. The RBM5 + 5 + 6/RBM5 + 6-specific primers used in the study described herein (LU15(2)/LS5) do not distinguish between

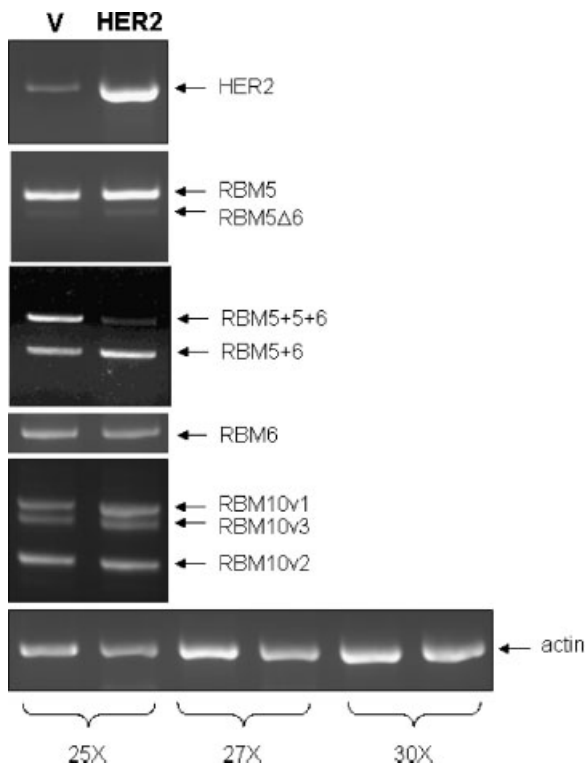

Fig. 4. The effect of HER2 upregulation on RBM5-related transcripts in MDA-MB-231 cells. Cells were transfected with either pcDNA3.1.myc.his (V) or pcDNA3.1.myc.his.HER2 (HER2), and HER2 RNA expression was confirmed by RT-PCR. Expression of each of the RBM5-related transcripts was monitored by RT-PCR, using gene-specific primers, as described in Materials and Methods. Actin expression was monitored using 25, 27, or 30 amplification cycles in the PCR reaction, to ensure that reactions were not saturated due to the use of excessive amounts of cDNA. Two percent agarose gel stained with 0.1 μg/ml ethidium bromide.

expression of RBM5 + 5 + 6 or a truncated RBM5 + 5 + 6 variant, termed Clone 26. Whereas full-length RBM5 + 5 + 6 RNA fits all the criteria for the process of nonsense mediated decay, and is therefore unlikely to encode protein, Clone 26 encodes a 21-kDa product (unpublished observations) that is pro-apoptotic [Sutherland et al., 2000]. These results are therefore highly significant in that they demonstrate that upregulation of the HER2 oncogene is related to decreased expression of a pro-apoptotic, tumor suppressor-related protein in a breast cancer environment.

The fact that neither RBM5Δ6 nor RBM10v2 showed decreased expression in the HER2-transfected MDA-MB-231 cell line may indicate (1) that the breast tissue results were only of borderline significance (which might explain the lack of any observed relationship when HER2 status was defined using the IB technique) or (2) that any possible HER2-mediated affect on RBM5Δ6 and RBM10v2 expression is inhibited in MDA-MB-231 cells.

DISCUSSION

The work described in this study demonstrates that, contrary to results expected based on the 17-gene metastatic signature study [Ramaswamy et al., 2003], where RBM5 RNA was downregulated, but anticipated from the results of the Oh et al. [1999] study, where RBM5 RNA was upregulated in primary breast tissue, RBM5 protein was indeed expressed, and in fact upregulated, in primary breast tumor compared to non-tumor cells. RBM10v1 protein was also upregulated in the tumor cells, expression being significantly positively correlated with expression of RBM5 protein. This strong expression correlation, considered in light of the high degree of sequence identify between the two proteins, is suggestive of a functional association. Two opposing functional associations can be envisioned: (1) RBM10v1 protein antagonises RBM5 function, and parallel expression prevents RBM5-related tumor suppressor activity, or (2) parallel expression of RBM10v1 protein is necessary for RBM5-related tumor suppressor activity. After all, RBM5 protein is ubiquitously expressed, but not pro-apoptotic unless overexpressed. If indeed, expression of both RBM5 and RBM10v1 are required for tumor suppressor activity, then the presence of both proteins in tumors would suggest that they are functionally inactive, for example, inactivated by a post-translational modification, or inactivated as a result of downstream signaling pathway interruptions. Preliminary evidence from our lab suggests that RBM10v1 is in fact a pro-apoptotic molecule. Other work from our lab suggests that RBM5 is phosphorylated and that dephosphorylation is associated with apoptotic activity [Shu et al., in press]. Future studies will be aimed at determining the phosphorylation status of both RBM5 and RBM10v1 protein in primary breast tumors.

The expression data also provide functional insights. For instance, based on the observation that the expression of RBM10v1 and the expression of RBM10v2 are very differently correlated with other RBM5-related factors, and expression levels are related to different pathological parameters (e.g., RBM10v2, but not RBM10v1, is highly significantly positively correlated with RBM6 RNA expression in relation to a number of indicators of poor prognosis), it suggests that RBM10v1 and RBM10v2 either have different functions, or similar functions in different niches. These findings are, however, in direct contrast to the work of Martinez-Arribas et al. [2006], who noted a significant relationship between the expression of both RBM10 variants at the RNA level ($P = 0.006$). Unfortunately, more detailed analyses relating to RBM10 protein expression await the generation of an antibody that can detect RBM10v2.

The expression data also provide some insight into possible mechanisms at play related to the expression of RBM5-related factors and cancer. The data clearly demonstrate that altered regulation of both protein and gene expression of RBM5-related factors is associated with oncogenesis. The elevated RBM5 protein levels seen in tumor tissue compared to the non-tumor tissue result from either increased translation or protein stabilization. The fact that RBM5 protein levels do not decrease in the primary tumors with associated metastases despite a reduction in RNA expression levels, suggests that in this subset of tumor cells, RBM5 protein degradation (perhaps involving ubiquitination pathways) is inhibited. Whatever the mechanism is that regulates RBM5 protein expression levels, it appears to be related to the mechanism regulating RBM10v1 protein expression levels,

since, regardless of tumor status or LNM status, the expression levels of both proteins remain significantly positively correlated.

In addition, the results suggest that alternative splicing has important repercussions for breast cancer development. For instance, downregulation of the specific RBM5 splice variant RBM5 + 5 + 6 was related to HER2 protein upregulation. Since the majority of upregulated HER2 in breast cancer stems from gene amplification, it is more likely that HER2 protein expression influences RBM5 + 5 + 6 variant expression than vice versa. This was confirmed by our in vivo analysis using the MDA-MB-231 human breast carcinoma cells, where upregulation of exogenous HER2 resulted in decreased expression of the RBM5 + 5 + 6 transcript. Disruption of apoptosis modulatory activity and potential tumor suppressor function is consistent with the potent oncogenic activity associated with HER2 expression.

Results from this study also helped to clarify the previously reported counterintuitive relationship between RBM5 and HER2 expression. In the study by Oh et al. [1999], it was reported that (a) upregulation of HER2 in both a breast and ovarian cancer cell line resulted in a moderate upregulation of RBM5 mRNA, and (b) a positive correlation existed between HER2 and RBM5 mRNA expression in 15 primary breast tumor specimens. The definition of HER2 overexpression was not indicated in this study, although the level of exogenous HER2 expression in the two cell line studies was reported to be equivalent to clinically relevant overexpression levels. HER2 RNA expression levels in the primary tumor specimens were visually, relatively described from Northern blots: "overexpression" was not defined. In our study, the experimental design was expanded to incorporate an examination of both RNA and protein, in non-tumor and tumor tissue, with clearly defined HER2 overexpression, in four times as many samples. HER2 overexpression was defined using two different methods, so as to incorporate clinical relevance with arguably more relevant scientific analysis, thereby providing a degree of confidence when both methods confirmed a correlation or relationship. The data reported in our study clearly demonstrate that not only was RBM5 protein expressed in the majority of tumors examined, but also it was upregulated compared to expression in non-tumor tissue. Interestingly, although a positive correlation was observed between RBM5 and HER2 protein expression in breast tumors, a positive correlation was not observed at the RNA level, unlike the observations reported by Oh et al. [1999]. Importantly, RBM5 protein expression did not correlate with HER2 levels in HER2 overexpressing tumors, but did correlate with HER2 levels in those tumors that did not overexpress HER2. In addition, the average level of RBM5 protein expression did not differ in relation to HER2 status in the tumor. Therefore, it cannot be concluded, as was suggested in the original study [Oh et al., 1999] that RBM5 contributes to the aggressive phenotype associated with overexpressed HER2, since it appears that the correlation between RBM5 and HER2 was lost in the HER2 overexpressors. These findings are more consistent with a tumor suppressor function for RBM5 than the Oh et al. study.

Perhaps the most striking observation from this study was the highly significant positive correlation between specific splice variants of RBM6 (RBM6A and/or RBM6Δ6) and RBM10 (RBM10v2) in relation to high tumor grade ($P = 0.000001$, $\rho_s = 0.873$, $n = 21$), large tumor size ($P = 0.000013$, $\rho_s = 0.777$, $n = 23$), high DCIS grade ($P = 0.000021$, $\rho_s = 0.874$, $n = 15$), and PR negative status ($P = 0.0000043$, $\rho_s = 0.888$, $n = 16$). Although no survival data were available to correlate with either the generally accepted prognostic indicators or the expression data, the results suggest that altered expression of RBM6 and RBM10v2 is an important aspect of breast tumor development.

Finally, although it is compelling to conclude that the downregulation of RBM5 RNA expression, as part of a 17-gene metastatic signature, has special significance with regard to tumor suppressor function, the broader view paints a very different picture. Our results demonstrate that whereas gene expression array signatures may be used as important diagnostic, prognostic, and/or predictive indicators, they may not represent causal factors inasmuch as gene expression represents only one facet of cellular physiology, all facets of which must be considered together to ultimately clarify the etiology of cancer and identify true therapeutic targets.

Without risking over-interpretation of the data, which was accumulated using semi-quantitative RT-PCR conditions that could have involved saturation of some samples (30–40 cycles), the results of this study suggest that

RBM5-related products are differentially expressed in relation to each other and in relation to a number of pathological parameters that are useful prognostic indicators. This differential expression suggests complex regulatory networks involving alternative splicing, tissue specificity (with reference to lobular versus ductal expression) and oncogenic expression (HER2). The generally non-overlapping nature of the expression of these RBM5-related products in relation to the individual pathological parameters suggests at least a certain degree of functional independence. The implication of these results is that the regulation of RBM5, RBM6, and RBM10 gene expression, including alternative splicing, is important to the pathogenesis of breast cancer, and that a better understanding of their regulation may reveal novel potential therapeutic avenues. Whether or not the expression patterns reflect a cause or consequence of breast tumor progression, remains to be determined. Further analysis, incorporating protein expression data for RBM6, RBM10v2, and alternative RBM5 variants, might also help to clarify any potential prognostic value relating to RBM5-related product expression in breast cancer. Thus, although this was largely an exploratory study, significant results, with some mechanistic confirmation, suggest that regulation of expression of this novel family of RNA binding proteins is important to breast cancer progression.

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