A Decreased Ratio of Laminin-332 β3 to γ2 Subunit mRNA is Associated with Poor Prognosis in Colon Cancer

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Abstract

Laminin-332 (Ln-332) is a heterotrimeric glycoprotein (α3β3γ2) unique to epithelial cells with crucial roles in signaling, adhesion, and migration. Altered localization or expression levels of Ln-332, particularly its γ2 subunit, are of prognostic value in a variety of cancers. However, the lack of standardized methodology and the limited quantification of previous study results have left unanswered questions, including the role of γ2 transcript variants and whether differential expression of this chain represents dysregulation of the whole heterotrimer. Herein, we test the hypothesis that mRNA changes in one or more Ln-332 encoding genes can be used to distinguish between early- and advanced-stage cancer specimens and shed light on mechanistic questions raised by previous studies. Statistical analyses of human microarray data from the publicly available expression project in Oncology (expO) dataset, including examination of the distributions of Ln-332 subunit mRNA levels, identified a significant decrease in the Ln-332 β3:γ2 mRNA ratio between normal (n = 10) and early-stage colon cancer (n = 29) specimens. The β3:γ2 ratio was further decreased in metastatic colon cancer (n = 41) compared with early-stage samples. Our findings raise the possibility that Ln-332 γ2 may be a therapeutic target against metastatic colon cancer because a lowered β3:γ2 ratio would reduce expression of heterotrimeric Ln-332 and increase monomeric γ2 secretion. Further, standardized, quantitative methods for patient prognosis and therapeutic choice could be developed based upon the Ln-332 mRNA changes we uncovered. (Cancer Epidemiol Biomarkers Prev 2009;18(5):1584–90)

Introduction

Cancer metastasis inherently requires local invasion (1, 2). In the case of carcinomas, local invasion itself is a multistep process involving degradation of basement membrane components that separate epithelial cells from underlying stromal constituents, followed by migration through the newly cleared pathway (3). One major basement membrane component that has been variously implicated in cancer progression is the heterotrimeric glycoprotein laminin-332 (Ln-332; refs. 4, 5). Ln-332 consists of three individual chains intertwined into a heterotrimer (α3β3γ2). Although each of these chains, α3 (*LAMA3*), β3 (*LAMB3*), and γ2 (*LAMC2*), has been reported to play a role in cancer progression (6), the γ2 chain is the most studied in this context. Numerous immunohistochemistry and *in situ* hybridization studies have shown γ2 chains to be localized at the leading edge of invading carcinomas, with its expression positively correlated to invasiveness and patient survival (6). In hepatocellular cancers, the γ2 chain is involved in epithelial to mesenchymal transition (7) and is strongly associated with metastasis (8). In head and neck cancer, Ln-332 γ2 is correlated to patient prognosis (9), as shown very recently by transcriptome analysis, which unveiled *LAMC2* (the gene encoding γ2) among a panel of up-regulated genes in oral tongue squamous cell carcinoma samples (10). Moreover, differential γ2 localization or expression levels have been shown to be of prognostic value in colorectal (11), anal (12), pancreatic (13), and gastric cancers (14).

Although there is clearly an involvement of γ2 in these various cancers, many important questions still remain unanswered. For instance, it is uncertain whether an increase in Ln-332 γ2 staining, seen in many studies, is due to an increased protein level or simply reflects differential localization or cleavage of the chain within the tumor. Because γ2 is specific to the Ln-332 isoform, it is frequently used as a surrogate marker for the presence of the entire heterotrimer; thus, expression of the other two chains is not always examined. The problem with this approach is that γ2 can be secreted both in heterotrimeric form and as a monomer (15), so without verifying the presence of the other two chains there is ambiguity over the context of γ2 chain expression.

Another concern is the wide variation in experimental assessment techniques for probing γ2 expression. Immunohistochemical staining, using various antibodies and fluorescent labels, has been the most widely used method for protein expression studies in human cancer tissues (6). For mRNA expression studies, *in situ* hybridization has been the most popular method employed, followed by Northern blotting. The problem with these techniques is that there is currently no accepted standard as to which protocol, label, or probe is used, creating a wide range of confusing results within the field. Further, distinction between the two γ2-encoding transcript variants (LAMC2 and LAMC2*) is rarely made. There is a report

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showing LAMC2 to be the more broadly expressed form in normal tissues (16), yet there are no published reports distinguishing between LAMC2 and LAMC2* expression in cancer. The LAMC2* expression pattern is important to examine, because the COOH-terminal cysteine residue missing from its protein product (17) is in a region predicted to be mandatory for stable heterotrimer formation (18, 19). Taken together, these issues make it impossible to directly compare experimental results of Ln-332 γ2 expression across studies from different laboratories. Nonetheless, the array of published reports is intriguing, as this molecule is clearly implicated in cancer progression, warranting further investigations.

Our primary goal in this study was to address whether the current reports of dysregulated γ2 expression in cancer tissue reflect increased synthesis of γ2, either as monomer or as a component of the Ln-332 heterotrimer. This aim required implementation of a method that: (*a*) examines all three chains simultaneously in any given sample; (*b*) provides a sensitive, reproducible, quantitative platform for detecting subtle differences that occur experimentally; and (*c*) distinguishes between transcript variants of Ln-332 γ2 (LAMC2 versus LAMC2*). The method that most closely fits these three requirements seems to be the highly standardized Affymetrix microarray platform commonly used for transcriptional analysis (20, 21).

The expression project in Oncology (expO) dataset (National Center for Biotechnology Information, GSE2109), compiled by the International Genomics Consortium, was chosen for data-mining due to the high number (n = 870) of tumors from different tissue types and various pathologic stages available at the time of analysis, which allowed us to test several hypotheses within the same dataset. Statistical analyses of mRNA expression data from this cancer dataset were done to search for significant changes in Ln-332 encoding genes among low-stage and metastatic cancers. These analyses revealed, for the first time, a link between decreased Ln-332 β3:γ2 expression ratio and metastasis in colon cancer.

Materials and Methods

Affymetrix Microarray Analysis. Microarray experiments were done per manufacturer's protocol by the International Genomics Consortium for their Expression Project for Oncology, using Affymetrix HG-U33 Plus 2.0 chips. This dataset is publicly available via the National Center for Biotechnology Information Gene Expression Omnibus website (http://www.ncbi.nlm.nih.gov/geo/), under accession number GSE2109. Expression data and associated patient information were downloaded by the Vanderbilt Microarray Shared Resource in May 2006, and normalized using the Microarray Suite 5.0 (MAS5.0) algorithm. Due to our interest in Ln-332, only the following probesets were analyzed: 202267_at (LAMC2), 207517_at (LAMC2*), 203726_s_at (LAMA3), and 209270_s_at (LAMB3).

The expO dataset (n = 870) includes patient tumor data from various pathologic stages (Pstage; 1a-d to 4a-d) and numerous tissue types (n > 8), allowing us to test several hypotheses within the same dataset. For this study, only samples with associated Pstage data listed were considered (n = 710). Further, because ''normal'' (i.e., noncancerous) controls were not a part of the expO study, a separate dataset (GSE4107; n = 10), obtained using the same methodology, was used as a baseline for our studies. Data downloaded by the Vanderbilt Microarray Shared Resource were provided as a single text file and subsequently organized into Microsoft Excel spreadsheets for data storage and analysis.

Statistical Analyses. Samples with Pstage 1a-2d were considered low-stage cancer. Samples listed as Pstage 4, or those cases where the isolation site differed from the primary onset site, were considered metastatic (late stage) cancers. Pstage 3 cases were omitted from this study due to controversy regarding potentially inappropriate classification of samples collected during this stage of disease. Separate Excel spreadsheets were generated for each histology type examined (i.e., low-stage carcinoma, metastatic carcinoma, noncarcinoma, and normal samples), which were further subdivided by tissue type to allow tissue-specific analyses to be done. Mean mRNA signal values and SD for each Ln-332 transcript were calculated on the $\log_{10}$ scale in SAS version 9.1.3 (SAS Institute, Inc.) and box-plots were generated.

For colon-specific analysis, comparisons were made between low-stage (n = 29) and metastatic (n = 41) colon carcinoma samples. To examine expression trends missed by simple examination of means, distributions of the expression data were also plotted. The relative frequencies of log-transformed signals were plotted using the density subroutine in *R*.[5] Additionally, the Ln-332 β3:γ2 chain ratios were examined. Differences of signal values on the log scale are mathematically equivalent to the log ratio, so differences in $\log_{10}$ Ln-332 chain signal values were determined using ANOVA techniques and the overall F-test for each tissue type, Ln-332 transcript, and interaction between tissue type and transcript. Ln-332 chain ratio distribution differences were tested using the Wilcoxon rank sum test statistic.

Results

Low-Stage and Metastatic Carcinomas from Mixed-Tissue Types Have Similar mRNA Levels of Ln-332 γ2 Chain. The simplest explanation for previous findings that increased γ2 expression is associated with poor prognosis is that γ2 protein levels, and presumably mRNA levels as well, are up-regulated. Because the γ2 chain is encoded by two mRNA variants, LAMC2 and LAMC2* (16), we felt it necessary to examine both transcripts to address whether one of them increases, there is a switch in transcript preference, and/or there is an additive effect of the two transcripts leading to an overall net increase in γ2 message. We tested these possibilities by direct comparison of mean LAMC2 (full length) and LAMC2* (truncated) mRNA levels, as determined by the expO study. All individual tumor mRNA samples had been analyzed by hybridization to Affymetrix HGU133 PLUS2.0 chips, as detailed in Materials and Methods.

The LAMC2 and LAMC2* mRNA expression levels of low-stage (n = 163) and metastatic (n = 151) carcinomas

[5] http://www.R-project.org

from various tissue types was first compared (Fig. 1A). Noncarcinoma levels were included as a pseudo-negative control here, because noncarcinomas are derived from nonepithelial tissue and should therefore have negligible Ln-332 levels. Noncarcinoma mean mRNA levels, for both transcripts, were significantly ($P < 0.0001$ for all comparisons except LAMA3 versus LAMAB3 with $P = 0.0229$) lower than other samples (Fig. 1A). Additionally, mean LAMC2* mRNA levels were drastically ($P < 0.0001$) lower than LAMC2 levels, regardless of histology (Fig. 1A), as observed in previous studies conducted in normal tissue (16). Although there were no significant differences ($P = 0.6640$) between γ2-encoding transcripts from low-stage and metastatic samples, there may have been changes in the other Ln-332 chains, which would also alter the amount of heterotrimer able to form. To test this possibility, we also compared α3 (LAMA3) and β3 (LAMB3) mRNA levels in the same low-stage and metastatic carcinomas used for γ2 (LAMC2/LAMC2*) transcript comparison (Fig. 1B). The LAMB3 transcript generally seemed to be more abundant than the other three transcripts (Fig. 1B). Both LAMA3 and LAMB3 mRNA levels, like LAMC2 and LAMC2*, were not significantly different ($P = 0.4049$) across low-stage and metastatic cancers (Fig. 1B). LAMA3 signal intensity levels of low-stage and metastatic carcinomas were 2.772 ± 0.448 and 2.705 ± 0.555, respectively. Similarly, LAMB3 signal intensity levels were 2.958 ± 0.510 and 2.958 ± 0.509, respectively.

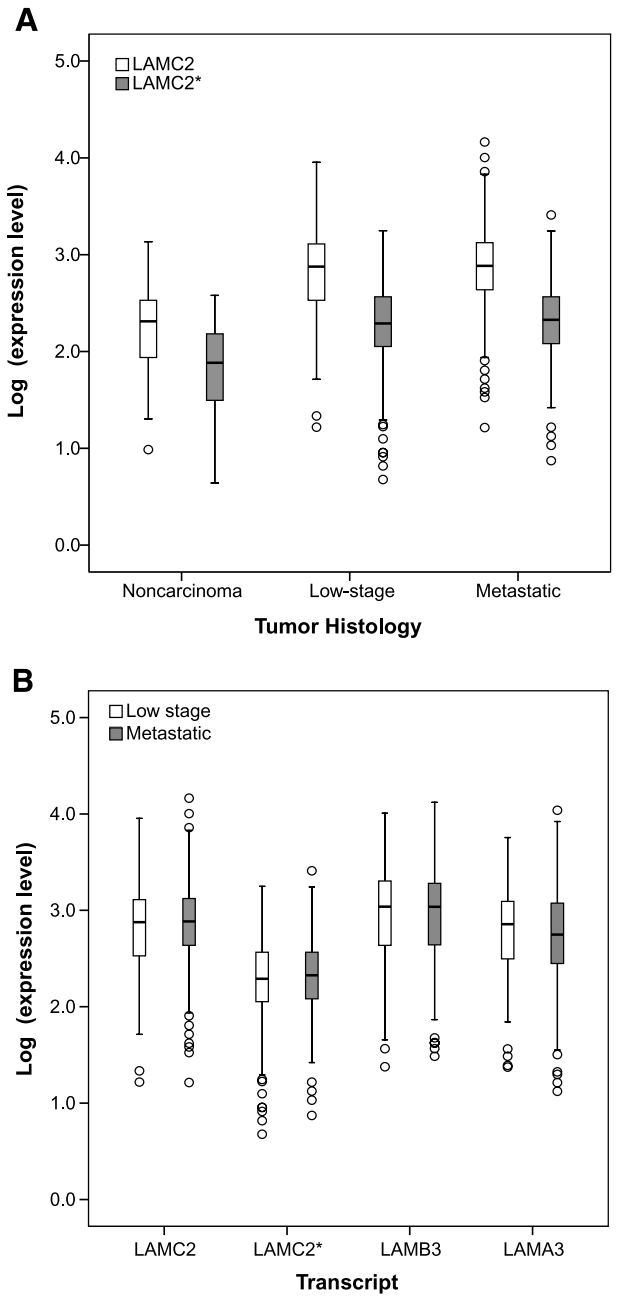

Figure 1. Box and whisker plots showing (**A**) log-transformed LAMC2 versus LAMC2* mRNA expression levels of noncarcinoma ($n = 56$), low-stage ($n = 163$), and metastatic ($n = 151$) cancer samples from expO microarray dataset (NCBI GEO GSE 2109) or (**B**) log-transformed LAMC2, LAMC2*, LAMB3, and LAMA3 mRNA expression of low-stage versus metastatic samples.

Taken together, these results show that large-scale global differences in Ln-332 mRNA chain levels either do not exist, or cannot be evaluated among several cancer tissue types in aggregate. To evaluate the latter, studies were subsequently focused in a tissue type–specific manner, so that inherently different basal Ln-332 expression levels among various tissues would not cloud analyses. Initial focus was on the colon, because colon cancer has extensive literature connections between γ2 dysregulation and disease prognosis. Further, the expO dataset contained a large enough number of colon samples to be adequately powered to detect statistically significant differences.

Low-Stage and Metastatic Colon Carcinomas Have Similar Ln-332 mRNA Mean Levels. Full-length γ2 (LAMC2) versus truncated variant (LAMC2*) expression in low-stage ($n = 41$) and metastatic ($n = 29$) colon carcinoma samples followed a similar trend as mixed-tissue cancer samples. That is, LAMC2* variant mRNA levels were again lower than LAMC2 levels but not significantly ($P = 0.1013$), and there was a negligible increase in LAMC2 signal in metastatic samples, as compared with low-stage samples (3.019 ± 0.2730 to 2.965 ± 0.2134, respectively; Fig. 2). As with the previous mixed-tissue analysis, colon cancer LAMB3 levels were highest, followed by LAMA3, LAMC2, and finally very low levels of LAMC2* were recorded. For low-stage samples, these values were 3.191 ± 0.2002, 2.978 ± 0.2456, 2.965 ± 0.2134, and 2.445 ± 0.3272, respectively. For metastatic samples, values were 3.152 ± 0.2985, 2.760 ± 0.4529, 3.019 ± 0.2731, and 2.446 ± 0.3221. Because there were no normal (non–cancer) tissues sampled in the expO dataset, we used normal colon samples from the National Center for Biotechnology Information GEO's GSE4107 dataset for a baseline comparison with expO's colon cancer samples. These samples were analyzed exactly as the samples in the expO dataset. Interestingly, LAMC2 and LAMC2* transcript levels of normal colon tissue were calculated to be 25% and 85% below even low-stage colon cancer

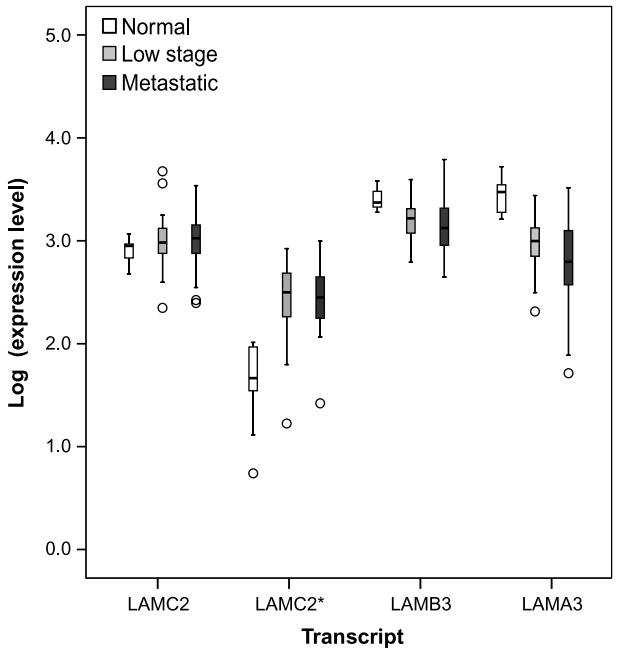

Figure 2. Log-transformed Ln-332 transcript levels from microarray of normal colon (n = 10), low-stage colon cancer (n = 41), and metastatic colon cancer samples (n = 29). Note that there is little difference between the trends of the low-stage and metastatic colon cancer samples.

samples, respectively (Fig. 2). Unlike colon cancer samples, LAMB3 levels in normal colon were not ranked highest (Fig. 2). In contrast, these samples had expression values equal to 2.735 ± 0.5020 for LAMC2, 2.206 ± 0.4501 for LAMC2* variant, 2.837 ± 0.5563 for LAMB3, and 2.680 ± 0.5377 for LAMA3 transcripts.

Ln-332 Heterotrimer Expression is Reduced in Metastatic Colon Tumors. Standard deviations among the signal intensity values of the colon cancer samples were high among each of the probe sets, raising the possibility that analyses based on the difference of mean expression levels (t-tests) alone might not be optimal. Therefore, the densities (relative frequencies) of the log-transformed mRNA signals were examined to compare shifts in the distributions between each chain. Compared with the distributions of low-stage colon cancer samples (Fig. 3A), metastatic tumors showed lower log expression levels for all three chains (Fig. 3B).

Ln-332 β3:γ2 Ratio is Reduced During Progression to Metastatic Colon Tumors. We next probed for possible changes in the way the γ2 chain is expressed. Heterotrimer formation first requires assembly of β3γ2 dimer, which is rapidly degraded if no α3 chain is present (22-24). The α3:γ2 ratio could, therefore, be informative for determination of Ln-332 heterotrimer levels. However, interpretation of this data would have been somewhat difficult, because the α3 chain is also a component of two other laminin heterotrimers whose levels would also have to be considered. To get around this, we chose to examine the β3:γ2 ratio because neither β3 nor γ2 chains are shared by other relevant laminins and thus only affect the Ln-332 heterotrimer. This approach also gives insight into γ2 monomer changes because, in the absence of the β3 chain, the necessary β3γ2 precursor does not form and the monomeric γ2 chain is free to be secreted (15). Therefore, we postulated that the β3:γ2 ratio could be used as a relative indicator of secreted heterotrimer versus γ2 monomer expression. The ratios of raw signal intensities were calculated for each patient, these values were log-transformed, and the relative frequencies of transformed ratios were depicted graphically for low-stage versus metastatic samples (Fig. 4A). A shift towards a lower β3:γ2 ratio was significantly associated with tumor aggressiveness, as given by the Wilcoxon rank sum test statistic (P = 0.0191; Fig. 4A). Importantly, the trend of a decrease in the mean, log-transformed β3:γ2 ratio spanned from a high value in normal colon (0.496 ± 0.132) to a lower value in low-stage cancer (0.226 ± 0.231), and finally to the lowest level in metastatic cancer (0.133 ± 0.228; Fig. 4B).

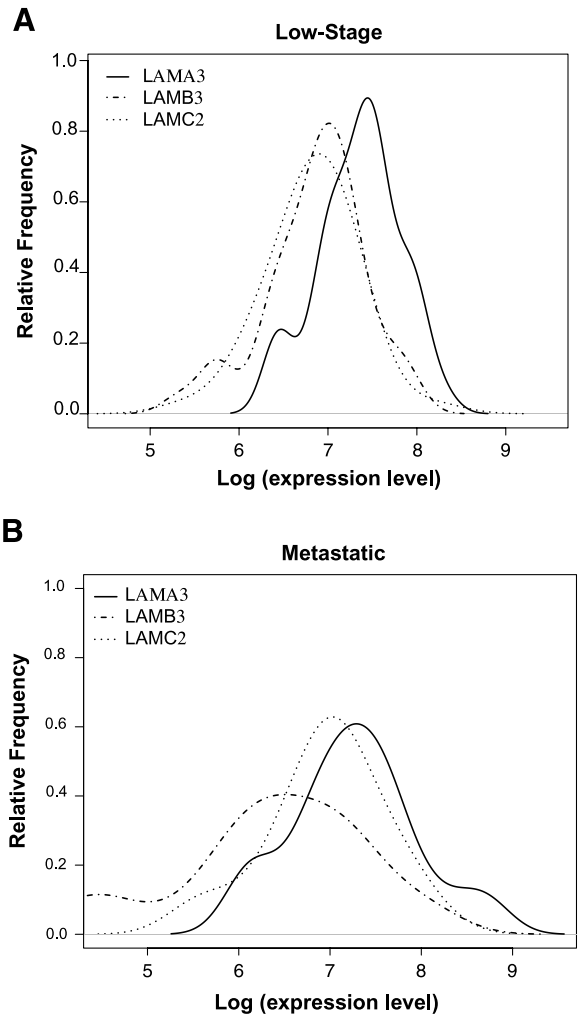

Figure 3. Distribution frequency analysis of log-transformed LAMA3, LAMB3, and LAMC2 transcript levels in low-stage colon tumors (n = 41; **A**) and metastatic colon cancer samples (n = 29; **B**).

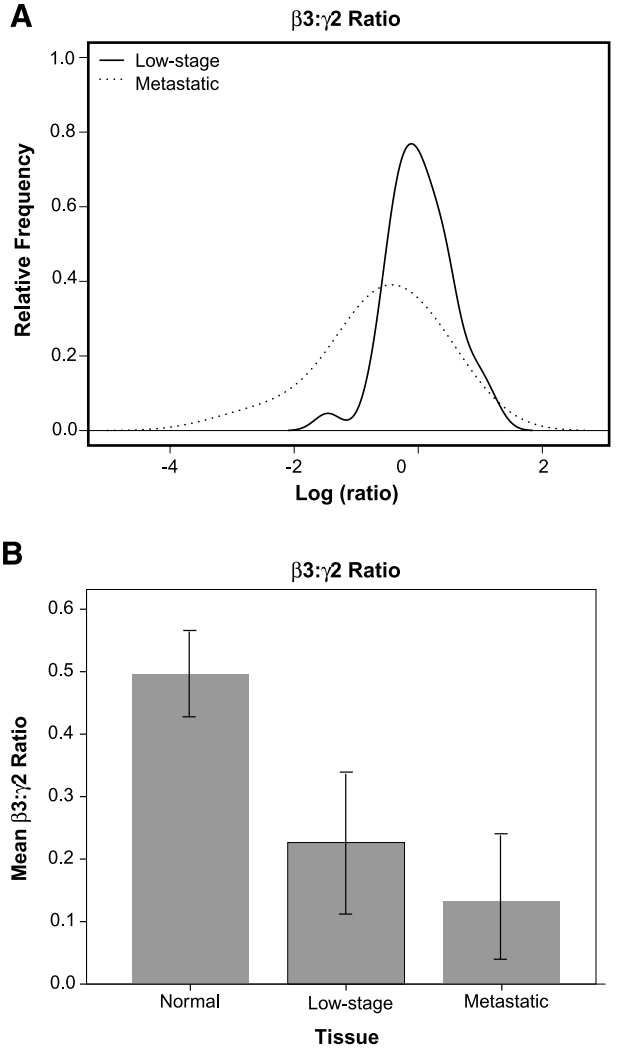

Figure 4. Log (β3:γ2) distribution frequencies indicate a lowered ratio of metastatic colon cancer as compared with low-stage colon cancer. **A.** Mean β3:γ2 ratio decreases during progression from normal colon (n = 10) to low-stage colon cancer (n = 41) to metastatic cancer (n = 29; **B**).

Discussion

Advanced analyses of a previously existing publicly available database, consisting of standardized Affymetrix mRNA measurements from various cancer tissue types, enabled us to examine critical aspects of Ln-332 expression largely overlooked in commonly used study methods (e.g., immunohistochemistry, *in situ* hybridization, Northern blotting). Previous studies were lacking in the areas of standardized methodology, quantification, and insight into the role of Ln-332 γ2 transcript variants (LAMC2 and LAMC2*), whose expression may be key in dysregulation of the whole Ln-332 heterotrimer (α3β3γ2). Because LAMC2* is missing the COOH-terminal exon (exon 23) of the full-length γ2 transcript (16) and no differential antibody exists to distinguish between these two protein products, transcriptome analysis is the only way to investigate the relative contribution of each γ2 transcript variant. In support of our transcript-focused assessment, it has recently been put forth in the literature that transcript-level assessment of microarray data is more reliable than gene-level analysis (25). In our case, examining the individual transcript variants did not reveal transcript switching across stages of cancer progression; it is important to stress, however, that the LAMC2* expression pattern needed to be examined because this variant is predicted to have a defect in stable heterotrimer formation (18, 19). Thus, if LAMC2* had been up-regulated, it would have indicated a tendency toward reduced Ln-332 heterotrimer formation and/or formation of unstable heterotrimers. The ability to rule this option out as a likely mechanism exploited by cancer cells led us to the conclusion that γ2 has a more complex role in cancer progression, not simply predicted by general transcript up-regulation.

The vast majority of studies reporting a positive correlation of Ln-332 γ2 with more aggressive tumor behavior have shown increased cytoplasmic staining, possibly due to protein up-regulation (6). As there was no appreciable alteration in total γ2-encoding transcript levels (LAMC2 plus LAMC2*) between early-stage and metastatic carcinomas, our data suggest there is unlikely to be a net change in γ2 protein production. We suggest these staining changes are more likely due to a protein localization switch or increased cleavage of the γ2 chain, leading to higher antibody staining. Distinguishing between these possibilities will require an in-depth protein-level study.

To examine relative contributions of Ln-332 heterotrimer versus monomeric γ2, LAMA3 and LAMB3 mRNA levels were examined in tandem with γ2-encoding transcript levels. The first step in laminin heterotrimer construction is the formation of βγ dimers, which are rapidly degraded if the α chain is not available for incorporation to form the more stable trimer (22-24). Further, because γ2 monomer can be secreted, any amount of γ2 present in excess of β3 can be postulated to represent the monomeric form; therefore, a shift to lower β3:γ2 ratio can be interpreted as a decrease in heterotrimer and a potential increase in γ2 monomer secretion. The major finding of this study was the association of lowered β3:γ2 mRNA ratio with colon cancer progression. That is, β3:γ2 ratios obtained for metastatic samples were significantly lower than those for low-stage samples, which were lower than normal colon samples. These results suggest that the context of γ2 expression may be as important as the level of expression and should be considered in future studies.

We propose that the mechanism through which the β3:γ2 ratio change gives cancer cells an advantage is through the modulation of Ln-332 heterotrimer and γ2 monomer amounts. The full mechanism by which Ln-332 chain ratios determine tumor behavior is likely complex, due to the dual nature of extracellular matrix (ECM) as a supportive and signaling structure. Altered expression of an ECM molecule in the basement membrane, specifically Ln-332, will therefore have several effects. First, a decrease in Ln-332 heterotrimers leaves fewer α3 molecules in the ECM to bind with the α6β4 integrin receptor, an interaction required for the formation of hemidesmosome structures necessary for

stable cell-matrix adhesion (26). This leads to detachment from the basement membrane, making migration easier. The problem would be further exacerbated by the accompanying increase in γ2 monomer. Our lab has previously shown that γ2 cleavage fragment DIII up-regulates matrix metalloproteinase-2 (MMP2) expression (27). Because MMP2 cleaves γ2, this would cause a positive feedback loop increasing the amount of DIII available to stimulate cell signaling through its unresolved novel epidermal growth factor receptor pathway, leading to adverse effects (27). Finally, an increase or decrease of Ln-332 in the ECM affects the number and type of cross-linkages in the basement membrane, because Ln-332 normally binds with Ln-311, Ln-321, and collagen molecules affecting the tensile strength of the structure (28). With fewer cross-linkages, the basement membrane will be weaker, allowing for easier invasion.

Although we have outlined a scheme whereby adhesion, signaling, and ECM composition all play complex roles that vary with γ2 expression context, the specific role of each of these individual areas will differ based on basal tissue–specific differences in normal ECM constituency, integrin expression pattern, and MMP activity. Unraveling this meshwork will require a comprehensive protein-level study of each of these areas, because transcript levels alone may give an inaccurate picture as they do not account for translational control mechanisms or the role of posttranslational modifications. Although beyond the scope of this investigation, these studies will be critical for understanding the invasion process and should aid in the design of targeted therapies against multiple interrelated points in the cascade.

Lastly, these data show that analyses must be done within one tissue group to pick up subtle changes in expression. However, given the proven and postulated involvement of Ln-332 in cancers of other distinct tissue types, the dysregulation of Ln-332 heterotrimer may well be applicable to tissues other than colon. The notion of mechanistic overlap is also supported by a growing body of literature reporting correlations between epidermal growth factor receptor (a signaling target of cleaved γ2 fragments) and γ2 expression with poor prognosis in a variety of cancers (29-34), making it tempting to speculate that the mechanistic scheme presented here is relevant beyond colon cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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