

Lung metastasis genes couple breast tumor size and metastatic spread

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The association between large tumor size and metastatic risk in a majority of clinical cancers has led to questions as to whether these observations are causally related or whether one is simply a marker for the other. This is partly due to an uncertainty about how metastasis-promoting gene expression changes can arise in primary tumors. We investigated this question through the analysis of a previously defined “lung metastasis gene-expression signature” (LMS) that mediates experimental breast cancer metastasis selectively to the lung and is expressed by primary human breast cancer with a high risk for developing lung metastasis. Experimentally, we demonstrate that the LMS promotes primary tumor growth that enriches for LMS⁺ cells, and it allows for intravasation after reaching a critical tumor size. Clinically, this corresponds to LMS⁺ tumors being larger at diagnosis compared with LMS[−] tumors and to a marked rise in the incidence of metastasis after LMS⁺ tumors reach 2 cm. Patients with LMS-expressing primary tumors selectively fail in the lung compared with the bone or other visceral sites and have a worse overall survival. The mechanistic linkage between metastasis gene expression, accelerated tumor growth, and likelihood of metastatic recurrence provided by the LMS may help to explain observations of prognostic gene signatures in primary cancer and how tumor growth can both lead to metastasis and be a marker for cells destined to metastasize.

cancer | genomics | oncogenesis

The consistent association of large tumor size, rapid growth rate, and metastatic risk in a majority of cases of clinical cancer suggests that the molecular bases of these phenomena may be linked (1–3). However, the nature of this link remains unresolved. Conventional models of metastasis envision rare metastatically competent variants emerging by chance as primary tumors grow, causally linking growth with likelihood of metastatic relapse (4, 5). In this view, genes that control primary tumor growth operate independently of stochastically acquired metastasis genes. Alternative models posit that prometastatic gene expression events are acquired early during tumorigenesis and may overlap with the genes that promote primary tumor growth, making tumor size a marker for metastatic risk (6). These alternative models form a teleological basis for using gene expression signatures from primary tumors to forecast whether patients are at high risk for micrometastatic disease. However, despite several reports on the success of gene signatures from primary tumors to predict development of distant spread (7–12), tumor size remains an independent prognostic factor on multivariate analysis (9). Thus, to what degree conventional versus alternative models can explain the acquisition of a metastatic phenotype remains unclear.

One of the difficulties in addressing the fundamental question on how metastasis gene expression events are acquired relates to the genetically complex nature of the phenotype itself. It has long been believed that there are numerous genes that control metastatic behavior due to the multiple steps required for distant growth and

the observation that tumors demonstrate tissue tropism (4, 5). However, despite the description of multiple microarray gene signatures that are derived by statistical approaches, the statistical association of genes with the complex genetics behind a “poor prognosis” clinical phenotype result in genes that primarily track with metastatic propensity but are of unknown relevance as mediators of metastatic behavior (13, 14). The absence of experimental evidence that these gene signatures are actual mediators of metastasis makes it difficult to test predictions on the basis of the conventional versus alternative hypothesis of metastasis. In contrast, functional screens for genes that mediate metastasis can yield gene sets that are functionally relevant to the metastatic phenotype that they control (15, 16). Based on such gene sets, questions can be asked about if and when metastasis gene expression events are acquired by the primary tumor and the relationship of these genes to parameters such as tumor size.

We previously used the human breast cancer cell line MDA-MB-231 in a mouse xenograft model to select cell subpopulations that are highly metastatic to lung (15). These populations share a gene expression signature that is associated with and mediates lung metastasis in a mouse model. Furthermore, these genes promote tumor growth within the mouse mammary gland, providing evidence that metastaticity can be linked to primary tumorigenicity. A subset of these genes, called the “lung metastasis gene-expression signature” (LMS), is expressed in a subgroup of human primary breast tumors with a pattern resembling the canonical expression profile of the lung metastatic cell lines. When tested on a cohort of 82 primary human breast cancers, a multigene classifier derived from the LMS predicted for patients at high risk for selective distant relapse to the lungs, but not to the bones. The expression of these genes in primary human tumors suggested a role both in primary tumor growth and as mediators of lung colonization from the circulation. By seeking further clinical validation that the LMS predicts for patients at high risk for lung metastasis, we have now uncovered experimental and clinical evidence that may explain how metastasis gene expression can be selected at the primary site. With this validation that the LMS can both mediate and predict lung metastasis, the influence of primary tumor growth in the context of conventional and alternative models of metastasis is addressed.

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Abbreviations: LMS, lung metastasis gene-expression signature; SAM, significance analysis of microarray; FDR, false discovery rate.

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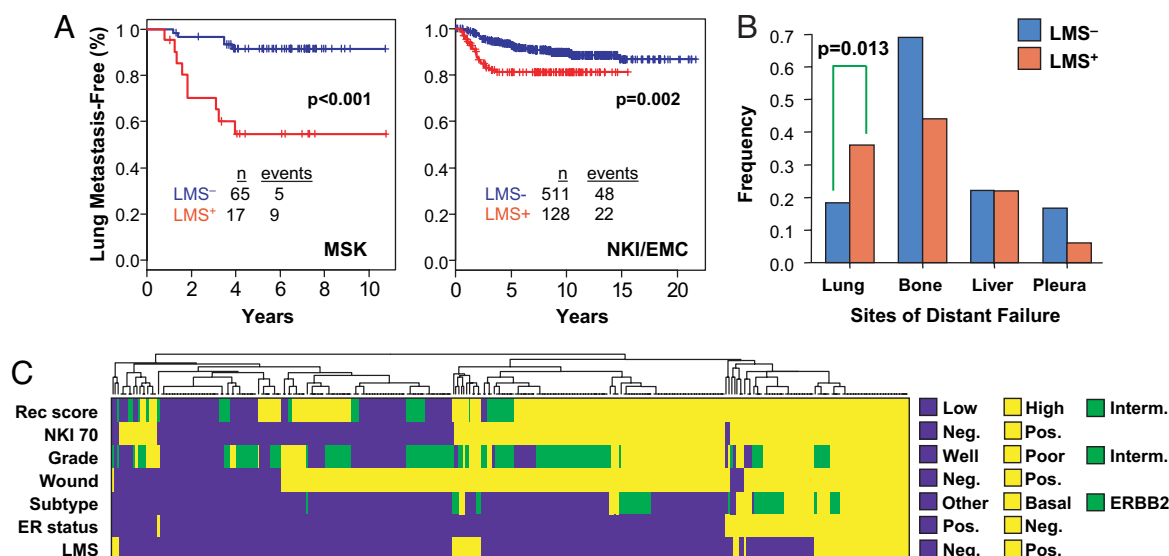


Fig. 1. Expression of the LMS predicts for increased risk of distant failure selectively in the lung and is associated with other markers of poor prognosis. (A) Kaplan-Meier curves representing the probability of cumulative lung metastasis-free survival for the MSK (Left) and combined NKI-295/EMC-344 (Right) cohorts. (B) Distribution of site(s) of first distant failure (simultaneous metastasis sites included) in the NKI-295/EMC-344 cohort according to LMS status. The *P* value for the difference in distribution for lung metastasis is shown and was calculated by a χ^2 test. Patients with LMS⁺ primary tumors are shown in red, and LMS⁻ tumors are in blue. (C) Hierarchical clustering was performed on the NKI-295 cohort with the indicated pathological and genomic markers consisting of the 70-gene prognosis signature (9, 10) (NKI 70), 16-gene recurrence score (18) (Rec score), molecular subtype (19) (Subtype), and wound response signature (17) (Wound). The legend for the color codes for each marker is shown. The luminal A, luminal B, and normal-like subtypes were grouped together as "Other" and consist of 5.8, 1.9, and 7.7% of LMS⁺ tumors, respectively.

Results

Validation That the LMS Predicts for Development of Lung Metastasis.

To validate that LMS expression by primary breast cancers can predict for risk of lung metastasis, two large cohorts of early stage breast cancer patients (NKI-295 and EMC-344) (9, 11) along with an expanded cohort (MSK-99) from an original series of tumors (15) were analyzed. The MSK cohort was more locally advanced compared with either the NKI-295 or the EMC-344 series (91% T2-T4 and 66% node positive compared with 47 and 49%, and 51 and 0%, respectively). This represents 738 primary tumors subjected to gene expression profiling across two microarray platforms. Hierarchical clustering with the 18 most univariately significant genes in the LMS classifier (15) shows their expression pattern among primary breast tumors [see supporting information (SI) Fig. 6]. Tumors classified as LMS⁺ show more uniform expression of these genes in a manner that resembles the cell line signature (weighted average Pearson correlation of LMS⁺ tumors to cell line signature is 0.27). These data demonstrate repeated observations of LMS expression by a subgroup of primary breast tumors.

Several statistical learning methods to classify tumors on the basis of LMS status performed similarly in predicting that LMS⁺ tumors are at high risk for developing lung metastasis (15). For simplicity and nonredundancy, here, we use only the nearest centroid classifier method. Results from this classifier demonstrate that LMS⁺ tumors from the MSK cohort have a significantly higher risk for lung metastasis compared with LMS⁻ tumors (Fig. 1A).

Of a combined total of 639 patients from the NKI-295 and EMC-344 series, 231 patients developed distant metastases; of these, 70 patients developed lung metastases after a median follow-up of 8.7 years. Compared with the MSK cohort, the prevalence of lung metastasis in both the NKI-295 and EMC-344 groups is expected to be low because most tumors were removed at an early stage. Nonetheless, LMS expression is significantly associated with a worse lung metastasis-free survival in the individual (data not shown) and combined NKI-295/EMC-344 cohort (Fig. 1A), regardless of whether the analysis was done on cumulative lung metastasis events (Fig. 1A) or site of first distant failure (SI Fig. 7A).

Multivariate analysis revealed the predictive ability of the LMS was independent of other standard prognostic markers (Table 1). Patients with LMS⁺ tumors selectively failed in the lung compared with bone and other visceral sites, including liver, and pleura (SI Fig. 7A). Although this was not associated with a higher rate of distant relapse in general or the likelihood that first distant failure involved multiple organs (data not shown), it was associated with a worse overall survival (SI Fig. 7B). An analysis of all sites of first distant failure revealed that compared with LMS⁻ tumors, LMS⁺ tumors are more likely to relapse in lung than in bone, liver, or pleura (Fig. 1B). Analysis with the NKI-295 cohort showed that LMS⁺ tumors mostly were estrogen receptor-negative (73%), grade 3 (69%), and of poor prognosis (92%) on the basis of either a previously described 70-gene expression signature (9, 10), wound response signature (17), or 16-gene Recurrence Score (18) (Fig. 1C). The majority (65%) belonged to the basal-like molecular subtype with a smaller fraction (19%) classified as the ERBB2 subtype (19). Thus, consistent with our preliminary findings (15), LMS⁺ primary breast cancers form a subgroup of tumors with other

Table 1. Multivariate Cox proportional hazard regression model for lung metastasis-free survival

Variable	HR (95% C.I.)	P value
LMS (pos. vs. neg.)	2.05 (1.1 to 3.83)	0.024
ER negative (yes vs. no)	2.04 (0.91 to 4.58)	0.082
Tumor size (per mm)	1.02 (1.00 to 1.04)	0.037
Lymph nodes (per node)	1.07 (0.89 to 1.29)	0.500
Age (per year)	0.97 (0.95 to 1.00)	0.048
Chemotherapy (yes vs. no)	0.67 (0.23 to 1.93)	0.450
Endocrine therapy (yes vs. no)	1.37 (0.40 to 4.75)	0.620

The covariates shown in bold are significant, with a *P* value of <0.05. Grade was excluded in this analysis due to 108 missing values out of 639 patients in the NKI-295/EMC-344 cohort. The final model contains 632 patients (seven deleted due to missing values for other covariates).

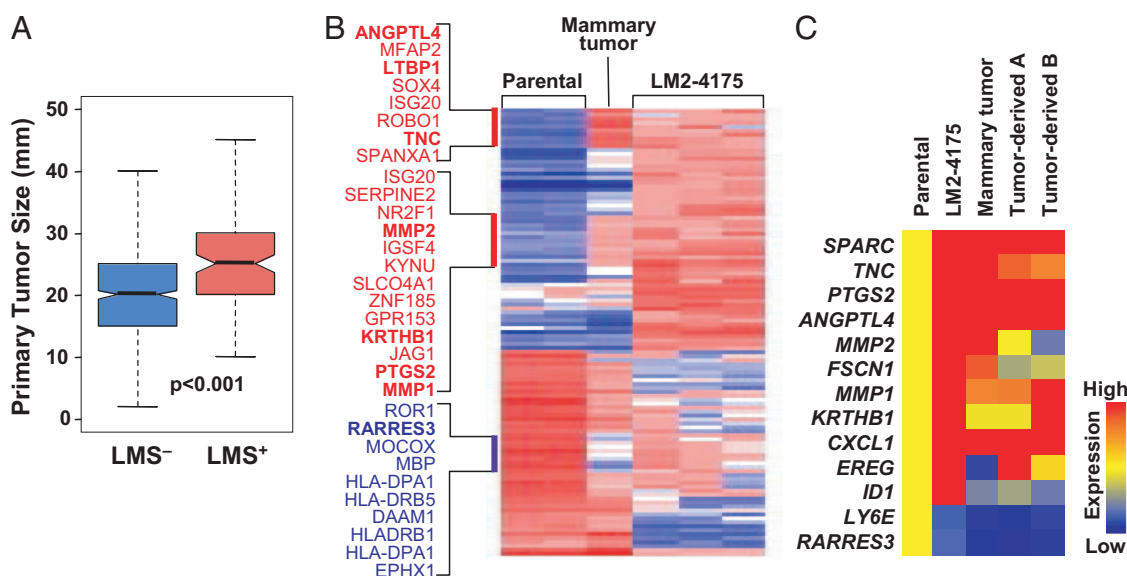


Fig. 2. LMS⁺ primary tumors are larger and can be experimentally selected for during primary tumor growth. (A) A box-and-whisker plot comparing the size distributions for LMS⁻ and LMS⁺ primary tumors in the NKI-295/EMC-344 cohort. Shown are the medians, 25th and 75th percent quartiles, and 1.5 times the interquartile range. *P* values for the box plots were calculated by using a Wilcoxon rank sum test. (B) Transcriptomic microarray profiling was performed on parental MDA-MB-231 cells, the LMS⁺ *in vivo* selected lung metastatic LM2 subpopulation (LM2-4175) (15), and cells derived from xenografted parental MDA-MB-231 mouse mammary fat pad tumors. Mammary tumor denotes a sample from which *in vivo* mRNA expression was assessed directly from a fresh frozen mouse mammary tumor. The heatmap corresponds to relative gene expression levels for 95 previously identified lung metastasis genes (113 probe sets), with red being high and blue indicating low expression. Gene labels are provided for gene clusters of interest due to partial selection during outgrowth of a parental MDA-MB-231 mammary tumor. Genes highlighted in bold are functionally validated mediators of lung metastasis or are included in the 18-gene LMS. (C) Confirmation of microarray-based gene expression levels by using quantitative RT-PCR analysis. Expression values for representative lung metastasis genes were normalized to parental MDA-MB-231 expression levels and displayed graphically as a heatmap. Tumor-derived A and B represent *in vitro* analyses of independent isolates of cells purified from dissociated MDA-MB-231 mammary tumors.

poor prognosis molecular markers and have higher rates of distant metastasis to the lung compared with most other distant sites.

The LMS Can Promote Primary Tumor Growth Resulting in Its Selection at the Primary Site. LMS genes can promote experimental tumor growth within the orthotopic mouse mammary gland (15). If clinically relevant, primary human breast cancers that are LMS⁺ will be larger at the time of diagnosis compared with LMS⁻ counterparts. Analysis of the median primary tumor size demonstrated that LMS⁺ tumors are larger than LMS⁻ tumors (Fig. 2A). In the absence of serial biopsies from the same patient, it is difficult to know whether LMS expression evolved as the tumor grew, resulting in LMS⁻ tumors becoming LMS⁺. To experimentally determine whether LMS genes can confer a selective advantage that results in an LMS⁻ tumor becoming LMS⁺ during tumor growth, the mouse model system was used. Mammary gland tumors were established from the parental MDA-MB-231 cell line, which harbors a small LMS⁺ subpopulation (< 1%, data not shown). Gene expression profiling (Fig. 2B) and quantitative RT-PCR analysis for LMS genes (Fig. 2C and SI Fig. 8) demonstrated an enrichment for LMS gene expression that includes genes that mediate lung metastasis, both in tumors generated from the parental population and in cell cultures derived from these tumors. These observations suggest that LMS⁺ cells within a breast cancer population have a growth advantage that can be efficiently selected during primary tumor progression.

Tumor Size Influences LMS-Related Metastasis. The ability of LMS⁺ tumor cells and metastatic mediators of the LMS to both promote primary tumor growth and be selected within the primary site suggest that tumorigenesis and metastasis can be mechanistically linked and tumor size alone would not independently influence LMS-related metastasis. However, previous work has shown that when LMS⁺ cells are grown within the mouse mammary gland and

then excised after reaching a size of 300 mm³, only about half of the mice develop lung metastasis, despite robust development of lung metastasis after direct inoculation of 2,000 cells into the mouse circulation (15). These results suggest that the primary site may be a significant barrier against distant dissemination even though the tumor is metastatically competent. To directly test this, LMS⁺ cells were grown as mammary tumors and circulating tumor cells were quantified by ex vivo colony forming assay. These results revealed that LM2 tumor cells do not initially intravasate into the circulation; however, progressive growth of the primary tumor leads to cells readily moving into the circulation (Fig. 3A). Once in the circulation, as few as 200 cells can form lung metastases (Fig. 3B). These results suggest that tumor growth may not necessarily contribute to metastasis solely by allowing for accumulation of metastasis genes, but rather may be required for intravasation of preexisting metastatically competent cells.

To determine whether the experimental results are paralleled by clinical observation, the link between LMS expression, tumor size, and metastasis was investigated with the NKI-295/EMC-344 cohort. To avoid model assumptions such as linearity and to control for standard prognostic markers and potential interactions, a random survival forest analysis (20) was used to examine how tumor size is predicted to influence metastasis among LMS⁺ primary tumors (Fig. 4A and SI Fig. 9). In this analysis, the estimated risk for metastasis is determined after controlling for standard prognostic markers and interactions. The estimated risk for each patient with the indicated tumor size reveals that for the LMS⁺ patients there is a sharp rise in risk with tumor sizes >2 cm. In contrast, LMS⁻ primary tumors showed no clear threshold for metastasis risk but rather display a steady rise beginning with tumors that are <1 cm. Similar results were observed when analysis was restricted to lung metastasis (data not shown). Stratification of LMS⁺ primary tumors into stage T1 (≤2 cm) versus T2 and larger (>2 cm) revealed that lung metastases are infrequent for the smaller LMS⁺ tumors (data not shown).



nostic modeling. There are important covariates that cannot be captured by measuring gene expression changes alone, and gene expression signatures derived from functional screenings can provide complementary information (14, 24).

The mechanism by which LMS⁺ primary tumors acquire the ability to intravasate after reaching a certain size is currently under experimental investigation. Recent work was revealed that the LMS genes *EREG*, *COX2*, *MMP1*, and *MMP2* constitute a vascular remodeling program that is coopted by tumor cells for the induction of tumor angiogenesis, intravasation, and lung extravasation (25). We note that despite the absence of a detectable change in gene expression as a function of either tumor size or lung metastatic risk in LMS⁺ tumors, we cannot exclude rare or diverse genetic or epigenetic changes that could contribute to metastatic behavior. In fact, experimental evidence has been provided for the existence of such gene expression events in regards to “metastasis virulence genes,” which principally control the rate rather than the likelihood of metastasis (15, 16).

The mode of progression to metastasis in LMS⁺ tumors may not apply to metastasis mediated by other genes or to other sites. Analysis of LMS[−] tumors demonstrates a steady rise in risk as a function of tumor size instead of a critical threshold size as seen in LMS⁺ tumors. Because LMS[−] tumors predominantly metastasize to bone, bone metastases gene-expression signatures may not correlate with tumor size despite correlating with clinical bone metastases and shorter survival. Thus, the present paradigm may only apply to fast-growing basal type tumors that metastasize to the lungs but not other more indolent but still lethal breast cancer syndromes. These issues highlight the complex genetic basis for metastasis and the historical difficulties in breaking ground on the credibility of different models. By providing confirmation of the biological and clinical relevancy of the LMS, this study leverages the known biology of a metastasis gene signature to address long-standing questions on how metastasis genes are engaged and to put forth new paradigms for future hypothesis testing.

Materials and Methods

Tumor Gene Expression Profiles. Maintenance of cell lines and xenografting of immunocompromised mice has been described (15). RNA extraction, labeling, and hybridization for DNA microarray analysis of cell lines and primary breast tumors have been described for the MSK-99 and NKI-295 cohorts (9, 15). The EMC-344 cohort consists of 286 samples previously described (11) and an additional 58 estrogen receptor-negative samples and is

available from the Gene Expression Omnibus (accession no. GSE5327). For quantitative RT-PCR studies, cDNA was synthesized from 1 μ g of total RNA. Gene expression levels were determined by using Taqman and the ABI Prism 7900HT (Applied Biosystems). β 2-Microglobulin was used as an endogenous control.

Data Analysis. New tumors from each cohort (MSK, EMC-344, NKI-295) were classified as LMS⁺ or LMS[−] based on a nearest centroid classifier as previously described (15). There were 61 patients in the NKI-295 that overlapped with the 78 patients used to define LMS class membership. For these patients, their original class assignments were used. Hierarchical clustering of normalized data with median centering of gene expression values was done by using the 18 most univariately significant genes of the LMS using TIGR MultiExperiment Viewer 3.1 (26). Kaplan–Meier survival analysis and Cox proportional hazards regression modeling was performed with the “survival” package 2.26 in the R statistical package 2.3.1 (www.r-project.org). Random survival forest analysis was performed with the “RandomSurvivalForest” package 1.0.0 for R by H. Ishwaran and U. B. Kogalur. SAM was done with the “samr” 1.20 package. All relevant clinical information for patients included in the cohorts has previously been published (11, 13, 17), or is provided in SI Table 1.

Intravasation. Parental or LM2 cells (15) were infected with pBabe retrovirus expressing a puromycin marker and injected into mammary glands of immunodeficient mice. Tumor volumes were measured as previously described (15). Animals bearing palpable tumors were killed, and 1–2 ml was bled through cardiac puncture. Red blood cells were removed by using ammonium chloride lysis buffer (Cambrex), and remaining cells were plated. After 48 h, adherent cells were grown in media with puromycin. After 10 days, colonies were stained with crystal violet blue and scored.

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