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Research article

Long non-coding RNA LCAL62 / LINCO00261 is associated with lung adenocarcinoma prognosis

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ABSTRACT

Background: More than half of non-small cell lung cancer (NSCLC) patients present with metastatic disease at initial diagnosis with an estimated five-year survival rate of ~5%. Despite advances in understanding primary lung cancer oncogenesis metastatic disease remains poorly characterized. Recent studies demonstrate important roles of long non-coding RNAs (lncRNAs) in tumor physiology and as prognostic markers. Therefore, we present the first transcriptome analysis to identify lncRNAs altered in metastatic lung adenocarcinoma leading to the discovery and characterization of the lncRNA \textit{LCAL62} as a prognostic biomarker.

Patients and methods: RNA-Seq, microarray, nanoString expression, and clinical data from 1,116 LUAD patients across six independent cohorts and 83 LUAD cell lines were used to discover and evaluate the survival association of metastasis associated lncRNAs. Coexpression and gene set enrichment analyses were used to establish gene regulatory networks and implicate metastasis associated lncRNAs in specific biological processes.

Results: Our integrative analysis discovered \textit{LCAL62} as the most down-regulated lncRNA in metastasis. Further low \textit{LCAL62} expression promoted aggressive phenotypes and regulated genes associated with metastasis (such as metastasis repressor \textit{FOXA2}). Low \textit{LCAL62} expression corresponded to poor overall patient survival across five independent lung adenocarcinoma cohorts (\(n=881\)) including our own nanoString validation cohort.

Conclusion: We discovered that \textit{LCAL62} was down-regulated in lung cancer progression to promote invasive phenotypes, and lower expression was significantly associated with poor patient outcome and aggressive lung adenocarcinoma.

1. Introduction

Non-small cell lung cancer (NSCLC) accounts for ~85% of all lung cancers and is the leading cause of cancer-related deaths worldwide [1]. Greater than 50% of patients present with metastasis at initial diagnosis [2]. Furthermore, most patients with early stage and locally advanced NSCLC eventually recur with metastatic disease, despite undergoing potentially curative therapies. The five-year survival rate for metastatic disease is ~5% [1]. Current inadequacies for stratifying patients with aggressive disease presents a strong rationale to systematically identify biomarkers of aggressive lung cancer to improve patient diagnosis and care.

To date lung cancer treatment and research have primarily focused on the deregulation of protein-coding genes, thereby missing potentially important non-coding RNAs. Long non-coding RNAs have emerged as a biologically and clinically relevant class of transcripts contributing to tumorigenesis [3, 4]. We recently conducted a first-of-its-kind \textit{ab initio} transcriptome assembly of publicly available RNA-Seq data from ~550 primary lung cancer patients to discover 111 lung cancer associated long non-coding RNAs (\textit{LCALs}) altered in lung cancer [5]. Just as this study

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established the lung cancer IncRNA landscape in primary tumors for researchers to further explore potential mechanisms [6] and biomarker utility [7, 8], a similar systematic approach in lung cancer metastasis would improve our understanding of disease progression. Despite the availability of multiple RNA-Seq data collections derived from primary tumors [5], none of these cohorts include metastatic cancer specimens. While previous technologies such as microarrays were designed primarily to monitor protein-coding gene expression levels, some platforms can be exploited to monitor the expression of a subset of IncRNAs [9]. Therefore, our study leveraged RNA-Seq, microarray, and nanoString expression platforms to identify IncRNAs associated with lung cancer metastasis and their use as biomarkers for stratifying patients with poor outcome to ultimately improve patient care.

Through our integrative analysis we discovered 281 IncRNAs differentially expressed in lung adenocarcinoma (LUAD) metastasis. We found that low expression of LCAL62 (aliases LINC00261, onco-IncRNA-17 and DEANR1), the most down-regulated IncRNA in metastatic disease, promoted aggressive phenotypes in vitro. Furthermore, we found and validated that low expression of LCAL62 was associated with poor overall survival in LUAD patients from five independent cohorts totaling 881 patients. We also observed a strong coexpression between LCAL62 and FOXA2, a known tumor and metastasis suppressor of lung cancer [10]. Taken together, LCAL62 could serve as a biomarker of aggressive disease and patient outcome in LUAD.

2. Materials and methods

2.1. Datasets and patients

Six public gene expression datasets representing 5,320 tumor samples (1,035 LUAD patients, 83 LUAD cell lines, and 4,121 other cancer patients) were used for the discovery of metastasis associated IncRNAs, functional prediction, and survival analysis of LCAL62. An independent cohort of 81 LUAD patients were enrolled at Washington University School of Medicine in St. Louis, MO (WashU cohort). Primary tumor specimens were evaluated for LCAL62 and FOXA2 expression using the nanoString nCounter platform. Informed consent was obtained from all patients and the protocol was approved by the Washington University Institutional Review Board (IRB). Dataset details are provided in Table 1.

2.2. Bioinformatics analysis

Microarray probes were repurposed by realigning their sequences against the transcripts to identify probes uniquely mapped to a lncRNA. TCGA RNA-Seq pre-alignments and microarray pre-normalized probe expression were used for subsequent analyses. Differential expression analysis was performed using the negative binomial model for RNA-Seq raw counts and the two-sample t-test for microarray data. Digital transcript counts from nanoString nCounter assays were normalized according to nanoString nCounter data analysis manual. The Kaplan-Meier product limit method and Cox proportional hazard model was used to evaluate association between gene expression and patient overall survival. More details of the bioinformatics methods can be found in the Supplementary Methods.

2.3. Experimental characterization

Experimental methods are detailed in the Supplemental Methods.

3. Results

3.1. Discovery of down-regulated IncRNA LCAL62 in metastatic lung adenocarcinoma

To identify IncRNAs altered in metastatic LUAD we leveraged a publicly available microarray gene expression dataset, referred to as the ‘Bittner cohort’ (GSE2109), that included 72 primary tumors and 10 unmatched metastatic tumors from brain (2), adrenal gland (3), cerebral tissue (1), small bowel (1), soft tissue of the arm (1), and secondary lung tumor (2). We identified 5,357 probe sets in the microarray platforms that uniquely mapped to 4,443 IncRNA genes compiled from multiple annotation sources (Supplementary Methods). Comparison of primary and metastatic tumors from the Bittner cohort identified 281 IncRNA genes differentially expressed in metastasis including 235 up-regulated and 49 down-regulated IncRNAs (Figure 1a). We found up-regulated IncRNAs known to promote tumor proliferation and metastasis in lung and other cancers such as H19 [11], BCAR4 [12], SNHG1 [13], and MIR17HG [14]. Our previous study, using three independent RNA-Seq cohorts, characterized 111 IncRNAs dysregulated in non-small cell lung cancer primary tumors compared to matched normal tissues, termed LCALs, including 78 IncRNAs dysregulated in LUAD [5]. We hypothesized that a subset of LCALs may be dysregulated in metastasis compared to primary tumors. To select a candidate for subsequent characterization we prioritized IncRNAs that are altered throughout lung cancer progression by overlapping the metastasis-associated IncRNAs with our previously identified primary-associated IncRNAs, LCALs. A total of 43 LCALs were uniquely annotated with at least one probe set from the Bittner cohort microarray. We discovered LCAL62 had the most dramatic decreased expression in metastasis (Figure 1a,b). Additional analysis confirmed that LCAL62 was downregulated in bone metastasis of patient derived xenografts (Figure 1c). LCAL62 was also down-regulated in primary tumors compared with normal tissues (Figure 1d).

Initial characterization of the TCGA LUAD cohort identified lower LCAL62 expression in proximal-inflammatory and proximal-
Figure 1. Low expression of LCAL62 associates with metastasis and cancer aggressiveness. (a) Volcano plot of the IncRNAs in the metastasis versus primary tumor comparison from the Bittner cohort. LCAL62 is the most down-regulated IncRNA in metastasis; (b) Expression of LCAL62 in patients (Bittner) and (c) patient derived cell lines and xenografts (GSE76194, P - patient primary tumor derived cell lines; M - matched bone metastases developed in mouse xenograft model); (d) Expression of LCAL62 in primary tumors (TCGA RNA-Seq) compared with normal tissues (N - normal, P - matched primary, UP - unmatched primary) and (e) grouped by expression-based subtypes (PI: proximal-inflammatory/squamoid, PP: proximal-proliferative/magnoid, TRU: terminal respiratory unit/bronchioid); (f) Cancer related gene sets enriched in LCAL62 GSEA analysis (top - enrichment plot of a representative metastatic gene set in melanoma negatively co-expressed with LCAL62, bottom - network of enriched gene sets). Nodes represent gene sets that are significantly correlated (red) or anti-correlated (blue) with LCAL62 expression, grouped by manually curated categories (dashed circles). Node size represents the number of genes in the corresponding gene set. Edge width represents the number of genes shared between gene sets.
proliferative expression-based subtypes (Figure 1e). To further investigate the involvement of LCAL62 in tumor progression and metastasis, we employed a ‘guilt-by-association’ approach that assumes a lncRNA is likely involved in similar biological processes as coexpressing protein-coding genes [15, 16]. We found that low LCAL62 expression was correlated with genes up-regulated in tumor growth and proliferation, tumor stage and metastasis, epithelial mesenchymal transition (EMT), and drug resistance (Figure 1f; FDR adjusted p < 0.001).

To further demonstrate if the lack of LCAL62 promotes aggressive disease, lung cancer cells were cultured with StemXVivo showing EMT hallmark proteins (E-cadherin and N-cadherin) altered during epithelial to mesenchymal transition. (b) Inducing epithelial to mesenchymal transition in the presence of StemXVivo supplement decreased LCAL62 expression in A549 (left) and HOP62 (right) cell lines as measured by quantitative RT-PCR (qRT-PCR); n of 2. Full non-adjusted images of the Western blots in this figure are provided in Fig. S1.

3.2. Low LCAL62 expression is associated with poor overall survival

Our ‘guilt-by-association’ analysis revealed a striking enrichment of gene signatures for lung cancer survival (Figure 5a) suggesting that lower LCAL62 expression is associated with poor clinical outcome. Building upon this, we evaluated the clinical significance of LCAL62 on patient overall survival within the TCGA LUAD cohort (n = 220, RNA-Seq) [17]. Most patients in TCGA LUAD cohort presented with early stage disease. As expected, there was an association between stage and outcome ($p < 0.001$, Table 2). We performed survival analysis stratifying patients by median LCAL62 expression and stage and found that decreased expression of LCAL62 was significantly associated with poor overall survival in LUAD patients ($p = 0.04$, HR = 1.9; Figure 5b). The survival effect was similar in early and late stages although 5% significance was not reached in stage III/IV due to the small sample size.

3.3. Microarray data across three independent cohorts confirms LCAL62 correlation with patient outcome

We confirmed association of LCAL62 expression with patient outcome by leveraging public microarray data from three independent LUAD cohorts totaling 583 patients (Figure 6a, Table 1). These cohorts included the Schabath cohort (n = 374) [18], Der cohort (n = 124) [19], and Rousseaux cohort (n = 85) [20] that leveraged two microarray platforms (Table 1). The majority of patients in these cohorts presented with early stage LUAD (Table 2). Low expression of LCAL62 was strongly and consistently associated with poor overall survival in all three cohorts (Schabath, $p = 0.0003$, HR = 1.7; Der, $p = 0.026$, HR = 1.9; Rousseaux, $p = 0.025$, HR = 2.0) (Figure 6a).

3.4. Orthogonal validation of LCAL62 correlation with patient outcome using nanoString

In addition to publicly available microarray datasets, we further validated the prognostic significance of LCAL62 in our independent WashU cohort (n = 81, stages I/II) using the nanoString nCounter platform. We confirmed low expression of LCAL62 was associated with poor overall survival in the WashU cohort ($p = 0.023$, HR = 1.9; Figure 6b). When patients from all cohorts were combined a significant association was observed between low LCAL62 expression and poor survival in both early and late stages ($p = 3.3 \times 10^{-9}$, HR = 1.9, Figure 6c). Overall, our analysis using three independent platforms (RNA-Seq, microarray, and nanoString) across five independent cohorts (n = 881) revealed a consistent association of LCAL62 expression with overall survival.

3.5. LCAL62 association with FOXA2

To identify LCAL62 target genes, we performed a coexpression analysis on six independent LUAD gene expression datasets (Table 1). The most highly coexpressed protein-coding gene with LCAL62 was FOXA2 (Forkhead Box A2), a gene encoding a transcription factor upstream of LCAL62 (Figure 7a) that is known to repress tumor growth and metastasis in multiple cancers including lung cancer [10]. Spearman correlation coefficient of expression between LCAL62 and FOXA2 ranged from 0.84 to 0.93 (Figure 7b). We further validated the strong correlation between LCAL62 and FOXA2 expression using the nanoString platform in our
independent cohort (WashU cohort, Spearman correlation = 0.80, Figure 7b).

Since cis-regulation of nearby genes is a common mechanism of lncRNA gene regulation [21] we investigated the relationship between LCAL62 and FOXA2. After silencing LCAL62 (at least 90% knockdown) with two independent siRNAs there was a 37.8% and 25.8% (*p < 0.05), respectively, decrease in FOXA2 expression in A549 cells (Figure 8a) and a 26.9% and 34.1% (*p < 0.0005), respectively, decrease in FOXA2 expression in HOP62 cells (Figure 8b). Interestingly, silencing FOXA2 also caused a decrease in LCAL62 expression (*p < 0.0005; Figure 8a,b).

Moreover, silencing FOXA2 (with 80% knockdown) in our lung cancer cell models increased cellular migration and invasion similarly to phenotypes observed in LCAL62-silenced cells (Figure 9). A previous study found that LCAL62 positively regulated FOXA2 in endoderm differentiation by recruiting the SMAD2/3 transcription factor to the FOXA2 promoter [22]. We hypothesized this regulatory mechanism would be conserved in the lung which is endoderm derived. In lung cancer models, we determined that LCAL62 bound to SMAD2/3 with a 20-fold and 5-fold enrichment in the A549 and HOP62 cell lines, respectively, as determined by RNA immunoprecipitation (RIP) (Figure 8c,d). Further, A549 cells with stably silenced LCAL62 showed decreased occupancy of SMAD2/3 at the FOXA2 promoter (Figure 10). These data highlight that LCAL62 may control transcription of FOXA2 through its interaction with SMAD2/3.

In contrast to the tissue-specific expression profiles commonly observed for lncRNAs [15, 23], we have observed that ~40% of LCALs...
were altered in multiple cancers suggestive of their conserved oncogenic roles across cancer types [5, 16]. In addition to LUAD, LCAL62 was down-regulated in lung squamous cell carcinoma (LUSC) under the gene alias onco-lncRNA-17 [5,16]. Therefore, we expanded our analysis to incorporate 12 cancer types using 4,938 TCGA RNA-Seq samples and found that the expression of LCAL62 was also decreased in thyroid cancer (THCA), kidney cancer (KICH), stomach cancer (STAD), and liver cancer (LIHC) (Figure 7c). Furthermore, coexpression analysis revealed that LCAL62 and FOXA2 were highly correlated (in both tumor and normal tissues) across multiple cancer types with LCAL62 differential expression (Figure 7d). LCAL62 and FOXA2 were the top correlated genes in LUAD, LUSC, STAD, and THCA. These results suggest LCAL62 has a conserved role regulating FOXA2 across cancers.

4. Discussion

Our comprehensive and systematic analysis of metastatic lung cancer is the first to show previously characterized non-coding RNAs in other cancers (i.e., BCAR4 and MIR17HG) [12, 14] are also up-regulated in metastatic lung cancer. Our systematic approach also revealed additional uncharacterized lncRNAs that could serve as the basis for subsequent mechanistic studies dissecting the role of lncRNAs in lung cancer metastasis. We prioritized LCAL62 since the lack of LCAL62 promoted aggressive phenotypes, associated with metastasis gene signatures, and correlated with patient outcome. We previously discovered LCAL62 as a down-regulated lncRNA in primary lung tumors via large-scale RNA-Seq analysis. While this current work was under review, several studies showed LCAL62 association with prognosis and function in vivo [24, 25, 26, 27]. However, these studies evaluated limited numbers of patients and utilized single transcriptomic platforms. Here we provide the most comprehensive and systematic evaluation of LCAL62 expression and association with prognosis in the largest compendium of patients to date (n = 1,116) from six multi-institutional and independent cohorts. Notably, low LCAL62 expression consistently correlated with patient outcome across 881 LUAD patients from five independent cohorts. Further supporting the robust nature of this association, we observed low LCAL62

![Figure 4](image-url)
Expression correlated with overall survival using microarray, RNA-Seq, and nanoString expression platforms. Within individual cohorts we observed a stronger association within early stage compared with late stage patients, however this may be due to the limited number of late stage patients. However, despite having only 69 and 58 late stage patients in the Schabath and TCGA cohorts, respectively, we still observed an association between low LCAL62 expression and poor overall survival. When the late stage patients from all cohorts were combined, a significant association of low LCAL62 and poor overall survival was observed, supporting that LCAL62 is associated with patient outcome independent of stage. Additionally, we showed that LCAL62 was associated with recurrence-free survival despite a limited number of patients (n = 387, 44%) with recurrence-free follow-up (p = 0.00045, HR = 1.8, combined cohort, Table 3).

Through computational prediction of LCAL62 function, we found gene signatures supporting an association of LCAL62 with metastasis. Our in vitro findings support our clinical observations by demonstrating that initiating EMT in lung cancer models resulted in a dramatic decrease in LCAL62 expression, and silencing LCAL62 expression resulted in increased cellular motility and invasiveness. Our analysis also revealed the consistent coexpression across 1,116 lung cancer patients between LCAL62 and the protein-coding gene, FOXA2, which is implicated as a metastasis suppressor in LUAD [10]. FOXA2 expression also had an association (although not as strong as LCAL62) with overall survival (p =

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**Table 2. Association of clinical variables with overall survival across cohorts.**

<table>
<thead>
<tr>
<th>Variable</th>
<th>TCGA n</th>
<th>Schabath n</th>
<th>Der n</th>
<th>Rousseaux n</th>
<th>WashU n</th>
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* 95% confidence interval.
1.6 × 10⁻⁵, HR = 1.6, combined cohort) and recurrence-free survival (p = 0.031, HR = 1.4, combined cohort, Table 3). The interplay between LCAL62 and FOXA2 was experimentally validated by silencing LCAL62 and observing decreased expression of FOXA2. Additionally, we determined that LCAL62 bound to SMAD2/3 to regulate FOXA2 expression in lung cancer, a relationship previously shown in definitive endoderm [22]. In contrast to the up-regulation of LCAL62 during endoderm differentiation, we observed a decrease in LCAL62 in metastatic samples potentially corresponding to dedifferentiation. However, subsequent mechanistic studies are necessary to determine how the loss of LCAL62 confers malignant phenotypes. We also believe this relationship extends beyond lung cancer. Our pan-cancer analysis revealed consistent coexpression between LCAL62 and FOXA2 in multiple cancer types suggesting a conserved regulatory interaction between these genes. Collectively, our
Figure 7. LCAL62 down-regulation and correlation with FOXA2 are conserved across cancer types. (a) UCSC Genome browser screenshot of the LCAL62 and FOXA2 region; (b) Correlation of expression of LCAL62 and FOXA2 in lung adenocarcinoma across cohorts (TCGA, CCLE - RNA-Seq; Schabath, Der, Rousseaux; Bittner - microarray; WashU - nanoString); (c) Down-regulation of LCAL62 and (d) correlation with FOXA2 in other cancer types (TCGA RNA-Seq, top - tumor, bottom - normal). Expression is measured by log2(FPKM+1) in RNA-Seq data and log2 (probe intensity) in microarray data; N - normal, P - matched primary, UP - unmatched primary.
Figure 8. LCAL62 regulates FOXA2 expression in lung cancer cell lines. qRT-PCR determined a decrease in FOXA2 expression upon silencing of LCAL62 relative to a scrambled control siRNA and normalized to the control housekeeping gene, RPL32 in (a) A549 and (b) HOP62 cell lines. RNA immunoprecipitation (RIP) assay coupled to qRT-PCR showed LCAL62 binding to SMAD2/3 relative to IgG control in (c) A549 and (d) HOP62 cells. As a positive control, U1 was enriched for its binding partner, SNRNP70, in RIP assay. *p < 0.05, **p < 0.0005, n of 2.

Figure 9. Low expression of FOXA2 increases aggressive phenotypes in lung cancer models. Increased cellular migration and invasion in (a,c) A549 and (b,d) HOP62 cells. qRT-PCR confirmed knockdown of FOXA2 in (e) A549 and (f) HOP62 cells for assays. *p < 0.0005 **p < 0.00001, n of 3.
in silico and in vitro results suggest that LCAL62 regulates FOXA2 to potentially help suppress metastasis across cancer types.

Overall, the poor patient outcome in advanced LUAD highlights a critical need for molecular biomarkers that can stratify patients with potentially help suppress metastasis across cancer types.

**Declarations**

**Author contribution statement**

Ha X. Dang, Nicole M. White, Christopher A. Maher: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Emily B. Rozyczki, Brooke M. Felsheim: Performed the experiments; Analyzed and interpreted the data.

Mark A. Watson, Ramaswamy Govindan: Contributed reagents, materials, analysis tools or data.

Jingqin Luo: Analyzed and interpreted the data.

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**Competing interest statement**

The authors declare no conflict of interest.

**Additional information**

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