Coding and non-coding RNAs produced at the RAMP2 locus are differentially expressed in non-small cell lung cancer.

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Non-small cell lung cancer (NSCLC) is a leading cause of death in the United States and worldwide[1,2]. We mined published microarray data[3,4,5] to discover genes associated with NSCLC. We identified significant differential expression of coding and non-coding RNA transcripts produced at the RAMP2[6] locus in tumors from patients with NSCLC. RAMP2 may be of relevance to the initiation, progression or maintenance of non-small cell lung cancers.

Keywords: non-small cell lung cancer, NSCLC, RAMP2, systems biology of NSCLC, targeted therapeutics in NSCLC.

Over 80% of patients diagnosed with non-small cell lung cancer (NSCLC) will expire within 5 years[7]. Improved understanding of the fundamental transcriptional biology of NSCLC tumors is important for discovery of novel therapeutic targets. We performed a systems-level, unbiased transcriptome analysis of human NSCLC tumors to identify genes whose expression was most different as compared to the lung by mining multiple independently published microarray datasets[3,4,5]. We observed significant differential expression of RAMP2 in NSCLC tumors, suggesting RAMP2 may be important to the biology of non-small cell lung cancers.

Methods

We utilized microarray datasets GSE74706[3], GSE33532[4], and GSE43458[5] for this differential gene expression analysis of NSCLC tumors in conjunction with GEO2R. GSE74706 was generated using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology (platform: GPL13497) with *n*=18 control lung tissue and *n*=10 NSCLC tumors. GSE33532 was generated using Affymetrix Human Genome U133 Plus 2.0 Array technology (platform: GPL570) with *n*=20 control lung tissue and *n*=10 NSCLC tumors. GSE43458 was generated using Affymetrix Human Gene 1.0 ST Array technology (platform: GPL6244) with *n*=30 control lung tissue and *n*=80 NSCLC tumors.

The Benjamini and Hochberg method of p-value adjustment was used for ranking of differential expression but raw p-values were used for assessment of statistical significance of global differential expression. Log-transformation of data was auto-detected, and the NCBI

generated category of platform annotation was used. A statistical test was performed to evaluate whether RAMP2 or RAMP2-AS1 expression was significantly between normal lung tissue and NSCLC tumor tissue using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for all statistical analyses (Version 8.4.0)(455).

Results

We harnessed the power of multiple, independently published microarray datasets[3,4,5] to determine in a blind fashion and at the transcriptome-level genes whose expression was most significantly perturbed in NSCLC tumors.

RAMP2 is differentially expressed in NSCLC.

We observed significant differential expression of the gene encoding the receptor activity modifying protein 2, RAMP2, in human NSCLC tumors when compared to normal lung[2]. When sorting each gene by significance of difference in expression between NSCLC tumor and the normal lung, RAMP2 ranked 210 out of 34183 total transcripts (Table 1). Differential expression of RAMP2 was statistically significant (Table 1; p=2.68E-11).

We queried a second dataset[3] to determine if we could validate RAMP2 differential expression in independent microarray data. We again observed significant differential expression of RAMP2 in human NSCLC tumors when compared to the lung (Table 2). When sorting each gene by significance of difference in expression between NSCLC tumor and the normal lung, RAMP2 ranked 146 out of 25906 total transcripts (Table 2). Differential expression of

RAMP2 in this second dataset was also statistically significant (Table 2; *p*=5.23E-14).

Analysis of a third microarray dataset[4] again revealed significant differential expression of RAMP2 in primary tumors from patients diagnosed with NSCLC. When sorting each gene by significance of difference in expression between NSCLC tumor and the normal lung, RAMP2 ranked 126 out of 33252 total transcripts (Table 3). Differential expression of RAMP2 in this third dataset was statistically significant (Table 3; *p*=3.66E-23).

An anti-sense transcript produced at the RAMP2 locus is differentially expressed in NSCLC.

Further analysis revealed significant differential expression of an anti-sense RAMP2 transcript (RAMP2-AS1) in tumors from patients with NSCLC as compared to normal lung tissue[4]. When sorting each gene by significance of difference in expression between NSCLC tumors and the normal lung, RAMP2-AS1 ranked 29 out of 33252 total transcripts (Table 4). Differential expression of RAMP2-AS1 in NSCLC tumors was statistically significant (Table 4; *p*=1.03E-28).

RAMP2 coding and non-coding RNA expression is significantly decreased in NSCLC tumors.

We obtained exact mRNA expression levels for the differentially expressed RAMP2 transcripts in each of three microarray datasets queried, in normal lung tissue and in primary NSCLC tumors. We also performed a statistical test to determine whether the difference in expression of RAMP2 was statistically significant when comparing NSCLC tumors to the normal lung. RAMP2 was expressed at lower levels across each dataset in NSCLC tumors

as compared to the normal lung (Figures 1-3); decreased tumor expression of RAMP2 was statistically significant in each case (Figure 1: $p<0.0001$; Figure 2: $p<0.0001$; Figure 3: $p<0.0001$). We calculated a mean fold change in RAMP2 expression of 0.7028 ± 0.0599 (Figure 2) and 0.8432 ± 0.0600 (Figure 3) respectively, when comparing NSCLC tumor to normal lung. RAMP2-AS1 was also expressed at significantly lower levels in NSCLC tumors when compared to the normal lung (Figure 4; $p<0.0001$). We calculated a mean fold change of 0.8857 ± 0.0353 in RAMP2-AS1 when comparing NSCLC tumors to the normal lung (Table 4).

Thus, we identified RAMP2 as among the genes most differentially expressed in human non-small cell lung adenocarcinoma and found that RAMP2 and RAMP2-AS1 were expressed at significantly lower levels in NSCLC tumors as compared to the normal lung.

Discussion

In conjunction with the G-protein coupled receptor calcitonin receptor-like receptor, CRLR, RAMP2 functions as part of the receptor for the vasodilatory peptide adrenomedullin[6,8]. RAMPs function in trafficking of CRLR to the plasma membrane. RAMP2 is expressed in the heart, skeletal muscle, placenta, lung, and pancreas[6]. RAMP2-deficient mice die mid-gestation resulting from edema and hemorrhage, with detachment of vascular endothelial cells from the basement membrane[8]. Edema was found to be a result of decreased expression of adhesion molecules including tight junction genes, leading to vascular permeability and leakage[8]. Investigators concluded that RAMP2 was important for angiogenesis and integrity of vascular

tissues[8].

One study of genes whose expression was lost in lung cancer as a result of promoter hypermethylation found that by immunohistochemical staining, 89.8% (44/49) of normal lung tissues were RAMP2-positive while only 22.1% (21/95) of lung cancer tissues were RAMP2-positive. Loss of RAMP2 expression was significantly correlated with tumor grade, with RAMP2-positive staining in 58.3% (7/12) of grade 1 tumors but only 10.4% (5/49) grade 3 and 4 tumors. Ectopic expression of RAMP2 in A549 and H1299 lung cancer cell lines resulted in marked reduction in colony forming ability *in vitro* and an increase in caspase-dependent apoptotic cell death. Thus, RAMP2 might possess tumor suppressive influence and decreased expression RAMP2 in lung cancers, by less sensitive means, has previously been documented.

In summary, we probed multiple independent published microarray datasets to discover differentially expressed genes in the leading cause of cancer death in the United States, non-small cell lung cancer, and discovered significantly decreased expression of coding and non-coding RNAs produced at the RAMP2 locus in tumors from patients with NSCLC. RAMP2 may be relevant to the biology of NSCLC.

References

1. Felip, E., Stahel, R.A. and Pavlidis, N.E.S.M.O., 2005. ESMO Minimum Clinical Recommendations for diagnosis, treatment and follow-up of non-small-cell lung cancer (NSCLC). *Annals of Oncology*, *16*, pp.i28-i29.

2. Siegel, R.L., Miller, K.D. and Jemal, A., 2015. Cancer statistics, 2015. *CA: a cancer journal for clinicians*, *65*(1), p.5.

3. Marwitz, S., Depner, S., Dvornikov, D., Merkle, R., Szczygieł, M., Müller-Decker, K., Lucarelli, P., Wäsch, M., Mairbäurl, H., Rabe, K.F. and Kugler, C., 2016. Downregulation of the TGFβ pseudoreceptor BAMBI in non–small cell lung cancer enhances TGFβ signaling and invasion. *Cancer research*, *76*(13), pp.3785-3801.

4. Meister, M., Belousov, A., Xu, E.C., Schnabel, P., Warth, A. and Hoofmann, H., 2014. Intra-tumor heterogeneity of gene expression profiles in early stage non-small cell lung cancer. *J Bioinf Res Stud*, *1*, p.1.

5. Kabbout, M., Garcia, M.M., Fujimoto, J., Liu, D.D., Woods, D., Chow, C.W., Mendoza, G., Momin, A.A., James, B.P., Solis, L. and Behrens, C., 2013. Ets2 mediated tumor suppressive function and met oncogene inhibition in human non–small cell lung cancer. *Clinical cancer research*, *19*(13), pp.3383-3395.

6. McLatchie, L.M., Fraser, N.J., Main, M.J., Wise, A., Brown, J., Thompson, N., Solari, R., Lee, M.G. and Foord, S.M., 1998. RAMPs regulate the transport and ligand specificity of the calcitonin-receptor-like receptor. *Nature*, *393*(6683), pp.333-339.

7. Heineman, D.J., Daniels, J.M. and Schreurs, W.H., 2017. Clinical staging of NSCLC: current evidence and implications for adjuvant chemotherapy. *Therapeutic advances in medical oncology*, *9*(9), pp.599-609.

8. Ichikawa-Shindo, Y., Sakurai, T., Kamiyoshi, A., Kawate, H., Iinuma, N., Yoshizawa, T., Koyama, T., Fukuchi, J., Iimuro, S., Moriyama, N. and Kawakami, H., 2008. The GPCR modulator protein RAMP2 is essential for angiogenesis and vascular integrity. *The Journal of clinical investigation*, *118*(1), pp.29-39.

9. Yue, W., Dacic, S., Sun, Q., Landreneau, R., Guo, M., Zhou, W., Siegfried, J.M., Yu, J. and Zhang, L., 2007. Frequent inactivation of RAMP2, EFEMP1 and Dutt1 in lung cancer by promoter hypermethylation. *Clinical Cancer Research*, *13*(15), pp.4336-4344.

Rank	ID	p-value	t	B	Gene
210	A_24_P116710	2.68E-11	-1.04E+01	15.8666947	RAMP2

Table 1: RAMP2 is differentially expressed in non-small cell lung cancer.

The rank of differential expression, probe ID, p-value of global differential expression, t, a t-statistic, B, the log-odds of differential expression when comparing tumor to lung, and gene are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
146	205779_at	5.23E-14	-12.887682	21.9733514	0.7028 ± 0.0599	RAMP2	receptor activity modifying protein 2

Table 2: RAMP2 is differentially expressed in non-small cell lung cancer.

The rank of differential expression, probe ID, p-value of global differential expression, t, a t-statistic, B, the log-odds of differential expression when comparing tumor to lung, the fold change in RAMP2 expression when comparing tumor to lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
126	8007348	3.66E-23	-12.603654	42.149738	0.8432 ± 0.0600	RAMP2	receptor activity modifying protein 2

Table 3: RAMP2 is differentially expressed in non-small cell lung cancer.

The rank of differential expression, probe ID, p-value of global differential expression, t, a t-statistic, B, the log-odds of differential expression when comparing tumor to lung, the fold change in RAMP2 expression when comparing tumor to lung, gene and gene name are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
29	8015706	1.03E-28	-15.1065	54.743093	0.8857 ± 0.0353	RAMP2-AS1	RAMP2 antisense RNA 1

Table 4: RAMP2-AS1 is differentially expressed in non-small cell lung cancer.

The rank of differential expression, probe ID, p-value of global differential expression, t, a t-statistic, B, the log-odds of differential expression when comparing tumor to lung, the fold change in RAMP2-AS1 expression when comparing tumor to lung, gene and gene name are listed in this chart.

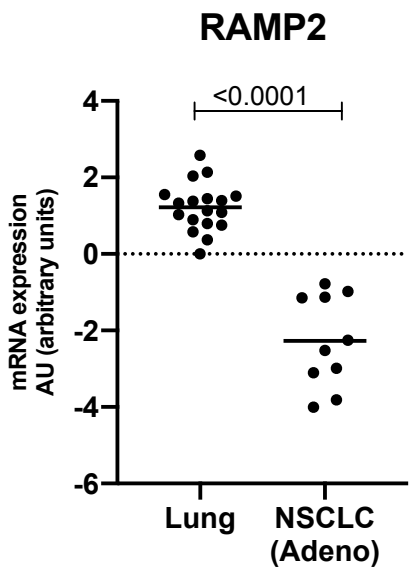

Figure 1: RAMP2 expression is significantly decreased in NSCLC tumors.

Expression of RAMP2 is graphically represented in control, normal lung tissue (left) and in primary tumors from patients with non-small cell lung cancer of the adenocarcinoma type (right); mean mRNA expression level is marked in each group and the result of a statistical test evaluating significance of difference in RAMP2 expression between the groups, a *p*-value, is listed above.

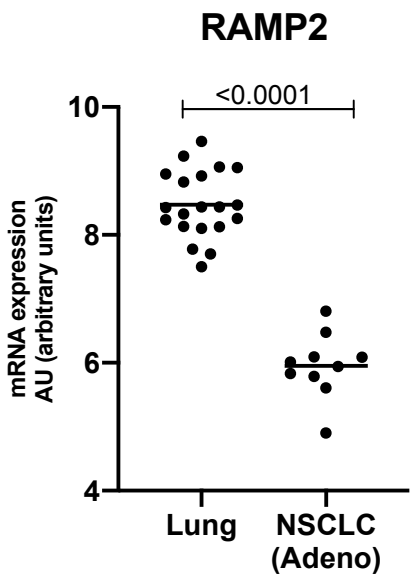

Figure 2: RAMP2 expression is significantly decreased in NSCLC tumors.

Expression of RAMP2 is graphically represented in control, normal lung tissue (left) and in primary tumors from patients with non-small cell cancer of the adenocarcinoma type (right); mean mRNA expression level is marked in each group and the result of a statistical test evaluating significance of difference in RAMP2 expression between the groups, a *p*-value, is listed above.

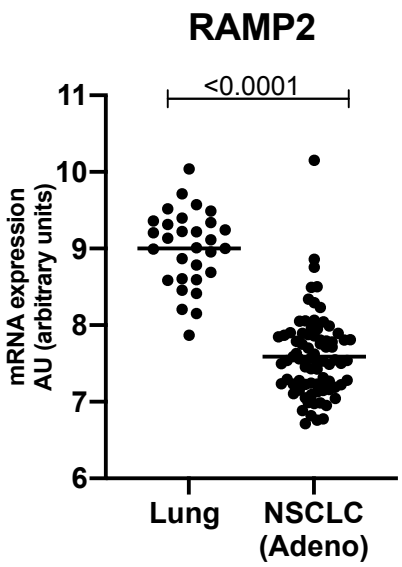

Figure 3: RAMP2 expression is significantly decreased in NSCLC tumors.

Expression of RAMP2 is graphically represented in control, normal lung tissue (left) and in primary tumors from patients with non-small cell cancer of the adenocarcinoma type (right); mean mRNA expression level is marked in each group and the result of a statistical test evaluating significance of difference in RAMP2 expression between the groups, a *p*-value, is listed above.

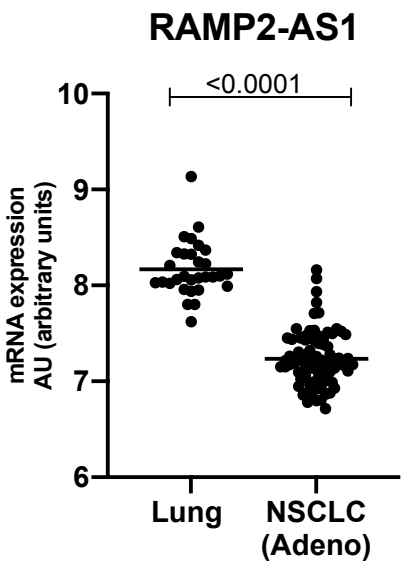

Figure 4: RAMP2-AS1 expression is significantly decreased in NSCLC tumors.

Expression of RAMP2-AS1 is graphically represented in control, normal lung tissue (left) and in primary tumors from patients with non-small cell cancer of the adenocarcinoma type (right); mean mRNA expression level is marked in each group and the result of a statistical test evaluating significance of difference in RAMP2-AS1 expression between the groups, a *p*-value, is listed above.