Expression of the interferon regulatory factor 6 in non-small cell lung carcinoma distinguishes squamous cell lung carcinoma from adenocarcinoma.

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Non-small cell lung cancer, classified as adenocarcinomas or squamous cell lung carcinomas, is the major cause of cancer death in the United States and worldwide¹. To understand the most significant transcriptional differences between adenocarcinomas and squamous cell lung carcinomas, we mined published microarray data from two separate studies^{2,3}. We identified interferon regulatory factor 6 (IRF6) as a distinguishing transcriptional feature of squamous cell lung carcinomas, suggesting the biology of IRF6 may be relevant to the pathways critical for the development or maintenance of squamous cell lung carcinomas but not of adenocarcinomas. IRF6 expression was significantly correlated with prognosis of patients with NSCLC, as patients with low tumor expression of IRF6 possessed significantly longer median overall survival than those with high tumor expression of IRF6. These analyses will also provide novel tools for diagnostic approaches and for guidance of treatment regimens for a cancer with dismal outlook.

Keywords: interferon regulatory factor 6, IRF6, non-small cell lung cancer, adenocarcinoma, squamous cell lung carcinoma, systems biology of NSCLC, targeted therapeutics in NSCLC, targeted diagnostics in NSCLC.

1 Non-small cell lung cancer is the single most common cancer in the United States and
2 the most common cause of cancer death in the United States¹. The two major types of non-
small cell lung cancer are adenocarcinoma and squamous cell lung carcinoma⁴.

3 Treatment approaches and prognosis can differ based on whether a patient is diagnosed
4 with adenocarcinoma or squamous cell lung carcinoma⁴, and current approaches to
distinguishing adenocarcinoma from squamous cell lung carcinoma rely on time-consuming
5 procedures involving immunohistochemical staining with multiple markers including TTF1, p40,
6 p63, CK5 and/or CK7 and subsequent readout by pathologists who will proceed to label each
section, in a binary fashion, as positive or negative for each marker⁵. To understand the
7 molecular nature of ACC and SCLC tumors in an unbiased fashion and at the systems level,
and to facilitate discovery of genes with utility in NSCLC diagnostics in a quantitative real-time
8 workflow providing a quantitative and independent measure rather than qualitative measure
based on pathologist interpretation, we performed comparative transcriptome analysis^{2,3} of the
9 two major types of NSCLC tumors: adenocarcinomas and squamous cell lung carcinomas.

10 This blind, systems-level approach identified the gene encoding interferon regulatory
factor 6, IRF6, as among the genes most differentially expressed when comparing
11 adenocarcinomas and squamous cell lung carcinoma tumors, with IRF6 expression
distinguishing squamous cell lung carcinomas from adenocarcinomas, suggesting IRF6 could
12 be a useful tool in NSCLC diagnostics.

13 **Methods**

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15 We utilized datasets GSE74706² and GSE33532³ for this comparative transcriptome
analysis of adenocarcinomas and squamous cell lung carcinomas. GSE74706 was generated
16 using Agilent-026652 Whole Human Genome Microarray 4x44K v2 technology; for this analysis,
we used $n=10$ adenocarcinomas and $n=4$ squamous cell lung carcinomas, and the analysis was
17 performed using platform GPL13497. GSE33532 was generated using Affymetrix Human
Genome U133 Plus 2.0 Array technology; for this analysis, we used $n=10$ adenocarcinoma
18 tumors and $n=8$ squamous cell lung carcinomas, and the analysis was performed using platform
GPL570.
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20 The Benjamini and Hochberg method of p -value adjustment was used for ranking of
differential expression but raw p -values were used to assess statistical significance of global
21 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
22 evaluate whether IRF6 expression was significantly between adenocarcinoma and squamous
cell carcinoma using a two-tailed, unpaired t-test with Welch's correction. We used PRISM for
23 all statistical analyses of differential gene expression in NSCLC tumors (Version 8.4.0)(455).
For Kaplan-Meier survival analysis, we used the Kaplan-Meier plotter online tool⁷ for correlation
24 of IRF6 mRNA expression levels with overall survival in $n=1925$ non-small cell lung cancer
patients.
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Results

We mined published microarray data from two separate studies^{2,3} to describe in an unbiased fashion and at the systems-level genes whose expression was most specific to either one of the two major types of non-small cell lung cancer: adenocarcinomas and squamous cell lung carcinomas.

IRF6 expression distinguishes squamous cell lung carcinomas from adenocarcinomas.

By comparing the global gene expression profiles of squamous cell lung carcinomas to adenocarcinomas, we found that the interferon regulatory factor 6 was among the most differentially expressed genes between the two major sub-types of NSCLC² (Table 1). When sorting each of the genes expression by microarray based on significance of change in expression between adenocarcinomas and squamous cell lung carcinomas, IRF6 ranked 86 out of 34183 total transcripts (Table 1). IRF6 differential expression between adenocarcinomas and squamous cell lung carcinomas was statistically significant (Table 1; $p=2.6E-06$).

In a separate dataset³, IRF6 was again amongst the most differentially expressed when comparing squamous cell lung carcinomas to adenocarcinomas in NSCLC (Table 2). When sorting each of the genes expression by microarray based on significance of change in expression between adenocarcinomas and squamous cell lung carcinomas, IRF6 ranked 65 out of 25906 total transcripts (Table 2). IRF6 differential expression between adenocarcinomas and squamous cell lung carcinomas was again statistically significant (Table 2; $p=2.39E-18$).

Interferon regulatory factor 6 is expressed at significantly higher levels in squamous cell lung carcinomas than in adenocarcinomas.

We obtained exact mRNA values for IRF6 to understand the magnitude and direction of difference in IRF6 mRNA expression between adenocarcinomas and squamous cell lung carcinomas in NSCLC. IRF6 was expressed at significantly higher levels in squamous cell lung carcinomas as compared to adenocarcinomas, and this difference was statistically significant (Figure 1 and Figure 2: $p<0.0001$ and $p=0.0005$, respectively). We calculated a mean fold change of 1.5362 ± 0.1160 in IRF6 expression when comparing squamous cell lung carcinomas to adenocarcinomas (Table 2).

Expression of IRF6 is associated with patient survival in NSCLC.

We performed Kaplan Meier survival analysis⁷ to determine if IRF6 expression was correlated with patient outcomes in NSCLC. We found significant correlation between expression of IRF6 and overall survival in patients with NSCLC (Figure 3 and Table 3); high IRF6 tumor expression was a negative prognostic indicator. While median overall survival was 98.5 months for NSCLC patients with low expression of IRF6, median overall survival was 52 months for NSCLC patients with high expression of IRF6. Correlation of IRF6 tumor expression with median OS with statistically significant (Figure 3; log rank p -value: $3.9e-06$; hazard ratio: 1.47 (1.25-1.74)).

Thus, by mining published microarray data from independent datasets, we found that IRF6 was among the genes whose expression most significantly distinguished squamous cell lung carcinomas from adenocarcinomas in NSCLC. Moreover, we found a statistically significant correlation between IRF6 tumor expression at the mRNA level and patient survival in NSCLC.

Discussion

To understand the most striking transcriptional features of each of the two major tumor types in NSCLC, we performed comparative transcriptome analysis of ACC and SCLC tumors using published microarray data, discovering interferon regulatory factor 6 as the among the most distinguishing transcriptional feature of squamous cell lung carcinomas.

IRF6 belongs to the family of interferon regulatory transcription factors that translocate to the nucleus and occupy interferon response regulatory elements in genomic DNA to transactivate expression of interferon-inducible genes following signaling downstream of infectious triggers, most typically viral infections, pathogen-associated molecular pattern (PAMP)-associated, or other stimuli⁸. IRF6 is translated from a message comprised of 10 exons and contains a winged-helix DNA-binding domain, an Smad-interferon regulatory factor-binding domain, or SMIR, which functions in protein binding⁹. Mice deficient in IRF1, IRF2, IRF3, IRF4, IRF6, IRF7, IRF8 and IRF9 are manifest no developmental phenotypes but all possess immune signaling-related deficits¹⁰⁻¹⁶. IRF6-deficient mice are the only mice with targeted deletion of an interferon regulatory factor manifesting development phenotypes - abnormalities in the skin, in the limbs and the craniofacial structure¹⁷. While the skin of wild-type embryonic day 17.5 (E17.5) embryos displayed wrinkling, this was remarkably absent from the skin of IRF6-deficient E17.5 embryos. Electron microscopy of wild-type skin displayed keratohyalin granules and a cornified outer layer, but this was absent in *Irf6*-deficient embryos. Toluidine blue is excluded from mature skin, as it does not penetrate cornified layers; toluidine blue was excluded from wild-type embryos but not from *Irf6*-deficient embryos. Proliferation and failure of differentiation of keratinocytes was found to be the source of an expanded spinous layer: while BrdU could be incorporated in both the basal and suprabasal layers of the epidermis in IRF6-deficient embryos¹⁷, BrdU was incorporated only in the basal layers of wild-type embryos; in addition, TUNEL staining was observed in epidermis from wild-type embryos but not from IRF6-deficient embryos¹⁷. Global gene expression analysis revealed that loricrin and filaggrin were significantly decreased in granular and cornified layers, and in the spinous layer, mice deficient in *Irf6* could not silence keratin 14 and p63¹⁷. In terms of limb defects, the forelimbs of *Irf6*-null mice were shortened and lacked visible digits, with a caudal projection that lacked visible hindlimb and tail. While *Irf6* was not expressed in the bone, the vertebrae, sternum, xiphoid process, digits and skull were all abnormal with a secondary palate cleft¹⁷. Thus, *Irf6* is critical for development of the skin, the limbs, the broader skeletal system and the palate¹⁷. Accordingly, mutations in the IRF6 gene are the cause of Van der Woude syndrome, an autosomal dominant cleft lip and palate with lip pits, and IRF6 mutations are also found in patients with Popliteal pterygium syndrome, a disorder resulting in orofacial defects, as well as abnormalities of the skin and the genitals¹⁸. Interactions between the transcription factor p63 and IRF6 are important for cleft palate development in mice¹⁹; other pathways that intersect with p63 and *Irf* in development, specifically in the development of the facial structure through support of epithelial cell death through apoptosis are *Pbx* and *Wnt*²⁰. A function for *Irf6* in keratinocyte proliferation, as

1 evidenced in mice deficient for *Irf6*, is supported hyper proliferative epidermis and soft tissue
2 fusion in mice modeling missense mutations found in patients with Van der Woude syndrome,
3 indicating *Irf6* is important for the transition from proliferation to differentiation of keratinocytes;
4 *Irf6* genetically interacts with 14-3-3 σ in this context²¹. In the context of cancer, during
5 epidermal development. *Irf6* is a Notch target in keratinocytes, and down-regulation of *Irf6*
6 expression supports tumor formation in mice induced by Ras, antagonizing keratinocyte
7 differentiation *in vivo*²². In squamous cell carcinomas, the expression of *Irf6* is significantly
8 down-regulated, CpG methylation of the *Irf6* promoter correlates with decreased expression of
9 *Irf6*, and decreased expression of *Irf6* correlates with tumor invasiveness and differentiation²³.
10 Genome-wide *Irf6* occupancy analysis demonstrated that *Irf6* could directly target genes
11 involved cell cycle, cell adhesion and cell-cell contact²³. Thus, *Irf6* is the only interferon
12 regulatory factor with functions in embryonic development, it is essential for development of the
13 skin, palate and skeletal structure, in humans, mutations in *Irf6* result in palate and orofacial
14 defects; *Irf6* can antagonize keratinocyte differentiation, its expression can be regulated by p63
15 and Notch, and *Irf6* down-regulation and promoter methylation has been described in
16 squamous cell carcinomas.

17 We found that the interferon regulatory factor 6 was the among the most differentially
18 expressed gene when comparing the tumors of patients with the two most common sub-types of
19 NSCLC: adenocarcinomas and squamous cell lung carcinomas. We also found significant
20 correlation between overall survival of NSCLC patients and IRF6 expression. IRF6 has value
21 as a diagnostic tool, as a prognostic indicator, and the biology of IRF6 may be of value in
22 understanding fundamental differences between the two major types of the most common type
23 of cancer, and the most common cause of cancer death in the United States and worldwide.
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Rank	ID	p-value	t	B	Gene
86	A_23_P748	2.6E-06	6.5990767	4.89926	IRF6

Table 1: Interferon regulatory factor 6 is among the most differentially expressed genes when comparing whole transcriptomes of adenocarcinomas and squamous cell lung carcinomas, the two major types of non-small cell lung cancer.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the two groups compared, and gene are listed in this chart.

Rank	ID	p-value	t	B	FC	Gene	Gene name
65	1552477_a_at	2.39E-18	12.7636467	31.494034	1.5362 ± 0.1160	IRF6	interferon regulatory factor 6

Table 2: Interferon regulatory factor 6 is among the most differentially expressed genes when comparing whole transcriptomes of adenocarcinomas and squamous cell lung carcinomas, the two major types of non-small cell lung cancer.

Rank of differential expression, probe ID, *p*-value with respect to differential expression, *t*, a moderated *t*-statistic, *B*, the log-odds of differential expression between the two groups compared, fold change of IRF6 when comparing squamous cell lung carcinomas to adenocarcinomas, gene and gene name are listed in this chart.

Figure 1: Expression of interferon regulatory factor 6 distinguishes squamous cell lung carcinoma from adenocarcinoma.

Messenger RNA (mRNA) levels of IRF6 in the tumors of patients with adenocarcinoma (“ACC”; left) and in the tumors of patients with squamous cell lung carcinoma (“SC”; right) are graphically represented here with mean mRNA levels marked and the result of a statistical test evaluating significance of difference in mRNA expression between the tumors of patients with adenocarcinomas and squamous cell lung carcinomas, a *p*-value, listed above.

Figure 2: Expression of interferon regulatory factor 6 distinguishes squamous cell lung carcinoma from adenocarcinoma.

Messenger RNA (mRNA) levels of IRF6 the tumors of patients with adenocarcinoma (“ACC”; left) and in the tumors of patients with squamous cell lung carcinoma (“SC”; right) are graphically represented here with mean mRNA levels marked and the result of a statistical test evaluating significance of difference in mRNA expression between the tumors of patients with adenocarcinomas and squamous cell lung carcinomas, a p -value, listed above.

Figure 3: Interferon regulatory factor 6 expression in the tumors of patients with NSCLC correlates with overall survival.

Depicted in this Kaplan-Meier plot is the probability of overall survival for $n=1925$ total patients stratified into two groups, based on low or high expression of IRF6 in patient tumors. The log rank p -value denoting statistical significance of difference in overall survival when comparing the two groups, as well as hazard ratio for this comparison is listed above. Listed below is the number of patients at risk (number of patients alive) per interval, after stratification based on IRF6 expression; in the first interval, number at risk is number of patients alive; in each subsequent interval, number at risk is the number at risk less those who have expired or are censored.

Low expression cohort (months)	High expression cohort (months)
98.5	52

Table 3: NSCLC patients with low tumor expression of IRF6 possess significantly greater overall survival than patients with high tumor expression of IRF6.

The median overall survival of $n=1925$ NSCLC patients based on stratification into low or high expression of IRF6 in tumors is listed in this chart.