NF-κB protein expression associates with 18F-FDG PET tumor uptake in non-small cell lung cancer: A radiogenomics validation study to understand tumor metabolism

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A B S T R A C T
Introduction: We previously demonstrated that NF-κB may be associated with 18F-FDG PET uptake and patient prognosis using radiogenomics in patients with non-small cell lung cancer (NSCLC). To validate these results, we assessed NF-κB protein expression in an extended cohort of NSCLC patients.

Methods: We examined NF-κBp65 by immunohistochemistry (IHC) using a Tissue Microarray. Staining intensity was assessed by qualitative ordinal scoring and compared to tumor FDG uptake (SUVmax and SUVmean), lactate dehydrogenase A (LDHA) expression (as a positive control) and outcome using ANOVA, Kaplan Meier (KM), and Cox-proportional hazards (CPH) analysis.

Results: 365 tumors from 355 patients with long-term follow-up were analyzed. The average age for patients was 67 ± 11 years, 46% were male and 67% were ever smokers. Stage I and II patients comprised 83% of the cohort and the majority had adenocarcinoma (73%). From 68 FDG PET scans available, average SUVmax and SUVmean were 8.3 ± 6.6 and 3.7 ± 2.4 respectively. Increasing NF-κBp65 expression, but not LDHA expression, was associated with higher SUVmax and SUVmean (p = 0.03 and 0.02 respectively). Both NF-κBp65 and positive FDG uptake were significantly associated with more advanced stage, tumor histology and invasion. Higher NF-κBp65 expression was associated with death by KM analysis (p = 0.06) while LDHA was strongly associated with recurrence (p = 0.04). Increased levels of combined NF-κBp65 and LDHA expression were synergistic and associated with both recurrence (p = 0.04) and death (p = 0.03).

Conclusions: NF-κB IHC was a modest biomarker of prognosis that associated with tumor glucose metabolism on FDG PET when compared to existing molecular correlates like LDHA, which was synergistic with NF-κB for outcome. These findings recapitulate radiogenomics profiles previously reported by our group and provide a methodology for studying tumor biology using computational approaches.

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1. Introduction

Radiogenomics is an emerging field dedicated to discovering new associations amongst imaging and gene features that will improve our understanding of human diseases and ultimately translate to improved patient care [1]. We have previously published a radiogenomics model of gene expression integrated with 18F-2-deoxy-2-fluoro-D-glucose (FDG) PET imaging that suggests NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) is a central node in tumor glucose metabolism and potentially related to patient prognosis [2]. In that study, we associated the gene expression of tumors for patients with non-small cell lung cancer (NSCLC) and FDG PET imaging data, and then examined the association of those FDG PET related genes and the imaging features themselves with outcome in separate cohorts. The result was a defined cluster of genes- or a metagene- of 508 individual genes that were associated with both FDG uptake and outcome. IPA network analysis (Ingenuity® Systems, Redwood City, CA) implicated NF-κB as a central node within this metagene and provided the rationale for us to proceed with further investigation since NF-κB is well established to play an important role in cancer development and FDG uptake is a surrogate for tumor glucose metabolism and may be prognostic clinically [3].

NF-κB [4,5] is an essential transcription factor that controls multiple cellular processes under normal and pathologic conditions, including tissue differentiation, inflammation, wound healing,
angiogenesis and proliferation [6]. It is present in all tissues and most common is in its cytosolic, bound form. Once released by inhibitor of nuclear factor kappa-B kinase (IkKB) [7], it then migrates to the nucleus to exert its influence on the cell.

Deregulated NF-kB signaling is oncogenic for multiple reasons [8]. Mitogens like cigarette smoke activate NF-kB [9], as do oncogenes like myc [10], and ras [11]. Downstream signaling of NF-kB activation involves cell matrix remodeling [12–14], cell adhesion [15,16], and chemotaxis [17], all of which are important for angiogenesis and metastasis. This has led to interest in targeting NF-kB clinically [18] and to studies assessing patient prognosis with increased NF-kB expression in cancer [19].

Major oncogenes or tumor suppressors like myc [20], and p53 [21–24] that regulate glucose metabolism, NF-kB [10,25], and in some cases both [25,26], have been reported. Exactly how NF-kB signaling interplays with the Warburg Effect [27,28] – or aberrant tumor aerobic glycolysis – and tumor cell metabolism in general has not been well studied, particularly in the clinical setting.

Here, we follow-up on our initial radiogenomics work, where we observed a potential relationship between NF-kB and PET FDG uptake, by creating a large tissue microarray (TMA) to examine if NF-kB immunohistochemistry expression relates to tumoral FDG PET uptake and prognosis in patients with NSCLC.

2. Methods

2.1. Study cohort

Patient samples were retrieved from surgical pathology archives at the Stanford Medical Center’s Department of Pathology and linked to a clinical database using the Cancer Center Database and STRIDE Database tools from Stanford [29]. We reviewed patients who had surgically treated disease and archival formalin-fixed, paraffin-embedded (FFPE) samples from 1995 through June 2010 for inclusion. Patients with samples of recurrent or metastatic disease only were excluded but treated samples (neo-adjuvant therapy) were allowed. Medical charts were reviewed to clinically annotate the tumor specimens with demographics, operative procedures, imaging data, and follow-up. Smoking history was defined as never, ever (quit more than one year ago or defined in the medical record as “former”) and current (active smoking or quit one year ago or less) as per the chart. Pathology reports were reviewed to confirm specimen pathology including stage, grade, histology and invasion status. For this study, tumor invasion was dichotomized as either (1) without any invasion be it pleural and/or lympho-vascular or (2) any invasion be it pleural and/or lympho-vascular. All aspects of this study were IRB approved prior to its initiation in accordance with the Declaration of Helsinki guidelines for the ethical conduct of research. A waiver of informed consent was obtained for the subjects in this study according to Stanford’s Institutional Review Board policy since this was a retrospective study of both alive and deceased patients, many of whom were lost to follow-up.

2.2. Follow-up

Follow-up data was available for this cohort from 1995 through 2010. Recurrence was defined by either imaging or biopsy and those who did not have at least 6 months of follow-up were censored for further analyses. Area of recurrence was defined as within the lung (local metastasis), in the lymphatics or pleura (regional metastasis) or in distant organ sites like the liver, bone, or brain (distant metastasis). The National Death Index (NDI) [30] was used to define vital status through October 30, 2010. Patients were not dead were assumed to be alive except for those who had left the country or were from other countries (who were censored) since the NDI relies on a social security number for vital status assessment. Synchronous tumors resected over time were eligible for prognostic assessment in patients with two primaries.

2.3. Tissue microarray (TMA) construction

The Stanford Lung Cancer TMA was developed from archival FFPE surgical specimens that contained viable tumor from duplicate slides that were reviewed by a board-certified pathologist (RW). The area of highest tumor content was marked manually for coring blocks corresponding to the slides. We then used single 0.6 mm cores to build the tissue microarray from this marked section. These cores were grouped by histology and stage and negative controls were taken from the West Lab that included a variety of benign and malignant tissues (65 cores) consisting of normal non-lung tissue (12 cores), abnormal non-lung tissue (13 cores), placental markers (23 cores) and normal lung (17 cores). Normal lung consisted of a specimen adjacent, but distinct, from tumor over the years 1995 through 2010, to assess the variability of staining by year. OligodT analysis was performed on the finished array to assess the architecture of selected cores and adequacy of tissue content prior to target IHC analysis. A co-registered Hematoxylin & Eosin slide was used as well to verify tumor location for cases where this was unclear on initial inspection. Since LDHA has been evaluated in NSCLC prognosis [31,32] and potentially is also related to FDG uptake due to its involvement in cancer related glycolysis [33,34], we evaluated this target in addition to NF-kBp65.

2.4. Immunohistochemistry (IHC)

IHC was performed using standard methods [35]. In general, antibody titration optimization was performed starting with the manufacturer’s recommendations followed by inspection of adequacy of staining on a separate cancer array with normal tissue controls. NF-kBp65 (Cell Signaling Technology, D14E12, XPlTm Rabbit mAb, #8242) was incubated at a 1:800 dilution using citrate antigen retrieval for final expression analysis. LDHA (Santa Cruz Biotechnology, sc-137243, LDH-A (E-9) Ab) was incubated at 1:400 dilution using citrate antigen retrieval. For NF-kBp65 and LDHA, qualitative scoring was graded as negative (≤20% tumor cells stained), moderate (>20% tumor cells stained) or high (>75% tumor cells stained) intensity using standard bright-field microscopy at 20–40× magnification. Cores that had diffuse weak uptake throughout the tumor were graded as “moderate” and only those with intense staining in the majority of the tumor were assigned a “high” intensity score. We did not score non-tumor cells such as stroma or inflammatory cells and we only assessed cytoplasmic staining intensity for both antibodies. All cores were evaluated by VSN and arbitrated by RW. Missing cores, cores with less than 20 tumor cells, or staining artifacts were treated as non-scorable. Analyses were performed only after all cores scoring had been finalized.

2.5. FDG-PET imaging data

FDG PET-CT images from 2003 through 2010 at Stanford University Medical Center that were most closely dated to the corresponding tumor pathology were retrieved to assess FDG uptake using a General Electric LS PET/CT with slice thickness 3–5 mm (GE, Milwaukee, WI, USA). Patients fasted for a minimum of six hours and a 370–550 mBq dose of FDG was administered. Fasting blood glucose was checked prior to FDG injection and patients were scanned approximately 60 min (range 45–65) after injection.

Only scans performed prior to resection or neo-adjuvant therapy were utilized leaving 88 in total after removing 2 with neo-adjuvant therapy administered prior to imaging. We extracted the maximum standard uptake value Suvmax, defined as the most intense
voxel of uptake within a region of interest (ROI) circumscribing the tumor, and the mean standard uptake value $S_{V\text{mean}}$, defined as the average voxel uptake across the same region of interest circumscribing the tumor, to assess FDG uptake within the primary tumor from which our TMA cores were sampled. The region of interest ranged from 1 cm to the entire cross-sectional area of the tumor. Patients who had synchronous tumors were eligible for analysis if each tumor had extractable FDG uptake information along with NF-kB expression data. We further dichotomized $S_{V\text{max}}$ at a positive value (>2.5) [36] for additional analysis.

### 2.6. Statistical analysis

Cohort characteristics were tabulated using the mean and standard deviation for continuous variables or number with percent for categorical or ordinal variables. Differences between groups (i.e., for recurrence or vital status) were calculated using a Student’s t-test or Wilcoxon test for continuous variables, and a Chi-squared or Fisher’s exact test for categorical variables. Categorical levels of NF-kB staining were compared with continuous $S_{V\text{max}}$ using a Wilcoxon rank sum test. Categorical NF-kB expression and dichotomized $S_{V\text{max}}$ were compared to variables of interest using a Kruskal–Wallis, Chi-squared or Fisher’s exact test. Kaplan–Meier (KM) curves were stratified by NF-kB, LDHA and combined NF-kB/LDHA expression scores for recurrence and death. Continuous $S_{V\text{mean}}$ and $S_{V\text{max}}$ values were associated with outcome along with other variables of interest using Cox proportional hazards (CoxPH) analysis for recurrence or death. All tests associations reported are for two-sided $p$-values and significance is defined as a $p$-value $<$ 0.05. Calculations and figures were generated using SAS (v 9.3; Cary, NC).
3. Results

Seventy-two patients from our previous radiogenomics cohort were included in this TMA. The TMA was constructed from 369 cores, from 359 patients, of which 4 were excluded since they were not primary lung tumors after detailed clinical review, leaving 365 cores for analysis (Table 1). Oligo(dT) analysis showed 99% of the samples were of adequate quality. NF-κB staining was predominantly cytoplasmic and stained weakly or not at all on 147 cores, moderately on 151 cores and highly on 47 cores (Fig. 1). Twenty NF-κBp65 cores were not interpretable due to artifact staining or tissue quality. LDHA cores stained strongly or moderately in 85 and 153 cores respectively and not at all in 114 cores (Supplement 1). Thirteen LDHA cores were not interpretable.

The average age for the cohort was 67 ± 11 years, and the majority of patients were stage I–II (83%), and Caucasian (62%) who smoked (67%) (Table 1). Average tumor diameter was 3.5 ± 2.0 cm, and most were moderately to poorly differentiated (83%) adenocarcinoma (73%). During follow-up, 105 patients recurred (29%) and 170 died (47%). The most common recurrence sites were lung (50%), lymph node (22%), bone (19%) and brain (15%). Differences by group for recurrence were noted for adjuvant therapy, stage, SUVmean and dichotomized SUVmax. For death, groups differed by age, ethnicity, stage and dichotomized SUVmax.

The mean SUVmax for available data was 8.3 ± 6.6 for 88 scans and 3.7 ± 2.4 for SUVmean. SUVmean and SUVmax varied by histology, with non-adenocarcinoma tumors having statistically higher uptake than adenocarcinoma tumors (p-value < 0.001 for both; Supplement 2). Increasing SUVmax corresponded to higher NF-κB expression (6.3 ± 5.2, 9.0 ± 6.8, and 13.0 ± 8.7) for each expression level respectively; p-value = 0.03) and the same was true for SUVmean (p-value = 0.02; Fig. 2). LDHA expression, on the contrary, was not significantly different for dichotomized SUVmax or SUVmean (Table 2). When dichotomized at a threshold value of 2.5, higher SUVmax was associated with smoking status, stage, tumor size, grade, histology, and tumor invasion Comparatively, increasing NF-κB expression was significantly associated with stage, histology and tumor invasion (Table 2). Similar to SUVmax, non-adenocarcinomas (squamous and other NSCLC histology) were associated with increased NF-κB expression when compared to adenocarcinomas (p-value = 0.02).

Outcome for this cohort of patients was as expected and in-line with reported literature estimates [37]. For example, increasing stage was associated with poorer outcome, five-year stage I survival was approximately 60%, and 64% of recurrences occurred within the first two years after resection (Supplement 3). As expected, well known prognostic variables like age, stage, tumor size, tumor invasion and tumor grade were significantly associated with recurrence or death (Table 3).

Increasing FDG uptake levels were strongly associated with five-year recurrence by univariate CPH models and showed a trend with death (p-value = 0.07 and p-value = 0.08 for SUVmax and SUVmean respectively) along with stage, tumor size, and tumor invasion (Table 3). For increasing LDHA expression there was a trend toward death and a significant association with recurrence (Table 3 and Fig. 3). KM plots stratified by high or low NF-κB expression showed a strong trend with increasing level of expression for death (p-value = 0.06; Fig. 3) that was synergistic with LDHA expression (high

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Table 2

<table>
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<th>n</th>
<th>SUVmaxc</th>
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<td>Tumor size (cm)</td>
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<td>Tumor grade</td>
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<td>Tumor invasion</td>
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<td>0.26</td>
<td>89</td>
<td>0.40</td>
<td></td>
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</tbody>
</table>

Associations are shown in bold when significant and shared when both biomarkers were significant, n, number assessed for calculation.

a See Table 1 for method of categorizing analytic variables on left.
b IHC for NF-κB and LDHA expression stratified by high (moderate & high IHC staining combined) or low expression.
c Threshold of 2.5 for SUVmax.

or low) (p-value = 0.03; Fig. 3). NF-κB expression showed no trend for recurrence for any level but again was synergistic with LDHA expression (p-value = 0.04; Table 3, Fig. 3). We found no significant associations for NF-κB levels by local, regional, or distant metastatic sites.

4. Discussion

We show here that NF-κB IHC expression is related to tumor glucose metabolism as measured by FDG PET. Additionally, we show that both of these biomarkers are related to advancing stage, histology and tumor invasion-variables that have established prognostic implications [37,38]. Our results support the recognized interplay of oncogenic, inflammatory and metabolic pathways in cancer by using a practical approach involving clinically relevant data. While previous studies have examined NF-κB expression and prognosis in other cancers [19] or in lung cancer as it relates to stage (which our study corroborates [39], no previous study has examined NF-κB expression along with FDG uptake and prognosis using a large database of surgically treated NSCLC patients.

Our previous radiogenomics study [2] examined FDG PET imaging features in the context of metagene expression profiles and tissue IHC recapitulated this computational approach in a consistent manner with a stronger signal noted for glucose metabolism than smoking, which is a known pro-inflammatory state [40]. Namely, protein level expression representing a complementary level of molecular information was analyzed in an extended, larger cohort of patients using an a priori biologically driven hypothesis from gene network analysis. This illustrates the potential utility of the radiogenomics approach, provides new data on NF-κB in lung cancer, and warrants further investigation on how NF-κB expression is mechanistically linked with tumor glucose metabolism.

Recent findings in the literature are beginning to unravel the connections between oncogenesis, inflammation, and tumor bioenergetics. TP53 is both an indirect and direct regulator of glycolysis via TIGAR (TP53 inducible regulator of glycolysis and apoptosis) [41,42] and G6PD [21], and it has been recently shown to regulate NF-κB via IκB kinase. Additionally, NF-κB is activated downstream of excess lactate production, a hallmark of the Warburg Effect [43]. Interestingly, of several studies that have analyzed lung tumor IHC expression as it relates to FDG PET (including ki67, VEGF, EGFR, GLUT1, and CAIX) [44–49], one study [50] confirmed that TP53 is related to both outcome and FDG uptake. TP53’s control of GLUT1 and GLUT4 [23] regulation supports this study’s clinical observation since FDG is taken up primarily through GLUT1, and could explain the associations that we found in our study as well.

Interestingly, LDHA tissue expression was associated with poorer outcome and did not associate with NF-κB expression or dichotomized FDG uptake but could show synergy with NF-κB for prognosis. LDHA and Lactate Dehydrogenase B (LDHB) are the primary subunits for LDHA, whose subtype is defined by location. Subunits two through five are composed primarily of LDHA, and LDH5 is well studied in the literature both at the tissue level and in circulation as a prognostic marker in lung cancer [31–34]. The lack of association with FDG uptake is surprising, given LDHA’s presumably stoichiometric relationship in glucose metabolism, however, other pathways may be at play given the added value of LDHA in combination with NF-κB, and we have no precedent in the literature to compare our findings. We therefore encourage the readership to follow-up on our results.

Table 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>Recurrence</th>
<th>Death</th>
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<tbody>
<tr>
<td></td>
<td>p-value</td>
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<td>Ethnicityb</td>
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<td>Smoking statusb</td>
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<td>TNM stageb</td>
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<td>0.98</td>
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<tr>
<td>T classification (1–4)b</td>
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<td>&lt;0.001</td>
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<tr>
<td>N classification (1–3)b</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tumor size (cm)</td>
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<td>&lt;0.001</td>
</tr>
<tr>
<td>Histology</td>
<td>0.97</td>
<td>1.01</td>
</tr>
<tr>
<td>Tumor invasionb</td>
<td>&lt;0.001</td>
<td>&lt;0.001</td>
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<tr>
<td>Gradeb</td>
<td>0.03</td>
<td>1.31</td>
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<td>SUVmax</td>
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<td>SUV</td>
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<td>LDHA (Low vs. High)b</td>
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<td>1.84</td>
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Significant values are in bold; HR, hazard ratio; CI, 95% confidence interval.

a According to AJCC VII.

b See Table 1 for levels.

c Moderate and high expression levels were combined for the low vs. high binary comparison for both NF-κB and LDHA.

d Defined as high expression levels for both markers (n = 66) vs. low expression levels for both markers (n = 162).
Fig. 3. Kaplan–Meier curves are shown by stage for (A) five-year disease free survival and (B) overall survival for $\text{SUV}_{\text{max}}$, NF-κB, LDHA and NF-κB/LDHA combined expression. The probability of event is on the y-axis and time-to-event is on the x-axis. The number of events is listed at the bottom of each graph and the p-value for significance between groups is shown within each figure. IHC expression levels were combined as high (moderate or high expression levels, yellow line) vs. low (green line) for simplicity of interpretation.
Strengths of our study include the novelty and large TMA we were able to examine with long-term follow-up and annotation, yet there are several important limitations to consider. The NF-kB superfamily is composed of two protein classes [51] that exist either with a C-terminal ankyrin repeat motif (Class I, NF-kB) or transmembrane activation domain (Class II, Rel; RelA/ p65). However, we assessed cytoplasmic NF-kBp65 only since the antibody clone we used preferentially bound this complex (Fig. 1). Because this is only one of the many protein complexes constituting “NF-kB,” we did not fully delineate its biology by defining the active nuclear state.

We do not think the results in this paper are clinically actionable at this point. However, we do provide a proof-of-concept for validating radiogenomics studies for the clinical investigator in addition to adding to the extant literature on NF-kB tumor biology. Validation in other external cohorts would be a key to moving these findings further toward the clinical lab, as well as more standardized pathology analyses [35]. While developing imaging tracers or drugs to NF-kB would be hindered by the ubiquity of this pathway in humans, other targets that are intimately related to NF-kB tumor metabolism with specificity could be clinically relevant.

Lastly, we only had FDG PET data for a quarter of this cohort, so there could be undetected bias in patients who were differentially excluded based solely on the fact that they did not have FDG PET imaging. Since our measurement of FDG uptake depended on a two-dimensional cross-section of tumor that did not necessarily co-register with the single 0.6 mm tumor core taken from the TMA, there may have been an erosion of statistical signal in our analysis, particularly in light of tumor heterogeneity.

5. Conclusion

NF-kB protein expression and PET FDG uptake of primary tumors in NSCLC appear to be associated with each other as well as with stage, histology and tumor invasion. Although NF-kB was not associated with LDHA expression, these two markers were synergistic for prognosis. These results validate a previously reported, discovery-based exploration of radiogenomic profiles in NSCLC and add to the growing literature linking cell bioenergetics and inflammation in a clinically relevant model.

Conflict of interest

None to declare for all authors.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.lungcan.2013.11.001.

References


