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Outcome Prediction Using Markers of Aerobic Glycolysis (the Warburg effect) Varies Between Tumor Regions in Stage I Non-Small Cell Lung Cancer

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Abstract

Lung cancer, even early stage disease, is an important cause of cancer related death in the US. The Warburg Effect, a phenomenon first described by Otto Warburg, occurs when tumor cells utilize glucose through glycolysis even in the presence of adequate oxygen (aerobic glycolysis). Previously described markers of the Warburg effect and altered tumor metabolism include hypoxia inducible factor 1 (HIF-1), pyruvate dehydrogenase kinase 1 (PDK-1), mammalian target of rapamycin (mTOR), carbonic anhydrase 9 (CA-9), hexokinase 2 (HK-2), and phosphorylated AMP-activated protein kinase (pAMPK). The presence of these antigens was assessed in peripheral and central regions of 58 resected stage I non-small cell lung carcinomas by tissue microarray (TMA) and immunohistochemistry (IHC). Using the median staining intensity as a cut off between high and low expression, peripheral and central antigen expression was correlated with overall and recurrence free survival in univariate and multivariate analysis.

In our study population high levels of HIF-1α in peripheral tumor regions were associated with worse overall and recurrence free survival. Central tumor expression of HIF-1α did not significantly correlate with outcome. A similar trend in the peripheral tumor was seen with PDK-1. In contrast, high levels of mTOR in central tumor cells were associated with improved overall survival. These findings suggest features of the Warburg effect even in early stage (small) lung tumors. Furthermore, they highlight the importance of assessing metabolic markers in the context of oxygen tension and tumor microenvironment. The significance of high HIF-1α expression may be different in relatively oxygenated tumor periphery than it is in the cells of more hypoxic tumor center.

Introduction

Lung cancer is the most common cause of cancer related death in the US [1], with 222,520 new cases and 157,300 deaths reported in 2010 [2]. For clinical purposes, lung cancer has traditionally been divided into the broad categories of small cell and non-small cell carcinoma. Non-small cell carcinomas represent the majority of cases, and encompass a broad range of histologic and molecular phenotypes that include squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Prognosis is relatively poor even for early stage non-small cell carcinoma (NSCLC), with Stage IA and IB 5 year survival reported to be 71.25% and 57% respectively [2]. Understanding the biology and metabolism of tumor cells helps determine factors related to tumor progression and spread, identifies potential new treatment targets and may help stratify patients to guide therapy.

Normal cells utilize glucose as fuel and metabolize it differently based on the availability of oxygen. When oxygen is present, glucose is metabolized through the tricarboxylic acid (TCA) cycle and electron transport chain to produce 36 adenosine triphosphate (ATP) molecules for each glucose molecule. In oxygen deprived tissue, glucose is converted to lactic acid through glycolysis, which yields only 2 ATP molecules per glucose molecule. Cancer cells have been shown to utilize glucose through glycolysis even in the presence of adequate oxygen. This process, known as the Warburg Effect (WE), is a primary reason for the increased utilization of glucose by tumor cells and the resulting production of lactic acid. Dr. Otto Warburg hypothesized that aerobic glycolysis in cancer cells was caused by derangement of energy metabolism due to DNA mutations which caused dysfunctional mitochondria and altered metabolic enzymes [3]. Activation of oncogenes or loss of tumor suppressor genes may contribute to the Warburg Effect in lung cancer cells [4,5]. Benefits of the WE are still not completely clear and there are several theories that attempt to explain causes and effects of the process. Based on their propensity for rapid expansion with an anatomically and physiologically abnormal vascular supply, tumors have an inherent potential for hypo-perfusion with hypoxia and necrosis. Aerobic glycolysis, although seemingly inefficient, has been shown to improve metabolic efficiency in tumor cells and rapidly proliferating cells when excess glucose is available [6]. Altered metabolism may thus serve to protect tumor cells in a labile oxygen environment. Furthermore, since many cellular functions including proliferation and survival are controlled to some degree by the availability of oxygen, inappropriate activation of hypoxia-related pathways may circumvent normal control mechanisms and

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help support a malignant phenotype. It is also likely that rapidly proliferating tumor cells must utilize glucose for needs other than ATP generation such as synthesis of amino acids, lipids and nucleotides [7].

There are many intermediates involved in energy metabolism, some with potential links to the WE. A subset of these markers were chosen for evaluation in this study (markers and rationale are summarized in Table 1). These include hypoxia inducible factor 1 (HIF-1), pyruvate dehydrogenase kinase 1 (PDK-1), mammalian target of rapamycin (mTOR), carbonic anhydrase 9 (CA-9), hexokinase 2 (HK-2), and phosphorylated AMP-activated protein kinase (pAMPK). Given the potential advantages provided by the Warburg phenotype and ties between these intermediates and other pathways relevant to malignancy, we sought to identify evidence of aberrant metabolism and evaluate any correlation between the levels of WE substrates and tumor characteristics, survival, and prognosis of resected pathologic stage I non-small cell lung carcinomas (NSCLC).

Materials and Methods

Ethics statement

The protocol for sample identification and tissue microarray construction was approved by the Institutional Review Board (IRB) of Roswell Park Cancer Institute (RPCI). Review of clinical information was conducted under an approval for electronic data review also granted by the IRB of RPCI, which waived requirements for patient consent.

Tissue microarray construction and immunohistochemical staining

Of all patients with Stage I NSCLC who underwent lung resection between the years of 1993-2000 at Roswell Park Cancer Institute (RPCI), those with adequate residual formalin fixed, paraffin-embedded tumor for tissue microarray (TMA) preparation were selected for study. The American Joint Commission on Cancer (AJCC) staging definitions (Sixth Edition) were used [8]. A final subset of patients (n=58) had sufficient corresponding clinical data for inclusion. Tissue specimens were provided by the Paraffin Archive of the RPCI Department of Pathology. Clinical data were obtained by review of existing medical records and included cancer recurrence, disease status at last follow up (alive with no evidence of disease, alive with recurrent disease, or deceased), cause of death and overall survival.

TMA blocks were prepared using standard methods [9]. From every patient, three 1.2 mm core samples each from central tumor, peripheral tumor, and adjacent benign lung tissue were included in the TMA (a total of nine cores per patient). Each of the antigens was evaluated by immune histochemical (IHC) staining of TMA slides. Prior to staining, slides were deparaffinized and all staining was completed on a Dako Autostainer (Dako®, Carpinteria CA) using diaminobenzidine (DAB) as a chromagen and hematoxylin as counter stain. Appropriate negative IgG controls were employed. Staining for HIF-1α (Novus Biologicals®, Littleton CO, dilution 1/10000) was accomplished by overnight incubation at 4°C after pretreatment with 3% H_2O_2 (10 minutes) and TRS buffer (pH 6.0, microwaved 10 min twice). Detection the next day utilized goat anti-mouse secondary antibody (15 min) with Elite ABC (Vector Labs®, Burlingame CA, 20 min), Amp reagent (Dako®, dilution 1/35, 10 min) and Peroxidase-Streptavidin conjugate (Invitrogen®, Carlsbad CA, 20 min). PDK-1 (Santa Cruz®, Santa Cruz CA, dilution 1/150) detection utilized pretreatment in TRS buffer, primary incubation for 1 hour, then detection using donkey anti-goat antibody (30 min) and Elite ABC (Vector Labs®, 30 min). mTor (Cell Signalling Technology®, Danvers MA, dilution 1/50) detection employed citrate buffer before overnight incubation and secondary incubation with Powervision Poly-HRP (Leica Biosystems®, Buffalo Grove IL, 30 min). CA9 (Santa Cruz®, dilution 1/150) was incubated for 1 hour, following pretreatment with citrate buffer and detection used Envision + (Dako®, 30 minute). HK-2 (Abcam®, Cambridge MA, dilution 1/17) utilized pretreatment with TRS buffer, incubation for 1 hour and detection by Envision + (Dako®, 30 minutes). Finally, pAMPK (Cell Signalling Technology®, dilution 1/50) used TRS pretreatment, 1 hour primary incubation and detection with goat anti-rabbit secondary antibody (30 min) and Elite ABC (Vector Labs®, 30 min).

Immunohistochemical stain scoring and statistical analysis

A pulmonary pathologist (PNB) evaluated IHC staining in a blinded fashion. The staining intensity in each TMA core was scored as 0 (absent), 1 (weak), 2 (moderate) or 3 (strong). The scores for replicate cores were combined into a mean score [10]. Staining was evaluated separately for peripheral and central tumor regions, and also across the whole tumor (central and peripheral together). The median staining intensity was determined for each substrate and tumor region and used as the cut-off point between 'high' (above median) and 'low' (median and below) level expression. Univariate and multivariate analysis utilized a Cox proportional hazards model. Kaplan-Meier methods were used to estimate survival distributions, recurrence free survival (RFS) and overall survival (OS). The log-rank test was used for comparison of the difference in time to event analysis. A 0.05 nominal significance was used in all testing. Statistical analysis and plots were completed using SAS, version 9.2, statistical software (SAS Institute Inc., Cary, NC).

Results

Clinicopathologic characteristics of the study population are outlined in Table 2. Mean patient age was 81.4 years (Median was 83 years, range 52 to 99 years), and there was an equal mix of male and female patients. The vast majority of study subjects (84%) were

Abbrev.	Marker	Cellular function
HIF-1α	Hypoxia Inducible factor 1	Increases during hypoxia and mediates expression of genes that modulate energy metabolism, angiogenesis and cell survival [5,11].
PDK-1	Pyruvate dehydrogenase kinase	Inhibits pyruvate dehydrogenase (PDH), blocking conversion of pyruvate to acetyl-CoA and preventing access to the citric acid (Krebs) cycle [5].
mTOR	Mammalian target of rapamycin	Integrates growth signals, energy status and other parameters to control protein synthesis, cellular growth and proliferation [11].
CA-9	Carbonic Anhydrase 9	Transmembrane protein modulates microenvironment pH, up-regulated under hypoxic conditions [10].
HK-2	Hexokinase 2	Phosphorylates glucose to glucose-6-phosphate, trapping it in the cell and feeding glycolysis [5].
pAMPK	Phosphorylated AMP activated protein kinase	Activated by low levels of ATP, it regulates energy homeostasis by increasing ATP production and stimulating catabolic pathways.

Table 1: Metabolic markers evaluated by immunohistochemistry.

		No. (%)
Sex	Male	29 (50)
	Female	29 (50)
Age	Mean	81.4
	Median	83.0
Race	Caucasian	47 (81)
	Other	11 (19)
Smoking Status	Active Smoker	15 (25.8)
	Ex-Smoker	34 (58.6)
	Never Smoker	5 (8.6)
	Unknown	4 (6.8)
Disease Status at Follow-up	No evidence of disease	26 (44.8)
	Recurrent disease	30 (51.7)
	Unknown	2 (3.4)
Cause of Death (n=46)	Death from lung cancer	27 (58.7)
	Death from other cancer	2 (4.3)
	Death from other causes	14 (30.4)
	Unknown	3 (6.5)
Tumor Histology	Adenocarcinoma	23 (36.2)
	Squamous Cell	21 (36.2)
	Large Cell	3 (6.4)
	Other NSCLC	11 (18.9)
Tumor Stage	IA	23 (39.6)
	IB	35 (60.3)
Tumor Grade (Differentiation)	Poor	30 (51.7)
	Well to Moderate	25 (43.1)
	Unknown	3 (5.2)

Table 2: Clinicopathologic characteristics of patient population.

active or prior smokers. Very few received neoadjuvant therapy prior to surgical resection (6%). Tumors were comprised of those with squamous (36%) and non-squamous (64%) histology. The majority (62%) of non-squamous tumors were adenocarcinoma. Mean patient follow-up was 77 months, during which 47% of patients died from lung cancer or related complications. At last follow-up, only 12 patients remained living (21%).

Based on univariate analysis, several markers showed a statistically significant association with overall survival and/or recurrence free survival. Representative examples of tumor staining are shown in Figure 1. High levels of HIF-1α in peripheral tumor regions were associated with worse OS with a hazard ratio (HR) of 3.07 (95% CI: 1.14-8.30, p=0.04). A similar but not significant trend was seen with high peripheral zone expression of PDK-1 (HR 2.02, 0.77-5.26, p=0.15). High levels of mTOR in the central tumor region were associated with improved OS (HR 0.36, 0.14-0.95, p=0.04); and a trend toward improved OS when assessed over the whole tumor (HR 0.41, 0.15-1.08, p=0.07). Worse RFS was related to high levels of HIF-1α in cells located in peripheral tumor areas (HR 6.52, 1.79-23.72, p=0.004). High pAMPK staining in the tumor center was associated with improved RFS (HR 0.29, 0.09-0.97, p=0.04). A similar trend toward improved RFS was also seen with high mTOR in central tumor areas (HR 0.35, 0.11-1.12, p=0.08). A statistically significant association with OS or RFS was not seen for HK or CA9 (data not shown). None of the clinicopathologic variables including smoking status (past or current), presence of chronic obstructive pulmonary disease (COPD), tumor grade, histologic tumor type, race, stage (IA vs IB) and surgery type (wedge vs lobectomy) proved significant in univariate analysis. Only a history of neoadjuvant therapy was associated with reduced overall survival (HR 5.6, 1.18-26.59, p=0.03).

Markers noted to be statistically significant in univariate analysis elevated with multivariate analysis. Clinicopathologic parameters included in the multivariate analysis were age, history of COPD, smoking status, grade, histology and surgical procedure. High peripheral HIF-1α continued to be associated with decreases in OS and RFS. Increased peripheral PDK-1 was associated with worse OS, but again not at a significant level. mTOR continued to have a positive effect on OS and RFS when observed at high levels centrally and in the tumor as a whole. pAMPK expression also had a positive effect on RFS, although this was statistical significant only in the central and whole tumor. HR and p-values for these associations are listed in Table 3.

The relationship between HIF-1α and PDK-1 staining and outcome was also apparent when generating unadjusted Kaplan Meier curves. These curves (Figure 2 and Figure 3) highlight the difference between assessing staining peripherally and centrally in these tumors. High expression of HIF-1α and PDK-1 centrally did not predict significantly different OS or RFS.

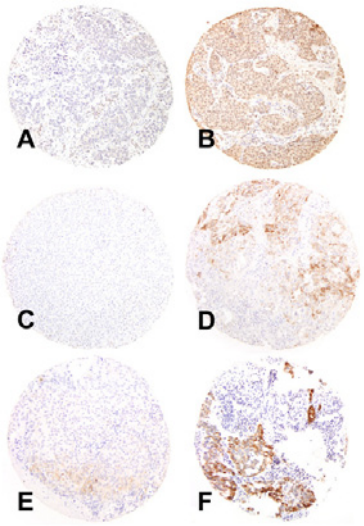

Figure 1: Representative immunohistochemical staining of markers HIF-1α, PDK-1 and mTOR. Variable expression of HIF-1α is illustrated by a poorly differentiated tumor (A) with negative to focally weak (1+) staining and a second tumor (B) with moderate (2+) staining of nuclei and cytoplasm. Similarly, PDK-1 staining in two tumors ranges from negative (C) to patchy and focally strong (3+) cytoplasmic expression (D). While one tumor (E) shows patchy and weak (1+) staining for mTOR, another (F) shows strong (3+) expression.

Marker	Tumor Location	Stain Intensity	RFS		OS	
		Median	HR* (95% CI+)	p	HR (95% CI)	p
HIF-1α	Central	1	0.73 (0.22-2.41)	0.60	0.67 (0.22-2.08)	0.49
	Peripheral	1	6.65 (1.14-38.97)	0.04	8.65 (1.78-42.06)	<0.01
	All Tumor	1	0.92 (0.29-2.89)	0.89	1.01 (0.35-2.96)	0.98
PDK-1	Central	1	0.63 (0.20-2.02)	0.44	0.77 (0.26-2.33)	0.65
	Peripheral	1	4.03 (0.65-24.93)	0.13	3.24 (0.84-12.46)	0.09
	All Tumor	1	1.89 (0.59-5.99)	0.28	1.62 (0.54-4.82)	0.38
mTOR	Central	0.5	0.20 (0.05-0.88)	0.03	0.23 (0.06-0.87)	0.03
	Peripheral	0.67	1.38 (0.25-7.54)	0.71	0.70 (0.16-3.11)	0.63
	All Tumor	0.5	0.10 (0.01-0.66)	0.01	0.12 (0.02-0.64)	0.01
pAMPK	Central	0	0.21 (0.05-0.86)	0.03	0.48 (0.15-1.56)	0.22
	Peripheral	0.17	0.54 (0.15-1.93)	0.34	1.78 (0.62-5.09)	0.28
	All Tumor	0.25	0.27 (0.08-0.90)	0.03	0.68 (0.24-1.95)	0.47

*Hazard ratio with 95% +Confidence Interval

Table 3: Multivariate impact of high (above median stain score) metabolic marker staining intensity on overall survival (OS) and recurrence free survival (RFS).

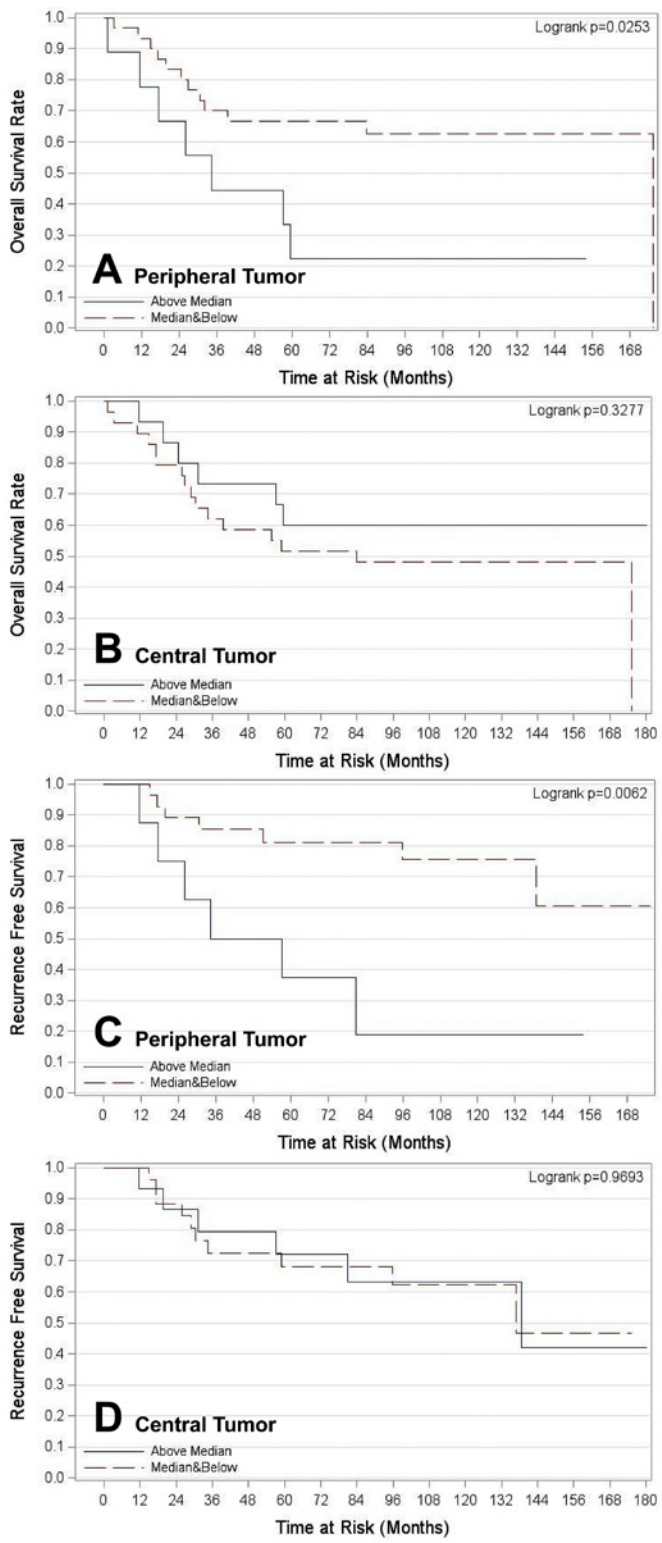

Figure 2: Unadjusted Kaplan-Meier Curves by HIF-1α staining intensity. Patients with high levels of HIF-1α staining (above median: solid line) in peripheral tumor show significantly worse overall survival (A) and recurrence free survival (C). By contrast, staining intensity of central tumor for HIF-1α shows no association with overall survival (B) or recurrence free survival (D).

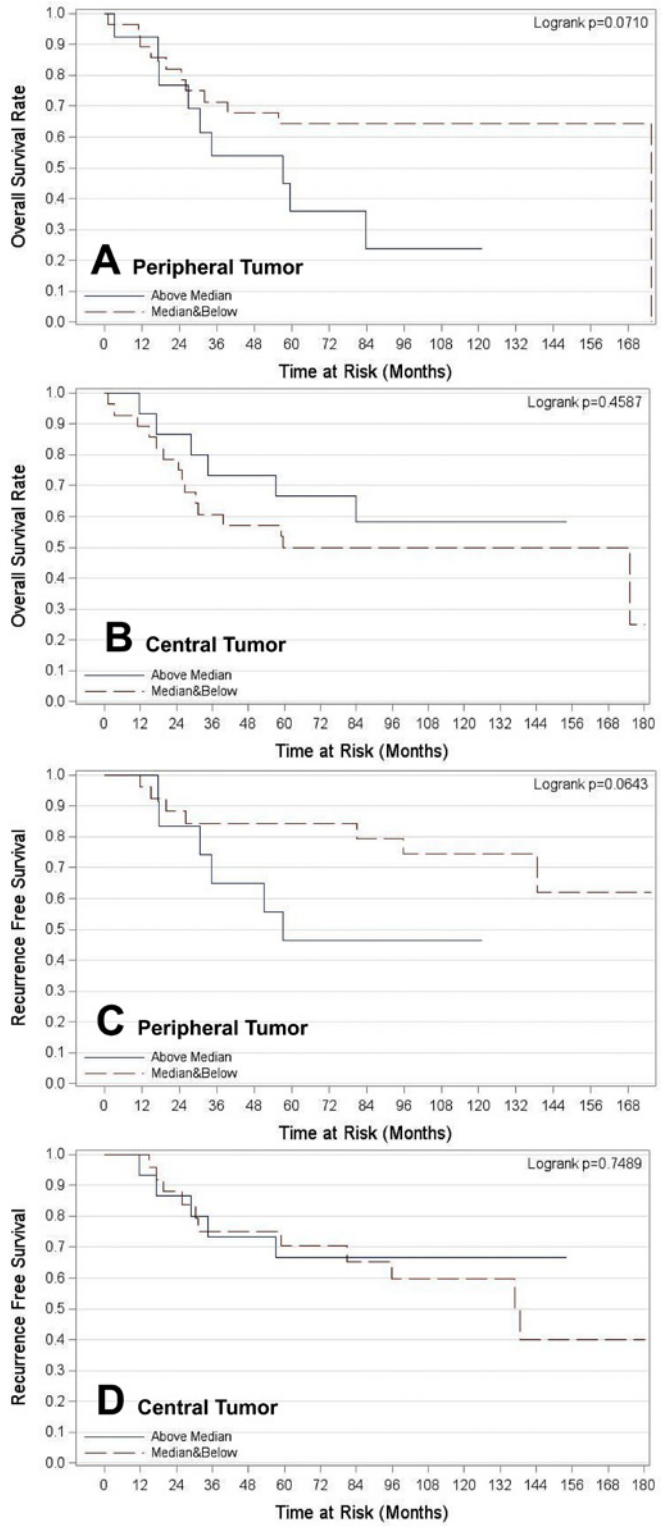

Figure 3: Unadjusted Kaplan-Meier Curves by PDK-1 staining intensity. Patients with high levels of PDK-1 staining (above median: solid line) in peripheral tumor show a trend toward worse overall survival (A) and recurrence free survival (C) that does not quite reach statistical significance. The PDK-1 staining intensity in central tumor shows no association with overall survival (B) or recurrence free survival (D).

Discussion

Tumors are a heterogeneous mix of neoplastic cells, inflammation and fibrovascular stroma. Although the lung is clearly a well oxygenated environment, the center of an invasive lung tumor does not necessarily have a stable oxygen supply. Lung tumor hypoxia has been demonstrated even in small (pT1) lesions [11]. Rapid tumor growth exceeding vascular supply or poorly formed and unstable tumor vasculature may cause drops in oxygenation and result in tumor hypoxia. It is to be expected, therefore, that many tumors will demonstrate areas of hypoxia and increases in molecular markers of hypoxia like HIF-1α and CA9. When these markers are increased even in the presence of oxygen, they can reflect aberrant changes in tumor metabolism. Additional proteins such as mTOR, pAMPK and HK-2 provide information on cellular energy status and glucose use. This study is, to our knowledge, unique since it looks specifically at these markers in the earliest stages of NSCLC and provides separate data on cells from the peripheral and central zones within the tumor. Staining intensity was chosen as the sole parameter for evaluation since even a small or developing subset of aberrant cells can potentially drive future behavior in an early tumor. We opted to divide tumors by the median level of expression thereby maximizing sensitivity in identifying tumors with a high expression level of the markers.

Under conditions of adequate oxygenation, the HIF-1α subunit of the HIF-1 heterodimer is degraded by proteosomal activity. Although HIF-1 levels and activity are normally induced by hypoxic conditions, HIF-1 may be increased by high levels of pyruvate even when oxygen levels are adequate [12]. Since pyruvate accumulates in the setting of aerobic glycolysis, this could create a positive feedback loop further stabilizing HIF-1. In turn, HIF-1 increases the activity of PDK-1 and effectively blocks entry of pyruvate into the citric acid cycle. Numerous research groups have suggested a potential role for the HIF-1α subunit as a prognostic marker either alone or in conjunction with other parameters [13-16]. In those studies, elevated levels of HIF-1α predict shortened RFS and/or OS. It seems likely that this effect reflects both the association of HIF-1 with hypoxia (in turn related to aggressive tumor growth) and HIF-1α as a marker of altered cellular energy metabolism even in the presence of adequate oxygen. The data from our study show a similar association between HIF-1α expression and worse RFS and OS. In this group of early stage NSCLC, however, it is high expression of HIF-1α in peripheral tumor cells that predicts shortened survival. Increased HIF-1α in the central tumor region or tumor evaluated as a whole (central and peripheral areas together) did not prove to be a significant predictor of outcome. Since the periphery of a relatively early (small) lung tumor is more likely to have an adequate oxygen tension, it may be that this background affords the most sensitivity in detecting HIF-1α abnormalities that reflect abnormal energy metabolism in the absence of hypoxia (Warburg effect). That the tumors with highest peripheral HIF-1α expression have a worse prognosis is potential evidence of a Warburg phenotype developing in these early tumors.

HIF-1 serves to trans-activate the *PDK* gene [17] and PDK-1 protein plays an important role in preventing pyruvate entry into the TCA cycle by inhibiting pyruvate dehydrogenase (PDH) mediated conversion of pyruvate to acetyl-CoA. Hazard ratios from our data clearly show a trend toward worse RFS and OS with high PDK-1 expression, but it does not quite reach statistical significance in the multivariate model. Once again, it appears to be tumors with higher (above median) peripheral expression of PDK-1 that behave more aggressively. Surprisingly we did not identify a significant correlation between expression levels of PDK-1 and HIF-1α in peripheral tumor (data not shown). The data of others has suggested that pyruvate mediated HIF-1α stabilization may be the result of reduced PDH in NSCLC [18], and the link between PDK-1 and HIF-1α in tumors is not straightforward. We did not evaluate PDH expression, but elevated tumoral HIF-1α outside the setting of hypoxia (peripheral tumor with improved oxygenation) could reflect aberrant PDH function rather than increased PDK activity.

An alternate perspective on these findings is suggested by recent work characterizing metabolic coupling of cancer epithelium and tumor associated stromal cells. In the proposed "reverse Warburg effect" [19] carcinoma cells induce aerobic glycolysis in the surrounding stromal cells by initiating a state of stromal oxidative stress. Production of reactive oxygen species in the form of hydrogen peroxide [20] induces stromal autophagy and aerobic glycolysis [21,22]. Via aerobic glycolysis, stromal cells produce abundant pyruvate and lactate as a source of energy for the surrounding carcinoma. Although our study does not examine marker expression in stromal cells, it is conceivable that elevated HIF-1α in the lung carcinoma cells is a reflection of elevated environmental pyruvate and/or hydrogen peroxide levels [23]. High peripheral HIF-1α staining could also, therefore, be an indication that a tumor is aggressively subverting energy metabolism in stromal cells at the invasive tumor front. This would clearly aid tumor growth and impact patient outcome. Presumably the energy balance between cancer cells and stroma is different in hypoxic central tumor where it is more difficult to consistently induce stromal aerobic glycolysis and tumor stroma is less substantial and metabolically depleted. Outside the setting of reverse Warburg, some data suggest that increased HIF-1α in cancer cells may actually limit tumor growth [24].

Some of the markers in this study are difficult to predict and understand in this limited data set because they have even broader roles in cellular physiology. In contrast to HIF-1α and PDK-1, we found that increased central and overall tumoral expression of mTOR and pAMPK predicted better survival. This result differs from those in a recent manuscript reporting that high levels of mTOR were associated with poor survival [25]. It is hard to directly compare our data with those, however, since that study includes a wider range of tumor stages (IA through IIB) with some positive nodal metastases and potentially greater tumor sizes. mTOR serves as a high level switch controlling protein synthesis and proliferation. Activity of mTOR controlled pathways can be inhibited by hypoxia in some tumor types, and this effect may be mediated by pAMPK [26]. The association between high mTOR and better survival we observed was strong in both univariate and multivariate models. The significance of this finding is unclear and warrants further investigation. pAMPk plays a critical role in sensing cellular energy status. When levels of ATP drop, activation of AMPk triggers catabolic activity including autophagy [27]. Under the reverse Warburg model cancer cells that are not effectively coupled to energy producing stroma would effectively be "starving" and induce autophagic activity to fulfill energy needs. Theoretically this could explain improved survival in tumors displaying high levels of pAMPk.

Our data suggest that increased expression of HIF-1α, PDK-1, mTOR and pAMPK may independently predict outcome in early stage NSCLC. More specifically, increased expression of HIF-1α and PDK-1 in the peripheral zones of tumor where adequate oxygenation is likely could reflect development of the Warburg effect and more aggressive behavior in a subset of tumors. Another explanation for these findings is that HIF-1α expression in tumor cells may be an indirect reflection of aerobic glycolysis in tumor stroma as part of the reverse Warburg effect. Either way, since staining for HIF-1α and PDK-1 in the central regions

or across the whole tumor did not significantly predict outcome in the same way, interpretation of these hypoxia-related biomarkers should probably take into account the context of tumor microenvironment. The biological meaning of elevated HIF-1α in a hypoxic central area of tumor is likely not the same as elevated expression in the normoxic tumor periphery. Although this study was not designed or powered to answer mechanistic questions related to the Warburg effect, it provides additional evidence that hypoxia, metabolism and the Warburg effect are linked together and influence NSCLC tumor behavior.

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References

1. American Cancer Society: Cancer Facts and Figures (2010) Atlanta, Ga: American Cancer Society, 2010. http://www.cancer.org/acs/groups/content/@nho/documents/document/acspc-024113.pdf. Accessed January 31, 2011.
2. National Cancer Institute: PDQ® Non-Small Cell Lung Cancer Treatment (2011) Bethesda, MD: National Cancer Institute. Date last modified 4/13/11 Available at:http://www.cancer.gov/cancertopics/pdq/treatment/non-small-cell-lung/healthprofessional. Accessed January 31, 2011.
3. Warburg O (1956) On the origin of cancer cells. Science 123: 309-314.
4. Kim JW, Dang CV (2006) Cancer's Molecular Sweet Tooth and the Warburg Effect. Cancer Res 66: 8927-8930.
5. Matoba S, Kang JG, Patino WD, Wragg A, Boehm M, et al. (2006) p53 Regulates Mitochondrial Respiration. Science 312: 1650-1653.
6. Vasquez A, Liu J, Zhou Y, Oltvai ZN (2010) Catabolic efficiency of aeobic glycolysis: The Warburg effect revisited. BMC Syst Biol 4: 58.
7. Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg Effect: The Metabolic Requirements of Cell Proliferation. Science 324: 1029-1033.
8. Greene FL, Balch CM, Page DL, Haller DG, Fleming ID, et al. editors (2002) AJCC Cancer Staging Manual, 6 ed. New York: Springer pp167-177.
9. Seligson DB (2005) The tissue micro-array as a translational research tool for biomarker profiling and validation. Biomarkers 10(supp): S77-82.
10. Liu CL, Montgomery KD, Natkunam Y, West RB, Nielsen TO, et al. (2005) TMA-Combiner, a simple software tool to permit analysis of replicate cores on tissue microarrays. Mod Pathol 18: 1641-1648.
11. Le QT, Chen E, Salim A, Cao H, Kong CS, et al. (2006) An Evaluation of Tumor Oxygenation and Gene Expression in Patients with Early Stage Non-Small Cell Lung Cancers. Clin Cancer Res 12: 1507-1514.
12. Lu H, Forbes RA, Verma A (2002) Hypoxia-inducible Factor 1 Activation by Aerobic Glycolysis Implicates the Warburg Effect in Carcinogenesis. J Biol Chem 277: 23111-23115.
13. Giatromanolaki A, Koukourakis MI, Sivridis E, Turley H, Talks K, et al. (2001) Relation of hypoxia inducible factor 1α and 2α in operable non-small cell lung cancer to angiogenic/molecular profile of tumors and survival. Br J Cancer 85: 881-890.
14. Park S, Ha SY, Cho HY, Chung DH, Kim NR, et al. (2011) Prognostic implications of hypoxia-inducible factor-1α in epidermal growth factor receptor-negative non-small cell lung cancer. Lung Cancer 72:100-107.
15. Hung JJ, Yang MH, Hsu HS, Hsu WH, Liu JS, et al. (2009) Prognostic significance of hypoxia-inducible factor-1 alpha, TWIST1 and Snail expression in resectable non-small cell lung cancer. Thorax 64: 1082-1089.
16. Lau SK, Boutros PC, Pintilie M, Blackhall FH, Zhu CQ, et al. (2007) Three-gene prognostic classifier for early-stage non small-cell lung cancer. J Clin Oncol 25: 5562-5569.
17. Kim JW, Tchernyshyov I, Semenza GL, Dang CV (2006) HIF-1-mediated expression of pyruvate dehydrogenase kinase: a metabolic switch required for cellular adaptation to hypoxia. Cell Metab 3: 177-185.
18. Koukourakis MI, Giatromanolaki A, Sivridis E, Gatter K, Harris AL (2005) Pyruvate Dehydrogenase and Pyruvate Dehydrogenase Kinase Expression in Non Small Cell Lung Cancer and Tumor-Associated Stroma. Neoplasia 7:1-6.
19. Pavlides S, Whitaker-Menezes D, Castello-Cros R, Flomenberg N, Witkiewicz AK, et al. (2009) The reverse Warburg effect. Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 8: 3984-4001.
20. Martinez-Outschoorn UE, Lin Z, Trimmer C, Flomenberg N, Wang C, et al. (2011) Cancer cells metabolically "fertilize" the tumor microenvironment with hydrogen peroxide driving the Warburg effect. Implications for PET imaging of human tumors. Cell Cycle 10: 2504-2520.
21. Martinez-Outschoorn UE, Whitaker-Menezes D, Pavlides S, Chiavarina B, Bonucelli G, et al. (2010) The autophagic tumor stroma model of cancer or "battery-operated tumor growth". A simple solution to the autophagy paradox. Cell Cycle 9: 4297-4306.
22. Martinez-Outschoorn UE, Trimmer C, Lin Z, Whitaker-Menezes D, Chiavarina B, et al. (2010) Autophagy in cancer associated fibroblasts promotes tumor cell survival: Role of hypoxia, HIF1 induction and NFkB activation in the tumor stromal microenvironment. Cell Cycle 9: 3515-3533.
23. Koppenol WH, Bounds PL, Dang CV (2011) Otto Warburg's contributions to current concepts of cancer metabolism. Nat Rev Cancer 11: 325-337.
24. Chiavarina B, Whitaker-Menezes D, Migneco G, Martinez-Outschoorn UE, Pavlides S, et al. (2010) HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells. Autophagy drives compartment-specific oncogenesis. Cell Cycle 9: 3534-3551.
25. Dhillon T, Mauri FA, Bellezza G, Cagini L, Barbareschi, M, et al. (2010) Overexpression of the mammalian target of rapamycin: a novel biomarker for poor survival in resected early stage non-small cell lung cancer. J Thorac Oncol 5:314-319.
26. Schneider A, Younis RH, Gutkind JS (2008) Hypoxia-Induced Energy Stress Inhibits the mTOR Pathway by Activating an AMPK/REDD1 Signaling Axis in Head and Neck Squamous Cell Carcinoma. Neoplasia 10: 1295-1302.
27. Kim J, Kundu M, Viollet B, Guan KL (2011) AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol 13:132-141.