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Carbonic Anhydrase IX Expression Correlates with FDG Uptake by Primary Non–Small Cell Lung Cancer

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Abstract

Tumor cells are characterized by an increased rate of glucose consumption and glycolysis. This increased glucose consumption leads to tumor acidification, which represents a major obstacle for several therapeutic strategies. Tumor cells have adapted to this acidification by upregulation of several H⁺-extruding transporter systems and proteins to cope with this compromised situation. One of these proteins is carbonic anhydrase IX (CA IX), which catalyzes the reversible hydration of carbon dioxide to carbonic acid outside the cell, leading to an acidic extracellular pH and a physiological intracellular pH. The aim of this article was to study semiquantitatively the expression of CA IX in non–small cell lung cancer (NSCLC) and to assess the existence of a possible relationship between CA IX expression and tumor FDG uptake, reflecting glucose metabolism. The levels and the extent of CA IX expression were estimated in immunohistochemical stained, formalin-fixed, paraffin-embedded tissue samples from 18 patients with NSCLC and compared with FDG uptake in FDG-PET imaging. We found a statistically significant correlation between CA IX Hscores and SUVmax and SUVmean values of the primary tumor. This relationship provides indirect evidence for cotranscription of glucose transporters and hexokinases that drive tumor hyperglycolysis and for CA IX governed by hypoxia-inducible factor-1 and suggests that, in the future, it may be possible to identify NSCLC patients who are most likely to benefit from CA IX targeting therapy on the basis of FDG-PET imaging.

Key words: CA IX, FDG-PET, NSCLC, pH

Introduction

As first described by Warburg more than 50 years ago, tumor cells utilize more glucose than normal cells and maintain a high glycolytic rate, even in conditions of adequate oxygen supply (aerobic glycolysis).¹ A major consequence of the Warburg effect and the accompanying aerobic glycolysis is tumor acidification because of the accumulation of lactic acid.^{2,3} Importantly, tumor acidification is associated with the acquisition of a metastatic phenotype and chemoresistance to weakly basic anticancer drugs such as vinca-alkaloids and anthracyclines.⁴ Although the acidification process results in

a drop of pH in the interstitial space, the intracellular pH in solid tumor cells remains close to the physiological value. This suggests that malignant cells extrude protons more avidly than their untransformed counterparts. Indeed, several H⁺-extruding transporter systems appear to be upregulated in cancer cells, such as the monocarboxylate carrier, which exports lactate and H⁺, the Na⁺-H⁺ antiporter, and the H⁺ channels.⁵

In addition to an increased H⁺ efflux, a wide variety of tumor types, including non–small cell lung carcinoma, show increased expression of the transmembrane zinc metallo-enzyme carbonic anhydrase IX (CA IX/CA 9). CA IX catalyzes

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TABLE 1. PATIENT RESULTS

Number	Gender	Age	T-stage	TNM-stage	FDG SUV _{mean}	FDG SUV _{max}	CA IX Hscore
1	F	55	3	2	5.4	6.2	3
2	M	58	3	3	4.6	5.2	3
3	M	79	2	2	13.5	16.7	4.5
4	M	82	2	1	12.1	13.0	6
5	M	78	2	1	19	22.1	7.5
6	M	79	2	4	0.6	0.7	0.0
7	F	73	2	13	8.4	10.1	7.5
8	M	69	2	1	6.3	7.6	4.5
9	M	78	2	3	5.2	6.6	0.0
10	F	56	2	2	6.1	7.2	1.5
11	M	52	2	3	5.8	6.7	2.0
12	M	80	2	3	8.1	9.6	4.5
13	M	62	2	4	9.1	10.9	2.5
14	M	61	2	4	5.4	6.4	1.5
15	M	61	2	1	15.2	16.1	9.0
16	M	65	2	3	11.0	12.9	3.0
17	M	65	3	3	6.4	7.5	5.0
18	F	57	2	4	14.0	15.0	4.5

SUV, standardized uptake value; CA IX, carbonic anhydrase IX.

the reversible hydration of carbon dioxide to carbonic acid outside the cell.⁶ Carbonic acid is subsequently transported inside the cell by $\text{HCO}_3^-/\text{Cl}^-$ anion exchangers, where it may buffer H^+ , while the H^+ generated remains outside the cell contributing to the buildup of the extracellular acid environment.^{5,7-9} Given the role of CA IX in the acidification of the extracellular tumor microenvironment and its neutralizing effect on the intracellular pH, and also the association of tumor acidification with tumor invasion and chemoresistance, CA IX is an attractive target for cancer therapy.

Importantly, the genes encoding for the glucose transporter and hexokinases that drive tumor hyperglycolysis and for CA IX are both under the control of hypoxia-inducible factor-1 (HIF-1).¹⁰⁻¹² Accordingly, the aim of this study was to study semiquantitatively the expression of CA IX in non-small cell lung cancer (NSCLC) and to assess the existence of a possible relationship between CA IX expression and tumor FDG uptake, reflecting glucose metabolism by primary NSCLC. Such a relationship, if existing, could provide a rationale for selection of those patients who have NSCLC and who may benefit from CA IX inhibition treatment, based on FDG-PET imaging.

Materials and Methods

Patients

Eighteen (18) patients in whom substantial biopsy material or a surgical resection specimen of a primary NSCLC was available and who had also undergone an FDG PET-CT scan at 1 week before the intervention or biopsy procedure were included. The diagnosis of NSCLC was histologically confirmed in all patients. CA IX expression was scored and related to FDG SUV_{mean} and SUV_{max} of the primary tumor.

FDG-PET

Whole body FDG-PET scans were acquired on a dedicated PET-CT scanner (Gemini, Philips), from the neck to the pelvis. Patients were required to fast for a minimum of 4 hours prior

to FDG injection. Blood glycemia was monitored with a portable capillary glucometer. Patients received a dose of FDG based on their body weight using the following formula: $([\text{body weight}/10] + 1) \times 37 \text{ MBq}$. The mean delay from FDG injection to imaging was 60 minutes, minimally 50 minutes. Images were acquired in a three-dimensional mode and reconstructed with attenuation correction (CT-based), using ordered subset expectation maximization. Data were processed using MIMVISTA software. Maximum FDG SUV was derived from a region of interest drawn over the tumor lesion on the slice containing the most intense tumor signal, using CT as a reference. The mean tumor SUV value was defined using region growing, using the voxel with the maximum value as seeding voxel and a threshold of 75% of the maximum value. The 75% threshold was chosen to largely exclude regions of tumor necrosis, which may significantly influence the mean SUV value.

FIG. 1. Immunohistochemical staining of CA IX ($\times 200$). Staining was typically confined to the cell membrane and had a strong intensity. CA IX, carbonic anhydrase IX.

FIG. 2. High FDG uptake in the primary non-small cell lung cancer of patient no. 15 and corresponding intense CA IX staining on histology. Arrow indicates the location of the primary tumor.

FIG. 3. Moderate FDG uptake in the primary non-small cell lung cancer of patient no. 12 and corresponding moderate CA IX staining on histology. Arrow indicates the location of the primary tumor.

Immunohistochemistry

Routinely processed, formalin-fixed, paraffin-embedded surgical pathology specimens were used for immunohistochemistry. Sections of 4 μ m thick specimens were mounted on SuperFrost[®] microscope slides (Menzel-Glaser), which were deparaffinized in xylene and rehydrated in a down-graded series of ethanol. After flushing in water, heat-induced antigen retrieval was performed for 20 minutes with the appropriate buffer (EDTA, pH=8.0), after which the tissue slides were cooled down for 20 minutes and then flushed in water for 10 minutes. The endogenous peroxidase activity was blocked for 5 minutes with 0.3% hydrogen peroxide (Dako) on each tissue slide. Primary antibody (anti-CA IX, ABCAM ab15086, 1/2000 in 1% bovine serum albumin (BSA)/phosphate-buffered saline) was then added and incubated for 1 hour at room temperature. After washing, the tissue sections were incubated for 30 minutes at room temperature with a labeled polymer-HRP antirabbit secondary antibody (Dako). The color reaction was developed using the chromogen 3,3-diaminobenzidine+ (Dako) for 10 minutes. After washing, the tissue sections were counterstained with Mayer's hematoxylin.

Phosphate-buffered saline with 1% BSA instead of the primary antibody was used as negative control on each slide to exclude false-positive responses from nonspecific binding of the secondary antibody. Prior to staining the specimens, an isotype control was performed to estimate the nonspecific binding of target primary antibodies to cell surface antigens. Nonspecific binding results from to Fc receptor binding or other protein-protein interactions.

Immunohistochemical analysis

The intensity and percentage of positive tumor cells in the immunoreaction were scored independently by two zexperienced observers. Only membranous staining was scored (intensity and percentage of positive cells). The percentage of tumor cells that were positive for the immunoreaction were scored as follows: 0% (score 0); 0%–20% (score 1), 20%–40% (score 2); 40%–60% (score 3); 60%–80% (score 4); and 80%–100% (score 5). Intensities of staining were categorized as absent (score 0), faint (score 1), average (score 2), or strong (score 3). An estimation of intensity and percentage of positive tumor cells was made after counting 10 high-power fields. A final histological score was calculated as following: $Hscore = [(a1 \times i1) + (a2 \times i2)]/2$, where i = the score of intensity, a = the score of amount of tumor cells that stained positive, and 1 and 2 refer to the scores of the two observers.

Statistical analysis

SPPS Windows, version 15.0, was used for statistical analysis. Correlation analysis was performed using the Spearman rank test (a p -value of <0.05 was considered significant).

Results

Patients

Patient results are shown in Table 1. There were 14 men and 4 women, and the mean age was 67 (range: 52–82). Four (4) patients presented with stage I disease, 3 presented with stage II disease, 7 patients presented with stage III disease,

FIG. 4. Scatterplot of the relationship between CA IX (CA9) Hscores and their corresponding FDG SUVmean values (Tmean).

and 4 patients presented with stage IV disease. FDG-PET data: FDG SUVmax values of the primary tumor ranged from 0.7 to 22.1 (mean: 10.0). FDG SUVmean values of the primary tumor ranged from 0.6 to 19 (mean: 8.7).

CA IX expression

Membranous tumor cell CA IX expression was detected in 16 (88%) patients (Fig. 1). Mean Hscore for CA IX expression in the total group was 3.9 (range: 0.0–9.0; SD: 2.8). CA IX expression was not related to tumor size (T-stage).

Correlation between CA IX Hscores and FDG SUVmax and SUVmean values

A statistically significant correlation was observed when plotting CA IX Hscores against FDG SUVmax and FDG SUVmean values of the primary tumor, respectively, $r = 0.716$ ($p = 0.001$) and $r = 0.758$ ($p = 0.0001$) (Figs. 2–5).

FIG. 5. Scatterplot of the relationship between CA IX (CA9) Hscores and their corresponding FDG SUVmax values (Tmax).

Discussion

The aim of this study was to investigate the expression of CA IX in NSCLC and to determine a possible relationship with FDG uptake in FDG-PET imaging.

In our series, 88% of the tumors investigated showed positive membranous staining for CA IX. Other studies investigating CA IX expression in NSCLC yielded varying numbers of positive cases, varying from 24.6% to 81.8%.^{13–17} In most of these studies, however, a different scoring methodology was used. Vermynen et al. differentiated between cytoplasmic, cytoplasmic with membranous reinforcement, and membranous only staining pattern and used a three-point scoring system for the intensity of staining. Immunostaining proved positive in 52 of 65 tumor samples, with the percentage of stained cells in positive tumors being highly variable.¹³ Giatromanolaki et al. only made a distinction between negative, weak cytoplasmic, and strong membrane/cytoplasmic staining. In their series, 39 of 107 cases studied showed strong membrane/cytoplasmic expression of CA IX.¹⁴ Swinson et al. assessed the percentage of cells with membranous and cytoplasmic expression and divided CA IX expression into quartiles depending on the percentage of cells that stained positive.¹⁵ Kon-no et al. classified NSCLC tumors as CA IX positive or negative using a cutoff of 20% of positive cancer cells showing an unequivocal strong membranous and/or cytoplasmic reaction; using this criterion, 33 of 134 cases studied were CA IX positive.¹⁶ Finally, in the study by Kim et al., a similar scoring system to the one used in the study reported was used and a comparable percentage of patients was reported, 54 of 75 patients (72%) showing CA IX expression.¹⁷ What was of interest, in four of five studies the prognostic relevance of CA IX expression was studied.^{14–17} In three of these four studies, namely the studies by Giatromanolaki et al., Swinson et al., and Kim et al., CA IX expression was related to poor prognosis in both univariate analysis and multivariate analysis, whereas in the study by Kon-no et al., CA IX expression was significantly associated with overall and disease-free survival in univariate analysis only.^{14–17}

In the series presented, a statistically significant correlation was observed between CA IX Hscores and SUVmax and SUVmean values of the primary tumor. This relationship provides indirect evidence for cotranscription of glucose transporter and hexokinases that drive tumor hyperglycolysis and for CA IX governed by HIF-1.^{10–12} To date, only one other study, that is, the study by van Baardwijk et al., reported simultaneously on FDG uptake and CA IX expression in patients with NSCLC. However, the purpose of this study was to address the prognostic value of FDG-PET imaging and several markers related to hypoxia (HIF-1 α and CA IX), proliferation (Ki-67), and glucose metabolism (GLUT1 and GLUT3). Accordingly, the scoring system used (a four-point score system, taking into account only the percentage of positive cells), was not optimized for correlation analysis, and hence, correlation analysis was not performed.¹⁸

Chemotherapeutic drugs that are weakly ionized, for example, vinca-alkaloids such as vinorelbine, which is currently used in an adjuvant setting as well as in advanced NSCLC, will enter cells by passive diffusion in their non-ionized form. In this form, they will tend to partition prefer-

entially across the cell membrane into the compartment where their ionized form predominates. Accordingly, increasing the pH of tumors will enhance the uptake of such agents by the tumor cell, increasing their therapeutic potential. To date, many classes of highly effective *in vitro* CA IX inhibitors have been developed and the pharmacological evaluation of some of them has recently begun (e.g., sulfonamide indisulam is currently being tested in phase II clinical trials).^{4,19,20} The relationship found between CA IX expression and FDG-PET in this study suggests that, in the future, it may be possible to identify NSCLC patients who are most likely to benefit from CA IX targeting therapy on the basis of FDG-PET imaging.

Conclusions

In conclusion, our results showed a statistically significant correlation between FDG uptake in FDG-PET imaging and CA IX expression, providing indirect evidence for cotranscription of glucose transporter and hexokinases that drive tumor hyperglycolysis and for CA IX governed by HIF-1. Based on this finding, studies assessing the predictive value of FDG-PET imaging for CA IX therapy may prove worthwhile to pursue.

Disclosure Statement

No competing financial interests exist.

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