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Levels of Human Equilibrative Nucleoside Transporter-1 are Higher in Proliferating Regions of A549 Tumor Cells Grown as Tumor Xenografts *In Vivo*

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Abstract

3'-Fluoro-3'-deoxythymidine (FLT) has been proposed for positron emission tomography (PET) – based identification of tumor chemosensitivity that is mediated by the human equilibrative nucleoside transporter-1 (ENT1). ENT1 facilitates transport of FLT into cells and elevated levels of FLT are associated with both larger FLT-PET signals and increased response to nucleoside-based chemotherapies. FLT-PET is also used as a measure of tumor proliferation. The present study examined the extent to which ENT1 levels vary in a proliferation-dependent manner in tumor cells *in vivo*.

Methods—The human adenocarcinoma cell line A549 was used to establish tumor xenografts in nude mice. FLT uptake was measured *in vivo* using PET, and further examined *ex vivo* using autoradiography. FLT uptake patterns were compared to immunohistochemical (IHC) analysis of ENT1 and the proliferation markers Ki67 and BrdU.

Results—Regional differences in FLT uptake matched differences in IHC proliferation markers. All cells stained for ENT1, but the staining intensity was twice as high for Ki67⁺ cells than for Ki67[−] cells.

Conclusions—Under *in vivo* conditions, proliferating regions of tumors show increased FLT uptake and higher ENT1 levels than nonproliferating tumor regions.

Keywords

FLT-PET Imaging; ENT1; Ki67; Tumor Cell Proliferation

Introduction

Preclinical and human imaging studies have established the potential of 3'-deoxy-3'-[F-18] fluorothymidine (FLT) as a positron emission tomography (PET)-based measure of cellular

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proliferation. The use of FLT uptake and retention as a surrogate marker for tumor proliferation is based on the assumption that thymidine kinase-1 (TK1, a cytosolic enzyme) activity up-regulated in S phase is the primary factor driving FLT uptake and retention in tumors [1]. While TK1 is an absolute requirement for radiotracer accumulation [2], variations in levels of the equilibrative nucleoside transporter-1 (ENT1), which facilitate FLT transport into cells, have been shown to modify intracellular peak levels of FLT [2, 3].

There are multiple chemotherapeutic agents such as cytarabine, 5-fluorouracil, and gemcitabine that are transported by ENT1 [4–7]. Higher ENT1 levels correspond to greater cell sensitivity to these nucleoside-based chemotherapies [4–7]. Higher levels of ENT1 have also been reported to be induced by treatment with these chemotherapies through their inhibitory effect on *de novo* nucleotide biosynthesis and subsequent compensatory upregulation of the salvage nucleotide synthesis pathway [8, 9]. These observations have led to the suggestion that FLT-PET imaging might be used to distinguish chemotherapy-sensitive tumors from resistant tumors by virtue of their higher levels of ENT1 [5, 8, 9].

In previous *in vitro* work by our group, we reported that plateau-phase cultures of A549 cells have lower levels of ENT1 than proliferating cultures of A549 cells [2]. Our observation and those of Cass et al. [10] and Coe et al. [11] suggest that ENT1 levels are proliferation dependent. To further test this hypothesis, A549 cells were grown as subcutaneous tumor xenografts in nude mice and histological measurements of ENT1 in proliferating and non-proliferating regions of the tumor were compared. Our results support the hypothesis that ENT1 levels are substantially higher in proliferating regions of tumors.

Materials and Methods

Radiotracer Synthesis

^{18}F -FLT was produced according to the method of Buck et al. [12] using 5'-O-benzoyl-2,3'-anhydrothymidine as the organic precursor. ^{18}F -FLT was purified by semi-preparative high-performance liquid chromatography, using a C_{18} column and solvent of 8% ethanol in water. Purified ^{18}F -FLT was made isotonic with phosphate buffered saline.

Cell Culture

The human lung adenocarcinoma cell line A549 was used for all studies. Cells were maintained in log phase growth in DMEM/F-12 media supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100U/mL penicillin, and 100 μg /mL streptomycin, in a fully-humidified atmosphere at 37°C with 5% CO_2 /95% air.

Research Animals

All animal experiments were performed according to procedures approved by the University of Washington Institutional Animal Care and Use Committee, protocol 3126-04. 12–17 week old male nude mice (The Jackson Laboratory, NU/J strain, stock# 002019) were used. Xenograft tumors were established by subcutaneous injection of approximately 3×10^6 A549 cells suspended in sterile saline into both posterior flanks of isoflurane-anesthetized animals. After injection of cells, tumors were measured 3 times per week with digital calipers and tumor volume was evaluated using the formula described by Jensen et al. [13]. Animals were used for subsequent studies when tumors reached a volume of 100–400 mm^3 . Additionally, tumor doubling time was calculated using GraphPad Prism Software (version 5.01; GraphPad Software), and only actively-growing tumors with doubling times between 4–20 days were included in the study.

Small Animal PET

Two to three hours prior to imaging, mice were administered 100mg/Kg of 5-bromo-2'-deoxyuridine (BrdU) via intraperitoneal injection. Mice were anesthetized with isoflurane, then administered 50 μ L of sterile saline containing 8–11 MBq of 18 F-FLT via retro-orbital injection [14]. One or two mice were positioned in an Inveon microPET scanner (Siemens), and dynamically imaged for 90 minutes using Inveon Acquisition software (Siemens) while maintained under 1–2% isoflurane. Following imaging, blood was collected by cardiac puncture and mice were euthanized. Tumors were collected and weighed, and radioactivity in the tumors and blood sample was measured using a Cobra II Auto Gamma γ -counter (Perkin Elmer). Tumors were processed further for autoradiography and/or immunohistochemistry as discussed below.

PET Image Analysis

Seventy to ninety minute summed PET images, empirically determined to have optimal tumor-to-background contrast, were analyzed using Inveon Analysis Workplace software (Siemens). Three-dimensional masks were set around tumors and regions of interest (ROIs) were defined by applying a threshold of 50% of the maximum intensity pixel value. To measure areas within a single tumor with the most and least activity (the tumor periphery and tumor interior, respectively) two additional sub-regions were created within the boundaries of the tumor ROI. This was done by creating two non-overlapping 3 $\times$ 3 $\times$ 3 voxel cubic regions, one centered on the maximum intensity pixel, and the other placed in the geometric center of the tumor. Values for mean activity (Bq/mL) from each ROI were used to calculate standardized uptake values (SUVs) as described in [15]. Activity measured in the post-scan blood sample was also converted to SUV using the same formula, and tumor SUVs were normalized by dividing by the blood sample-derived SUVs.

Autoradiography

Following PET scans, excised tumors were cut in half with a scalpel. Half of the tumor was placed in formalin for immunohistochemistry and the other half used immediately for autoradiography. Tumor tissue was embedded in Optimal Cutting Temperature compound (Sakura Finetek, Torrance, CA), then flash-frozen in liquid nitrogen. Three to five non-adjacent 10 μ m sections were cut from the frozen blocks in a cryomicrotome at -20°C and collected onto charged slides. Slides were exposed to phosphor imaging screens (Imaging Screen-K, Kodak) overnight at room temperature to capture distribution of radioactivity in the sections. Digital images were produced from the phosphor screens using a Molecular Imager FX scanner (Bio-Rad) and Quantity One Software (Version 4.0, Bio-Rad). Images were analyzed using Alice image-processing software (Perceptive Informatics, Inc., Boulder CO). In all cases, radioactivity was clearly higher in the tumor periphery compared to the tumor interior. To compare these areas, a region was manually drawn to encompass the high-intensity pixels of the periphery, and a second region was manually drawn to encompass the remaining area of the tumor interior. Data from these analyses were expressed as mean pixel intensity per region.

Immunohistochemistry

Tumors were fixed in 10% neutral-buffered formalin for 24–48 hours and then stored in 70% ethanol until embedding in paraffin. Adjacent 5 μ m sections of paraffin-embedded tissue were collected onto charged slides, and subsequent procedures were performed on a Leica Bond Automated Immunostainer. Slides were deparaffinized and rehydrated through graded alcohols. Antigen retrieval was carried out with sodium citrate, pH 6.0, for 10min at 100°C . Endogenous peroxide activity was blocked with 3.0% hydrogen peroxide for 5min. The following primary antibodies were applied to the slides at room temperature: ENT1

(Rabbit polyclonal; Lifespan Biosciences, Seattle, WA; catalogue number LS-B3385; 1:200 dilution, 15min), BrdU (Rat monoclonal; Abcam, Cambridge, MA; catalogue number AB6326; 1:2,000 dilution, 30min), and Ki67 (Mouse monoclonal; Dako, Carpinteria, CA; catalogue number M7240; 1:100 dilution, pre-incubated 20min with biotinylated goat anti-mouse Fab fragments, blocked with mouse serum, then applied to tissue for 60min). Slides were washed, then incubated in the appropriate reagents or secondary antibodies at room temperature: ENT1 (Goat-anti-Rabbit Poly-HRP polymer; Leica, Buffalo Grove, Illinois; catalogue number DS9800, 8min), BrdU (Rabbit-anti-Rat polyclonal; Vector Laboratories, Burlingame, California; catalogue number AI-4001; 1:300 dilution, 8min), and Ki67 (Streptavidin-HRP; Jackson ImmunoResearch, West Grove, PA; catalogue number 016-030-084, 1:2,000 dilution, 30min). Color development was carried out by 10min incubation in diaminobenzidine, followed by counterstaining in hematoxylin. Slides were dehydrated through graded alcohols, cleared in xylene, and mounted in permanent mounting media. Optimal antibody concentrations were determined experimentally, and appropriate isotype controls were included for each antibody used.

Microscopy and Evaluation of Immunohistochemical Staining

Slides were digitized using a NanoZoomer Digital Pathology System (Hamamatsu, Bridgewater, NJ) and 4–5 non-overlapping 20x fields of the tumor periphery and tumor interior were saved as independent “JPG” files. Samples were assessed for staining differences between the tumor periphery and tumor interior for each of the immunohistochemical markers.

For Ki67 and BrdU, image processing software was used to count both the total number of cell nuclei in each image, and the number of positively-stained nuclei. Data were then expressed as percent of positively-stained nuclei, derived from these counts. To count total number of nuclei, files were first loaded into Photoshop software (Adobe, version 11.0.2, San Jose, CA) and converted to grayscale images. These images were then loaded into ImageJ Freeware (version 1.44, National Institutes of Health, USA) and analyzed with the ITCN Automated Nuclei Counter plug-in for ImageJ (version 1.6, University of California, Santa Barbara, CA). To count only positively-stained nuclei, Photoshop software was first used to select and delete unstained background and then images were converted to grayscale and analyzed with ImageJ as described above. To ensure consistent selection of unstained background between images, the background staining color range was determined from isotype controls and saved in Photoshop as an “AXT” file, and this file was applied when selecting background color for deletion. To validate the automated cell counting method, positively-stained nuclei were manually counted on all images and compared to automated counts with an unpaired t-test. Manual and automated counts did not differ significantly ($p > 0.05$).

For hENT1 staining, 20x images of 300 cells were evaluated. Visible nuclei were counted in each image and assigned a score from 0 to 3⁺ to indicate staining intensity (0 – no staining, 1⁺ – light staining, 2⁺ – moderate staining, 3⁺ – dark staining). Four interior and five peripheral regions were scored for each tumor, and all were analyzed by two independent scorers. An H-Score was then calculated using the following formula:

$$\text{H-Score} = (\% \text{ at } 0) \times 0 + (\% \text{ at } 1^+) \times 1 + (\% \text{ at } 2^+) \times 2 + (\% \text{ at } 3^+) \times 3$$

To rule out regional variations in Ki67, BrdU, or ENT1 staining due to cell death, adjacent sections stained with hematoxylin and eosin (H&E sections) were examined for tumor necrosis. Tumors with a large necrotic core but viable cells in the tumor periphery were excluded from the final data set. Necrotic areas were defined as regions with intense pink

staining, few or no purple-stained cell nuclei, or a high number of small cells with abnormal nuclei or apoptotic bodies. By contrast, areas of viable cells were defined as regions with little pink staining and densely-packed cells with purple-stained nuclei.

Statistics

Measurements of FLT uptake and immunohistochemical markers were compared between tumor periphery and tumor interior regions by averaging data between samples and comparing means with t test or Mann-Whitney rank sum test while Pearson correlation and linear regression analysis was used to test the relationships between immunohistochemical markers, FLT uptake, and other variables (Sigmaplot 11.0, Systat Software, Inc., Chicago, IL). An α level of 0.05 was used for defining statistical significance.

Results

Measurement of FLT Distribution *in vivo* and *ex vivo*

The distribution of FLT was measured with FLT-PET imaging in 15 tumors grown in the flanks of mice *in vivo*. FLT signal was not uniform for the tumors. In all cases, FLT uptake was observed primarily in the tumor periphery. SUV values for peripheral and interior regions were quantified and the results averaged for all tumors (Table 1). Significantly higher ($p < 0.0001$) FLT uptake was found in the tumor periphery. The mean percentage increase for the peripheral SUV values over the interior SUV values was 37.5 ± 3.7 . The range of increases was from 17% to 73%.

FLT distribution was analyzed in greater detail in 3 tumors *ex vivo* by preparing autoradiographs from thin tissue sections. Similar patterns of FLT uptake were seen in autoradiography images and their corresponding *in vivo* PET images (Figure 1). The FLT signal was primarily concentrated in the periphery of the tissue sections.

Image analysis software was used to quantify differences in radioactivity between tumor periphery and interior on autoradiographs. Three to five independent slices were analyzed for each tumor, and results were averaged. The mean percentage increase for the peripheral intensity values over the corresponding interior values were 88.3, 20.8, and 25.4 for tumors coded 'M34L', 'M53L', and 'M54L', respectively.

Immunohistochemical Measurement of Tumor Proliferation

Immunohistochemical measurements were made on 6 tumors (Table 2). The tumor sizes ranged from 83mm^3 to 256mm^3 (mean = $153.2 \pm 27.3\text{mm}^3$). Tumor 'M53L' was the largest tumor analyzed. Tumor doubling times ranged from 8.7 to 19.7 days (mean = 14.1 ± 1.9 days). Tumors 'M19' and 'M53L' had the slowest growth rates. There was no significant correlation between tumor volume and doubling time ($p > 0.05$).

The percentage of tumor defined as periphery in the slices used for immunohistochemical measurements ranged from 36.6–74.0 (Table 2). The tumor with the largest percentage analyzed as periphery was 'M38L' while that tumor with the smallest percentage analyzed as periphery was 'M53L'.

Ki67 staining (Figure 2B, F, J) identifies actively-proliferating cells in all phases of the cell cycle [16]. Staining for Ki67 was significantly higher in the tumor periphery versus interior ($p < 0.001$), consistent with a higher density of proliferating cells in the tumor periphery. To rule out staining differences due to cell death, adjacent H&E sections were examined for tumor necrosis. In all tumors used for analysis, both tumor periphery and tumor interior areas contained mostly viable cells, and no significant necrosis (Figure 2D, H, L). The mean

fraction of Ki67-stained cells in the periphery was 0.27 ± 0.03 (range = 0.13 – 0.37), while the corresponding value for the interior was 0.09 ± 0.02 (range = 0.04 – 0.13).

BrdU incorporation (Figure 2A, E, I) was used as an indicator of cellular proliferation in the tumor sections. BrdU staining identified cells in S-Phase during the 4–5 hour period between BrdU administration and tissue collection. In all tumors analyzed (5 out of 6), there were significantly higher proportions of BrdU-stained cells in the tumor periphery ($p < 0.001$). The mean fraction of BrdU-stained cells in the periphery was 0.11 ± 0.016 (range = 0.05 – 0.15), while the corresponding value for the interior was 0.02 ± 0.005 (range = 0.01 – 0.04). Many of the BrdU-labeled cells in the tumor interior appeared clustered around blood vessels.

As only Ki67⁺ cells should be in cycle, we calculated a labeling index as a ratio of BrdU-labeled cells to the total number of Ki67⁺ cells. The labeling index for the peripheral regions of the tumors analyzed ranged from 0.32 to 0.43 (mean = 0.40 ± 0.01) while that for the interior ranged from 0.10 to 0.43 (mean = 0.28 ± 0.06). For 2 of the 5 tumors analyzed, ‘M19’ and ‘M29L’, the labeling indices of the periphery and interior regions was essentially the same (Table 2), suggesting that growth rates among the cycling cells in these regions of each tumor were also similar. For ‘M38L’, ‘M38R’, and ‘M53L’, the labeling indices in the interior were lower than that seen in the periphery, which would be expected if the growth rates among cycling cells in these regions were slower than cells in the periphery.

There was a significant linear correlation between the fraction of cells labeled with BrdU and the fraction of cells expressing Ki67 (Figure 3A, $r^2 = 0.94$, $p < 0.001$). There was no significant correlation between Ki67 levels and tumor size or doubling time (Figure 3B, C; $p > 0.05$) even when the periphery and interior were analyzed separately.

Immunohistochemical Measurement of ENT1

Tumor sections adjacent to those used for proliferation analysis were stained for ENT1. There were obvious differences in ENT1 staining intensity across tumors, with robust staining in the tumor periphery and less in the interior (Figure 2C, G, K). H-scores were significantly higher ($p < 0.001$) for peripheral regions of the tumor as compared to interior regions (Figure 3D). The peripheral tumor H-scores were approximately double that observed for the interior regions of the same tumor (mean = 2.2 ± 2.8 fold, range = 1.3–3.2-fold). The mean H-score for the peripheral regions was 180.8 ± 12.9 and that for the interior was 88.7 ± 13.9 . ‘M53L’ was the slowest growing tumor with the smallest fraction of tumor identified as peripheral. The periphery of this tumor also had the smallest Ki67⁺ fraction and H-score of any other tumor periphery. ENT1 levels were significantly higher in tumor regions where Ki67⁺ cells made up more than 15% of the total cell number ($p < 0.001$). There were significant linear relationships between H-scores and the fraction of cells expressing Ki67 ($r^2 = 0.74$, $p < 0.001$) and between H-scores and the fraction of BrdU⁺ cells (Figure 3E, $r^2 = 0.83$, $p < 0.001$).

Discussion

We previously reported that plateau-phase *in vitro* cultures of A549 cells have lower levels of ENT1 than proliferating cultures of A549 cells [2]. We now report that elevated levels of ENT1 were found in proliferating regions of A549 tumors grown *in vivo*. Small subcutaneous tumors of A549 cells grown in nude mice consistently demonstrated regions of higher ENT1 staining co-localizing with regions of high cell proliferation as identified by staining for BrdU incorporation and Ki67. Based on the observation that ENT1 levels are higher in proliferating cells than in non-cycling, growth-arrested cells, we conclude that control of ENT1 levels is partially proliferation dependent; Ki67[−] (G₀) cells have

approximately half as much ENT1 as Ki67⁺ (cycling) cells. As FLT uptake was also greater in the proliferating regions of tumors, these observations strengthen the rationale for using FLT to predict tumor response to therapies such as 5-fluorouracil and gemcitabine, treatments that preferentially target growing cells. These results also highlight the ability of FLT to distinguish proliferation differences within tumors, emphasizing its potential power as an early marker of tumor response to treatment.

Previous observations from Cass et al. [10] and Coe et al. [11] reported that ENT1 levels are cell cycle phase dependent, with higher levels associated with S phase. We observed a significant linear relationship between H-scores and BrdU⁺ cell numbers (Figure 3E), where the fraction of BrdU⁺ cells appeared to discriminate differences between tumor H-scores better than Ki67⁺ fraction (Figure 3D), which would be consistent with the reports of Cass et al. [10] and Coe et al. [11].

Even though only a single tumor cell line was used, there were variations in each of the growth-related parameters. Some of the differences in growth parameters were related to tumor size and tumor doubling time, but there was no way to predict the growth parameters for all tumors based on either of these values. For example, 'M19' was a small tumor that grew slowly but had comparatively high Ki67⁺ proportions in its peripheral regions. And while both Ki67⁺ and ENT1 levels were 2–3-times higher in peripheral versus interior regions of 'M53L', they were much lower than peripheral regions in the 6 other tumors.

The A549 cell line used in this study has intact TP53 function, and this is likely to be related to the predictable G1/G₀ phase growth-arrest behavior seen in these cells under *in vitro* conditions [2]. The immunochemistry results with Ki67 are consistent with a similar arrest of A549 cells in G1/G₀ *in vivo* [16]. Disruption of TP53 function, as often occurs in solid tumors might potentially complicate the link between proliferation and ENT1 levels. Treatment with radiation or certain cancer chemotherapies might further complicate the link between proliferation and ENT1. Further studies on this topic should aid in the interpretation of FLT-PET data for the analysis of tumor treatment response.

Conclusions

ENT1 levels are approximately twice as high in actively proliferating regions of tumors grown *in vivo*. These regions also show higher proportions of Ki67⁺ and BrdU⁺ cells and increased FLT uptake *in vivo*. Proliferation-dependent changes in ENT1 correlate with FLT uptake, strengthening the rationale for using FLT as a tracer of both cell proliferation and relative ENT1 levels.

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Figure 1.

Ex vivo autoradiography studies, examining distribution of ^{18}F -FLT in 3 separate tumors. Grayscale images: *in vivo* FLT-PET images, coronal sections of whole mouse and independent, non-adjacent slices of tissue used in analysis. False-colored images: demonstration of higher ^{18}F -FLT concentration at tumor periphery.

Figure 2.

IHC results from a single representative tumor. Panels A, B, C, and D are from adjacent 5 μ M sections. First column (A–D): 1.25x views of the whole tumor stained for (A) BrdU, (B) Ki67, (C) ENT1, and (D) H&E. Scale bars in A–D = 1mm. Red insets indicate areas of tumor exterior magnified in second column (E–H), blue insets indicate areas of tumor interior magnified in third column (I–L). Second column (E–H): 20x views of tumor exterior, stained as described above. Third column (I–L): 20x views of tumor interior, stained as described above. Scale bars in E–L = 100 μ m.

Figure 3.

Quantitative analysis of IHC results comparing exterior and interior regions of the same tumor. (A) BrdU versus Ki67⁺ fractions. (B) Tumor doubling time (days) versus Ki67⁺ fraction. (C) Tumor size (mm³) versus Ki67⁺ fraction. (D) Ki67⁺ fraction versus H-Score. (E) BrdU⁺ fraction versus H-Score.

Symbols: Tumor M10 (circle), M19 (inverse triangle), M29L (square), M38L (diamond), M38R (triangle), M53L (hexagon). Peripheral tumor regions (filled symbols) and interior (open symbols).

Table 1

FLT-PET SUVs measured in A549 tumors grown in nude mice

Tumor ID	SUV - Total	SUV - Periphery	SUV - Interior
34L	2.29	2.97	1.95
34R	1.73	2.09	1.58
38L	1.95	2.51	1.59
38R	2.12	2.19	1.74
52L	1.74	2.40	1.58
52R	1.87	2.68	1.19
M53L	1.37	2.06	0.56
M53R	1.45	2.02	1.35
M54L	2.01	2.78	2.01
M55L	4.00	5.19	4.30
M55R	3.70	4.86	3.13
M57L	3.84	5.05	3.15
M57R	4.16	5.47	3.43
M58R	5.74	7.53	5.21
M59L	1.90	2.69	1.54
Average	2.66	3.50	2.29
Standard Error	0.33	0.43	0.33

Table 2

Summary of Individual A549 Tumor Characteristics

Tumor ID	Tumor Volume (mm ³)	Doubling Time (days)	Periphery Size (% Tumor)	LI ¹ Exterior	LI ¹ Interior
M10 ²	107.2	15.5	49.9	ND ³	ND ³
M19	132.4	19	46.1	0.415	0.410
M29L	83.2	8.7	59.3	0.387	0.427
M38L	126.8	12.3	74.0	0.364	0.105
M38R	213.7	9.3	56.5	0.412	0.240
M53L	256.1	19.7	36.6	0.428	0.237

¹ BrdU Labeling Index

² M10 was A549-LXSN [17] while all the other tumors were the A549 parent line

³ No BrdU Labeling data