Endogenous Two-Photon Fluorescence Imaging Elucidates Metabolic Changes Related to Enhanced Glycolysis and Glutamine Consumption in Precancerous Epithelial Tissues

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Abstract

Alterations in the balance between different metabolic pathways used to meet cellular bioenergetic and biosynthetic demands are considered hallmarks of cancer. Optical imaging relying on endogenous fluorescence has been used as a noninvasive approach to assess tissue metabolic changes during cancer development. However, quantitative correlations of optical assessments with variations in the concentration of relevant metabolites or in the specific metabolic pathways that are involved have been lacking. In this study, we use high-resolution, depth-resolved imaging, relying entirely on endogenous two-photon excited fluorescence in combination with invasive biochemical assays and mass spectrometry to demonstrate the sensitivity and quantitative nature of optical redox ratio tissue assessments. We identify significant differences in the optical redox ratio of live, engineered normal and precancerous squamous epithelial tissues. We establish that while decreases in the optical redox ratio are associated with enhanced levels of glycolysis relative to oxidative phosphorylation, increases in glutamine consumption to support energy production are associated with increased optical redox ratio values. Such mechanistic insights in the origins of optical metabolic assessments are critical for exploiting fully the potential of such noninvasive approaches to monitor and understand important metabolic changes that occur in live tissues at the onset of cancer or in response to treatment. Cancer Res; 74(11); 3067–75. ©2014 AACR.

Introduction

Metabolic alterations contribute to the development of numerous diseases, including obesity, diabetes, and cancer (1). Utilization of major metabolic pathways, such as glycolysis, glutaminolysis, and oxidative phosphorylation, is altered in cancer cells to meet the bioenergetic and biosynthetic demands associated with tumor growth (2–4). The mechanistic bases of these alterations are studied intensively as they could provide important targets for the development of novel diagnostic and therapeutic modalities (5). Such studies are typically performed in two-dimensional (2D) cell culture models via invasive assays that often preclude analyses as a function of time. Studies with methods that allow for dynamic metabolic assessments within relevant live tissues and/or 3D tissue models by noninvasive techniques are critical to monitor metabolic changes during disease development or in response to novel treatments.

Two-photon excited fluorescence (TPEF) microscopy is well-suited for such studies because of its high resolution in 3-dimensions and the ability to noninvasively detect morphologic and functional cellular changes in living tissues (6–9). This is particularly true for metabolic studies, as two key metabolic coenzymes, reduced nicotinamide adenine dinucleotide (NADH) and oxidized flavin adenine dinucleotide (FAD), are naturally fluorescent (10) and serve as optical biomarkers to estimate the redox state of a cell (11). However, quantitative correlations between optical redox measurements and redox estimates acquired via traditional invasive means, such as enzymatic assays or nuclear magnetic resonance and mass spectrometry measurements, have been elusive. Such correlations are not trivial because the endogenous cellular fluorescence detected within the excitation and emission wavelengths used to detect NADH and FAD overlaps with fluorescence from other cellular chromophores, such as keratin, lipofuscin, and retinol (8, 9, 12, 13). In addition, other flavins (14) or NADH-like molecules such as NADPH (15) may also contribute to signals detected in the same excitation/emission wavelength ranges. Furthermore, changes in NADH, FAD, and redox-related optical biomarkers have been interpreted almost exclusively on the basis of expected alterations in the relative levels of oxidative phosphorylation and glycolysis (11). While these are the main energy-producing pathways in the cell, more detailed studies that examine the potential role of other processes, such as fatty

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acid biosynthesis or glutaminolysis, in detected optical metabolic readouts have been lacking.

The goal of this study was to examine the sensitivity of tissue optical redox ratio measurements to distinct metabolic changes that can potentially occur at the onset of cancer development. To achieve this goal, we imaged live, 3D engineered epithelial tissue equivalents (EETE) that provided a well-controlled, reproducible tissue model that enabled us to examine the relationship between the optical redox ratio and metabolic alterations associated with glycolysis and glutaminolysis. Normal stratified squamous epithelial tissues were constructed using primary human foreskin keratinocytes (HFK). The precancerous tissues were based on cells that serve as good models of cervical squamous intraepithelial lesions. Specifically, we used HKc/DR cells that are HPV16-immortalized HFKs, that are differentiation-resistant and do not growth arrest in response to TGF-β. While these cells are nonmalignant, they have an mRNA expression profile that reflects many cellular and molecular alterations characteristic of cervical cancer (16). To determine whether the observed changes may be directly linked to expression of HPV oncoproteins in cells or whether they are selected for during the process of immortalization, we also used nonimmortalized HFK populations with stable expression of the HPV16 E6 oncoprotein (16E6 HFKs), the HPV16 E7 oncoprotein (16E7 HFKs), or the HPV16 E6 and E7 oncoproteins (16E6E7 HFKs). The HPV16 E6 and E7 oncoproteins interfere with cellular signal pathways, including the p53 and pRB tumor suppressors, respectively, that are frequently inactivated during carcinogenesis (17). Hence, the results obtained with HPV16 E6 and/or E7 expressing HFK EETEs may provide insights that might be relevant for a range of epithelial tumors that have mutations in these pathways.

Using three different types of EETEs, we sought to establish a direct correlation between an optical redox ratio of FAD/(NADH + FAD) fluorescence and corresponding redox ratio assessments based on invasive mass spectrometry measurements. We hypothesized that alterations in the relative levels of glycolysis or glutaminolysis that often occur during the onset of cancer would result in distinct optical redox ratio changes. We identified diverse optical redox changes in the different EETE types and performed enzymatic assays and mass spectrometry measurements to elucidate the potential relationships among the optical redox ratio, oxidative phosphorylation, glycolysis, and glutaminolysis. Thus, we demonstrated the potential of optical redox measurements to track quantitative concentration-based redox changes and provided a more detailed metabolic context for the interpretation of distinct optical redox alterations that may occur at the onset of cancer.

**Materials and Methods**

**Engineered epithelial tissue equivalents**

Five distinct types of 3D EETEs were created using established methods (18) and the following types of human primary epithelial cells: HFKs used as normal control cells (C HFKs); HFKs with stable expression of HPV16 E6, HPV16 E7, and HPV16 E6/E7 generated by infection with appropriate retroviral vectors as described previously (19); and the HKc/DR cells, derived from PHK16-0b/HKc/HPV16d-1, a permanent cell line established by immortalization of HFKs derived from a single donor by expression of a head-to-tail dimer of the full-length HPV16 genome followed by selection for TGF-β and differentiation resistance (20, 21). HKc/DR cells are nonmalignant, but mRNA expression analysis has shown that they share many of the transcriptional aberrations that are detected in cervical carcinoma cells (16). HKc/DR cell line authentication was performed through short tandem repeat (STR) analysis, which confirmed that the DNA profile matched PHK16-0b cells.

**TPEF data acquisition**

Three-dimensional stacks of TPEF images were acquired from live EETEs using a commercial Leica TCS SP2 confocal microscope equipped with a Tisapphire laser (Spectra Physics). Samples were placed on glass coverslips, excited with 755, 800, and 860 nm (TPEF) light, and imaged using a 63×/1.2 NA water immersion objective. TPEF Z-stacks were acquired in 2 μm depth increments. The microscope was equipped with a microincubation chamber that kept samples in a controlled environment at 37°C and 5% CO2. TPEF images were acquired by non-descanned PMTs with a filter cube containing a 700-nm short-pass filter (Chroma SP700bp), a dichroic mirror (Chroma 495dcxr), and emitter bandpass filters centered at 460 nm (Chroma 460bp40) and 525 nm (Chroma 525bp50). One to four regions from 50 independent EETE tissues (nHKc = 12, n16E6 = 11, n16E7 = 8, n16E6E7 = 15, nHKc/DR = 4) were imaged and assessed. All TPEF fluorescence intensity images were normalized to fluorescein concentrations (micro-molar) to account for changes in PMT gain and laser power as described previously (22).

**Quantitative analysis of endogenous fluorescence**

The overall data analysis approach is depicted schematically in Fig. 1. In epithelial tissues, natural fluorescence emanates mainly from NADH, FAD, and keratins (8, 13, 15). It is important to isolate the keratin-positive fluorescent pixels to accurately quantify the intracellular NADH and FAD fluorescence contributions. This was achieved using linear discriminant analysis (LDA) based on a combination of the fluorescence intensity images that we detected at 460 and 525 nm using 755, 800, and 860 nm excitation (see Supplementary Methods). The LDA equations were used to classify each pixel in every TPEF image automatically as either keratin or non-keratin.

Pixels with fluorescence that were not classified as keratin were identified as pixels that could contain NADH and/or FAD fluorescence. Specifically, the signal detected at 755 nm excitation and 460 nm emission was attributed to NADH, whereas the signal detected in the 525 nm range upon 860 nm excitation was attributed to FAD, consistent with previous studies (9, 12, 22). The redox ratio was computed on a pixel-by-pixel basis as the normalized fluorescence intensity contributions from FAD over the sum of the intensity contributions from NADH and FAD.

As most EETEs contained upper layers largely occupied by keratin and very few cells, we report redox ratio assessments that started at optical fields within the superficial layers containing cells in at least 80% of the area and continued to the deepest sections covered with cells in at least 90% of the...
field. The pixels of some of the deeper layers that appeared dark and comprised less than 10% of these optical sections were presumably occupied by the underlying collagen and were identified as pixels not occupied by cells by the LDA algorithm. Mean redox ratio estimates for each layer were derived only from the cell-occupied pixels (i.e., pixels occupied by keratin at the top or collagen at the bottom layers were excluded) to ensure that the reported redox ratios represent accurately the metabolic state of the cells. The average redox ratio was calculated on the basis of individual pixel redox ratio estimates from each optical section acquired within a stack that spanned the epithelium. The values representing the redox ratio as a function of depth for each stack of images were interpolated to 45 points to report summary statistics on depth-dependent redox ratios acquired from EETEs with varying thicknesses.

For the calculations reporting layer-based mean values, the interpolated redox ratio was divided into three equal sections representing signal primarily from the superficial (S), para-basal (P), and basal (B) layers.

To enhance cellular feature visualization, the intensities of redox ratio color maps were weighted by the sum of the normalized intensity of the NADH and FAD fluorescence channels. After this weighing, all 3D image rendering was done in OsiriX (v3.0.2). This processing was done for visualization purposes only.

**Biochemical analysis**

To validate the specificity and sensitivity of the optical redox ratio to the actual NADH, NAD⁺, and FAD concentrations, intracellular metabolites were extracted and measured through liquid chromatography/tandem mass spectrometry (LC/MS-MS). To extract intracellular metabolites, tissues ($n = 14$) were cut in half before imaging. The epithelium was peeled from the dermis and flash-frozen in preparation for a modified version of a previously established extraction protocol (23).

**Biochemical assays of tissue media**

To assess tissue substrate consumption, fresh media were provided to the EETE cultures 24 hours before imaging. Fresh media and cultured media were collected 2 to 3 hours before imaging and frozen at −80°C. Samples were defrosted, and standard glucose (Pointe Scientifc, G7519-1L) and lactate reagents (Trinity Biotech, 753-11) were used to measure the glucose and lactate concentration in the fresh and cultured media with a microplate reader. Glutamine was quantified from the media using LC/MS-MS as described above. Glutamine consumption measurements made 24 hours before imaging were normalized by thickness of the epithelial cell layer measured optically to compare consumption data across EETE groups.

**Immunohistochemical staining**

Following TPEF imaging, tissues were fixed to be stained with hematoxylin and eosin (H&E) or antibodies against keratin 10 (K10), keratin 14 (K14), involucrin (Inv), loricrin (Lor), and proliferation marker [proliferating cell nuclear antigen (PCNA)]. Antigen retrieval was performed by
incubating sections in a citrate buffer (10 mmol/L citric acid, 0.05% Tween 20, pH 6.0) at 95°C for 20 minutes. Primary antibodies were used at the indicated dilutions: K10 (1:200, Abcam ab9026), K14 (1:200; Abcam ab7800), involucrin (1:200, Abcam ab53112), loricrin (1: 80, Sigma-Aldrich AV41738), and PCNA (1:250, Abcam ab29). Secondary antibodies were used at the indicated dilutions: goat anti-rabbit (1:200; Abcam ab96885) and goat anti-mouse (1:200, Abcam ab96879). Slides were mounted using Vectashield H-1200 Mounting Medium with DAPI (Vector Labs) and imaged with a Leica DFC340 FX camera.

Statistical analysis

To assess statistical significance of the redox ratio differences among groups, we used a one-way ANOVA and Dunnet post hoc tests in Jmp 10 SAS. The average redox ratio of individual EETEs was computed from the average redox ratio of individual image volumes, and these EETE-averaged values were analyzed in the ANOVA. Similarly, a one-way ANOVA was used for the analysis of the G/L and glutamine assays. The significance of Pearson correlation coefficients between measurements was determined on the basis of the null hypothesis that R = 0.

Results

Redox ratio images enable visualization of functional biochemical differences between normal and precancerous EETE models

Depth-dependent, TPEF images of EETEs relying entirely on detection of endogenous fluorescence acquired at a combination of excitation (755, 800, and 860 nm) and emission (460 ± 20 nm and 525 ± 25 nm) wavelengths were analyzed to identify and quantify the signals emanating primarily from NADH, FAD, and keratin (Fig. 1). The intensity-based TPEF images detected at each one of these excitation/emission ranges reveal subcellular morphologic and organizational features that change as a function of depth (see Supplementary Fig. S1 for an overlay of the 755/460 and 860/525 nm images). These features are also prominent in the corresponding images that are extracted from analysis of the detected fields and display functional biochemical information regarding keratin content and localization as well as the optical redox ratio, defined as FAD/(NADH + FAD) fluorescence (Fig. 2). For example, the terminally differentiated keratinocytes detected in the superficial C HFK EETE layer have a dark nucleus surrounded by a large fluorescent cytoplasmic region (Fig. 2 and Supplementary Fig. S1, leftmost column). The bright cytoplasmic region of the
cells within the deeper parabasal layers is smaller and this trend continues with the cells of the basal layer. The cells are represented by green/yellow color hues that correspond to redox ratio values in the 0.55 to 0.6 range. Small amounts of endogenous keratin-associated fluorescence are detected in all layers, but most of this signal is confined to the upper cornified layer that is easily visible in the corresponding 3D projection of all the optical sections acquired across the depth of this C HFK EETE (Fig. 2B and Supplementary Movies S1 and S2).

The profile of these depth-dependent morphologic and endogenous TPEF changes observed for the HFK EETEs is very different for HKc/DR EETEs that model high-grade cervical precancerous lesions (Fig. 2, rightmost column, and Supplementary Movies S3 and S4). These EETEs consist of cells with fluorescent cytoplasmic regions, surrounding prominent dark nuclei throughout the depth of the epithelium, which is generally thinner and lacks a well-defined keratin layer but has fairly prominent keratin-associated fluorescence in all layers. The redox ratio of these cells has values (0.45–0.5 range) that are lower than all other tissues as indicated by the blue color hues.

This loss of depth-dependent morphologic changes is also evident in 16E7 and 16E6E7 HFK EETEs, even though the 16E6E7 HFKs appear larger throughout the depth of the EETE. In addition, the 16E7 and 16E6E7 HFK EETEs have a well-defined keratin-associated fluorescence layer that is easily visible in the 3D optical stacks and projections (Fig. 2B and Supplementary Movies S5–S8). The 16E6 HFK EETEs on the other hand exhibit depth-dependent cell morphologic changes that are similar to those of the C HFK EETEs (Fig. 2A and B and Supplementary Movies S9 and S10). Furthermore, the 16E6, 16E7, and 16E6E7 HFK EETEs exhibit redox ratios that have consistently higher redox values than C HFK or HKc/DR EETEs (0.58–0.65 range represented by green/yellow color hues).

These differences in keratinization and depth-dependent cell morphologic features are also evident in traditional H&E-stained sections of these EETEs (Fig. 2C). In addition, immunohistochemical staining for the proliferation marker PCNA is confined to the basal layer only for the C and 16E6 HFK EETEs, which exhibit the most prominent depth-dependent morphological changes (Fig. 2D and Supplementary Fig. S3). In contrast, proliferative cells are found in more superficial layers in the other EETEs. The PCNA staining–based differences in proliferation are consistent with the staining patterns of markers for different stages of squamous differentiation such as cytokeratin 14 and 10, involucrin, and loricrin (Supplementary Fig. S2). Taken together, these results illustrate that significant morphologic differences are detected among the different EETE types we examine, and these differences are visualized along with distinct functional biochemical readouts in the endogenous TPEF-based images.

**HPV16 E6- and/or E7-expressing HFKs exhibit distinct optical metabolic biomarkers**

The depth-dependent redox ratio trends depicted in Fig. 2A are quantified in Fig. 3. Identification of keratin-associated fluorescence, which is often much brighter than that of NADH and FAD, was critical for consistent redox ratio estimates. The mean redox ratio values are shown for C HFK, 16E6 HFK, 16E7 HFK, 16E6E7 HFK, and HKc/DR EETEs from the superficial (S) to the parabasal (P) and basal (B) layer (Fig. 3A). The overall redox ratio of the HKc/DR EETEs is significantly lower than that of the C HFK EETEs (P < 0.037; Fig. 3B). Interestingly, the 16E7 HFK and 16E6E7 HFK EETEs exhibit an increased redox ratio compared with C HFK EETEs (P < 0.013), suggesting that metabolic alterations induced when E7 is expressed alone or co-expressed with E6 are distinct from those occurring in HPV16-immortalized HFKs (i.e., HKc/DRs). The 16E6 HFK EETEs have also a slightly higher redox ratio compared with...
C HFK EETEs but the difference is not statistically significant ($P = 0.38$). We note that the mean redox ratio of the superficial layer alone follows closely the overall trends reported (Fig. 3B) and leads to even more significant differences between the C HFK and the HKc/DR, E7 and E6E7 HFK EETEs ($P < 0.014$; Fig. 3C). This finding suggests that assessment of the full epithelial thickness may not be necessary to detect differences between normal and precancerous lesions. In addition, this quantitative analysis demonstrates that significant redox ratio differences are detected not only between normal and precancerous tissues but also between different types of precancerous tissues.

The optical redox ratio is correlated with metabolic biomarkers assessed via invasive biochemical assays

To determine whether the observed optical differences in redox ratios represented actual biochemical alterations, we determined the relative concentrations of $\text{NAD}^+\text{, NADH and FAD}$ in a subset of the optically examined EETEs ($n = 14$) using LC/MS-MS. The redox ratios determined using the two different methods were indeed significantly correlated, as shown in Fig. 4A ($R = 0.726$, $P = 0.003$). Interestingly, the optical redox ratio, defined as the ratio of FAD fluorescence to (NADH + FAD) fluorescence, also correlated strongly with the chemically determined ratio of NAD$^+$ concentration to (NADH + NAD$^+$) concentration (Fig. 4B; $R = 0.777$, $P = 0.001$).

After having established that the redox ratio determined by our noninvasive spectroscopic imaging method on live tissues is consistent with traditional biochemical measurements, we investigated the biochemical basis of the redox ratio differences between the EETEs. As lactate is the end product of anaerobic metabolism of glucose, we determined the ratio of glucose uptake relative to lactate production (G/L) using enzymatic assays to assess oxidative versus nonoxidative metabolism. The G/L value is lowest for the HKc/DR EETEs (Fig. 4C; $P = 0.0056$), indicating that they exhibit the highest glycolytic activity relative to oxidative phosphorylation. Overall, the G/L values are correlated with the mean optical redox ratios shown in Fig. 3B ($R = 0.5436$, $P = 0.0445$). In addition, we examined the role of glutamine as a carbon source, as glutamine metabolism can fuel the tricarboxylic acid (TCA) cycle in cancer cells either to produce energy via oxidative phosphorylation or to synthesize lipids, especially under hypoxic conditions (24, 25). We found that glutamine consumption was highest for the E6E7 and 16E6E7 HFK EETEs, which also exhibited the highest optical redox ratios (Fig. 4D; $P \leq 0.001$). To strengthen the association between the distinct optical redox ratio changes we observe in EETEs that exhibit enhanced levels of either glycolysis or glutaminolysis, we performed studies using standard HFK monolayer cultures that were exposed either to low-oxygen-containing media (to enhance glycolysis) or to glucose-free media (to decrease glycolysis and enhance glutaminolysis; Supplementary Methods). Enhanced glutamine consumption in glucose-free media was confirmed by LC/MS-MS (Supplementary Fig. S4). Consistent with our studies using EETEs, we found that increased levels of glycolysis relative to oxidative phosphorylation led to a significant decrease in the optical redox ratio, whereas increased levels of glutamine consumption relative to glycolysis led to a significant increase in the redox ratio (Supplementary Fig. S4). Therefore, these studies demonstrate that the optical redox ratio enables accurate determination of the redox state of live cells and it is sensitive to distinct metabolic alterations.

Discussion

In this study, we demonstrate the direct correlation between the optical redox ratio defined as the ratio of FAD/(NADH + FAD) fluorescence to the biochemical redox ratio based on determination of the relative concentrations of FAD and NADH using LC/MS-MS. This optical redox ratio is a sensitive biomarker of metabolic changes that occur within 3D precancerous EETEs that are characterized by enhanced levels of either glycolysis or glutamine consumption relative to normal epithelial tissues. Specifically, we find that an increase in the rate of glycolysis, as assessed by a decreased G/L value, is associated with a lower redox ratio, whereas increased levels of glutamine consumption are related to significantly higher levels of the redox ratio.

To make quantitative optical redox ratio assessments that reveal differences between tissues with subtle morphologic and cellular anomalies, it was important to identify the bright

Figure 4. Biochemical analysis validates optical redox ratio and provides insights regarding changes in the contributions of different metabolic pathways for normal and precancerous tissues. The optical redox ratio, defined as FAD/(NADH + FAD) is highly correlated with the corresponding redox ratio (A) and the redox ratio defined on the basis of the traditional redox biomarkers, NAD$^+$ and NADH (B), based on LC/MS-MS measurements from the same tissues. Assessment of glucose consumption to lactate production (C) and glutamine consumption ($\mu$mol/tissue volume; D) reveals significant differences between normal and precancerous tissues. Bar plots display the mean and SEM. $^1$, $P < 0.05$ from the C HFK EETE group.
keratin-associated fluorescence and exclude it from the redox calculations. The crosslinks that form between the keratin filaments as cells differentiate are likely major contributors to this fluorescence (13). This is supported by the qualitative agreement in the localization patterns between this nonmitochondrial endogenous fluorescence and staining with markers of terminal squamous differentiation, such as involucrin and loricrin (Supplementary Fig. S2). Nevertheless, lipids and/or lipofuscin may also fluoresce in this region, and more detailed spectroscopic studies would help elucidate its origins in more detail (8, 12, 26).

The optical redox ratio, FAD/(NADH + FAD), assessed following keratin fluorescence removal, is strongly correlated to the biochemical redox ratio defined on the basis of LC/MS-MS measurements of FAD and NADH concentrations (Fig. 4A). Moreover, we find that the optical redox ratio is strongly correlated to the canonical definition of cellular redox ratio based on the concentrations of NAD$^+$ and NADH, that is, NAD$^+$/[NADH + NAD$^+$]. This is significant, as NADH and NAD$^+$ are the primary cofactor pair that together participate in oxidation–reduction reactions central to cellular energy metabolism. The strong correlation of the optical redox ratio to the biochemical redox ratio of NAD$^+$ and NADH is likely due to the unequal contribution of different flavoproteins to the detected fluorescence (14). In fact, it has been shown that at least 50% of flavin-associated cellular fluorescence emanates from FAD bound to lipoamide dehydrogenase (LipDH) complexes, whose concentration in turn should correlate to local NAD$^+$ concentrations (14). Therefore, despite possible limitations associated with endogenous fluorescence-based redox assessments, the optical redox ratio we assess using the signals detected in two emission bands centered at 460 and 525 nm, at three excitation wavelengths (755, 800, and 860 nm) is a robust and relevant metabolic indicator.

Interpreting the observed redox ratio differences among tissue types is not trivial, as there is a complex network of biosynthetic and bioenergetic pathways that affects the NADH and FAD cofactor pools (27). Enhanced glycolysis relative to oxidative phosphorylation is expected to lower the redox ratio due to accumulation of NADH, as oxidative phosphorylation is coupled to NADH oxidation. Indeed, we observed a decreased redox ratio when we exposed standard monolayer HFK cultures to low-oxygen media. Thus, the lower redox ratio that we observe in the HKc/DR EETEs is consistent with enhanced levels of glycolysis relative to oxidative phosphorylation when compared with control EETEs. While enhanced glycolysis may not be the only possible contributor to the observed decrease in the optical redox ratio, this explanation is supported by the significantly lower G/L values for the HKc/DR EETEs compared with the control EETEs (Fig. 4). The lower redox ratios indicative of higher rates of glycolysis in our precancerous HKc/DR EETEs are consistent with numerous studies that have established enhanced aerobic glycolysis, a phenomenon often referred to as the Warburg effect (2–4), as a hallmark of several cancers, including cervical carcinoma (28).

Interestingly, the 16E7 and 16E6E7 HFK EETEs have significantly higher redox ratios throughout the depth of the epithelium in comparison to the normal EETEs. This increase suggests that cells in these tissues oxidize NADH more rapidly or reduce NAD$^+$ more slowly. One possible explanation may be impaired glucose catabolism and elevated glutamine consumption. The use of glutamine as a substrate for the TCA cycle and the production of biosynthetic substrates has been found to occur in cancer cells and tumors that do not have adequate access to products of glycolysis (24, 25) and this pathway has been identified as a promising target for cancer therapeutics (29–31). In fact, HPV16 E7 has been reported to inhibit M2-PK, which would result in limited availability of pyruvate (32). The latter metabolite is the main glucose-derived substrate for the TCA cycle in most noncancerous cells. Thus, in the absence of pyruvate, E7-expressing cells could resort to glutamine as a TCA cycle carbon source. The products and intermediates of the TCA cycle can be used either for fueling oxidative phosphorylation or for various biosynthetic pathways (22). In this light, an increase in the redox ratio within cells with limited access to pyruvate could reflect a decrease in glycolysis and corresponding elevated TCA cycle activity and oxidative phosphorylation fueled by glutamine. While an increase in the optical redox ratio is not expected always to be associated with enhanced glutamine consumption, the observed rates of glutamine consumption, assessed from LC/MS-MS, and G/L ratios for the E7 and E6E7 HFK EETEs are consistent with this explanation. Our studies with HFK monolayer cultures further support the association between enhancements in glutamine consumption and the optical redox ratio. In addition, the fact that the redox ratio increases with enhanced glutamine consumption further supports our hypothesis that the TPEF signal we detect at 460 nm is primarily sensitive to NADH and not NADPH; NADPH levels likely increase during glutaminolysis (24), and if the optical redox ratio were sensitive to this increase, its value would decrease.

We note that the redox ratio is highest in the superficial layers of all EETEs with the exception of 16E6 and 16E6E7 HFK EETEs (Fig. 3). The HPV E6 oncoprotein has been reported to facilitate the evasion of programmed cell death through the degradation of apoptosis-related proteins p53, Bak, and/or myc (33). As p53 and myc are central modulators of glucose and glutamine catabolism (24, 33–35), it is possible that by coordinating their activity, HPV16 E6 expression may enable cells to avoid cell senescence and continue biosynthesis in the upper layers of the epithelium. This may be the origin of the unique depth-dependent redox patterns within 16E6 and 16E6E7 HFK EETEs, even though more detailed studies are needed to test this possibility.

The emphasis of the presented work has been placed not only on the use of the optical redox ratio as a tool to improve understanding of metabolic changes but also on the application of this technology to noninvasive early cancer diagnosis. It is interesting to note that the redox ratio differences within the superficial layer of the EETEs, which is always readily accessible for interrogation by optical methods, are reflective of the differences we observe when analyzing the full depth of the EETEs. This may be important for the implementation of the described methods in a clinical setting, where rapid image acquisition is needed. The development of appropriate probes...
(36), as well as the emergence of TPEF imaging systems that are already being used clinically (37, 38), suggest that translation and more rigorous testing of the approach we have established is feasible.

In summary, these studies demonstrate that TPEF imaging can be used for quantitative assessment of the redox state of epithelial tissues and that such measurements represent valid and reliable, noninvasive approaches to detect alterations in cancer-relevant metabolic pathways in live cells. The ability to provide metabolic assessments without disturbing cell or tissue function with high spatial resolution in live tissues will enable studies that quantitatively assess functional metabolic changes either during cancer development or in response to treatment.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: A. Varone, J. Xylas, K. Münger, I. Georgakoudi
Development of methodology: A. Varone, J. Xylas, K.P. Quinn, G. Sridharan, C. Alonzo, I. Georgakoudi

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