Acquired resistance to EGFR tyrosine kinase inhibitors alters the metabolism of human head and neck squamous carcinoma cells and xenograft tumours

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Background: Acquired resistance to molecularly targeted therapeutics is a key challenge in personalised cancer medicine, highlighting the need for identifying the underlying mechanisms and early biomarkers of relapse, in order to guide subsequent patient management.

Methods: Here we use human head and neck squamous cell carcinoma (HNSCC) models and nuclear magnetic resonance (NMR) spectroscopy to assess the metabolic changes that follow acquired resistance to EGFR tyrosine kinase inhibitors (TKIs), and which could serve as potential metabolic biomarkers of drug resistance.

Results: Comparison of NMR metabolite profiles obtained from control (CAL 5 ) and EGFR TKI-resistant (CAL 8 ) cells grown as 2D monolayers, 3D spheroids or xenograft tumours in athymic mice revealed a number of differences between the sensitive and drug-resistant models. In particular, we observed elevated levels of glycerophosphocholine (GPC) in CAL R relative to CAL S monolayers, spheroids and tumours, independent of the growth rate or environment. In addition, there was an increase in alanine, aspartate and creatine + phosphocreatine in resistant spheroids and xenografts, and increased levels of lactate, branched-chain amino acids and a fall in phosphoethanolamine only in xenografts. The xenograft lactate build-up was associated with an increased expression of the glucose transporter GLUT-1, whereas the rise in GPC was attributed to inhibition of GPC phosphodiesterase. Reduced glycerophosphocholine (GPC) and phosphocholine were observed in a second HNSCC model probably indicative of a different drug resistance mechanism.

Conclusions: Our studies reveal metabolic signatures associated not only with acquired EGFR TKI resistance but also growth pattern, microenvironment and contributing mechanisms in HNSCC models. These findings warrant further investigation as metabolic biomarkers of disease relapse in the clinic.

Epidermal growth factor receptor (EGFR/HER1) is over-expressed in 95% of head and neck squamous cell carcinomas (HNSCC) and high levels are correlated with poor patient prognosis and decreased overall survival (Grandis et al, 1998; Ang et al, 2002), suggesting that the disease is EGFR-driven and making a strong case for targeting EGFR in HNSCC. Disappointingly, however, clinical evidence to date shows that EGFR-targeted agents, including monoclonal antibodies and tyrosine kinase inhibitors (TKIs), have little efficacy in HNSCC as a monotherapy, raising the possibility that alternative survival pathways may be activated in
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this disease (Box et al, 2013a,b). To address this, new strategies for targeting EGFR in HNSCC are being evaluated clinically including the use of pan-HER inhibitors and rational drug combinations that target several signalling pathways simultaneously (Agulnik, 2012).

A key challenge for TKIs (including those targeting EGFR) in cancer is the development of acquired resistance, with many patients showing disease progression not long after the initial response (Wagle et al, 2011; Lovly and Shaw, 2014). Thus, the elucidation of underlying molecular mechanisms, as well as early biomarkers of patient relapse, is key for identifying subsequent therapies that may delay or indeed prevent further disease progression, and ultimately improve long-term survival.

Studies have shown that treatment failure is, in many cases, due to cancer cells acquiring new genetic and molecular alterations that enable them to survive in the face of therapeutic stress (Holohan et al, 2013). Additionally, transformed tumour cell metabolism may be equally important in this regard since alterations in key processes such as glucose, glutamine, nucleotide, fatty acid and phospholipid metabolism allow cancer cells to adapt to their growth demands and thrive in a hostile microenvironment (Bailey et al, 2012; Ward and Thompson, 2012). Several oncoproteins and oncogenic signalling pathways directly regulate vital components of the metabolic network, thus providing a link between the molecular abnormalities of cancer and the transformed metabolic phenotype (Deberardinis et al, 2008; Vander Heiden et al, 2009; Vousden and Ryan, 2009).

Altered metabolism is a hallmark of cancer (Hanahan and Weinberg, 2011) and metabolic features of cancer are being explored for diagnosis and staging as well as for informing on disease response and resistance to therapy (Beloueche-Babari et al, 2010; Beloueche-Babari et al, 2011). In this context, metabolic imaging and/or profiling techniques such as positron emission tomography, nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry play an important role in translating findings from pre-clinical studies to humans.

We have developed a model of acquired resistance to EGFR TKIs based on the human HNSCC cell line CAL 27, and shown that a resistant derivative (CALR) exhibits a distinct protein expression signature compared with its sensitive counterpart (CALS) (Box et al, 2013a). Because of the central role that metabolism plays in tumour biology (Hanahan and Weinberg, 2011), we explored here the metabolic features of EGFR TKI sensitivity in CAL5 and CALR cells grown as 2D monolayers, 3D spheroids and tumour xenografts by using NMR spectroscopy. Our aim is to assess the value of 3D cultures, compared with 2D or xenograft tumour models, for the discovery of potential metabolic signatures underlying resistance that could potentially be developed as minimally invasive metabolic biomarkers of EGFR TKI resistance, and to interrogate the influence of the microenvironment on metabolic readouts.
GLUT-1 (Merck Millipore) or β-actin as a loading control (Abcam).

**Glycerophosphocholine-phosphodiesterase (GPC-pd) assay.** GPC-pd enzymatic activity was monitored by 1H NMR by using a modification of a previously described method (Iorio et al., 2010). 3 × 10⁷ cells were pelleted, washed and homogenised then sonicated at 4 °C in 0.5 ml 100 mM Tris-HCl (pH 7.2) buffer containing 10 mM DTT and 1 mM EDTA in D₂O. Following a 30 min centrifugation at 12 000 r.p.m., supernatants containing the cytosolic fractions were transferred to a 5 mm NMR tube. GPC-pd was assayed at 30 °C by monitoring the 1H NMR-observed build up of choline over time (35 min) following the addition of exogenous GPC and MgCl₂ at a final concentration of 1.2 mM and 10 mM, respectively.

**Statistical analysis.** For comparison of metabolite levels, Student’s t-test was used with P-values of ≤ 0.05 considered to be statistically significant. Data represent the mean ± s.e.

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**RESULTS**

**Acquired resistance to EGFR TKI in HNSCC alters tumour metabolism.** We have previously reported that CALR cells exhibit a faster growth rate compared with CALS when grown as subcutaneous xenograft tumours but not when cultured as monolayers in vitro (Box et al., 2013a). Interestingly, when grown as 3D spheroids, CALR grew more rapidly than CALS cells with average spheroid volumes at day 7 of 31 ± 3 and 17 ± 4 × 10⁶ μm³ (n = 5 biological repeats, P = 0.02), respectively, indicative of a more aggressive growth phenotype as observed in vivo.

We hypothesised that our cell line models with distinct growth properties would possess different metabolic characteristics. Thus, prior to assessing metabolic differences associated with the resistance phenotype we explored the extent of any baseline metabolic variations between the three growth conditions: 2D, 3D and xenograft tumours, within each cell line. As shown in Figure 1A, unbiased multivariate analysis with PCA of the 1H NMR spectral data indicated that the 2D cells, 3D spheroids and tumours exhibit separate clustering within each cell line, consistent with a distinct metabolic phenotype. The clustering was maintained even when data from CALS and CALR were merged, suggesting strong model-dependent patterns. The score scatter plots indicate that the variation along the PC1 axis is driven by differences between the 2D and tumour data vs the spheroid data while the variation along the PC2 axis is driven by differences between the 2D vs tumour data with spheroid data overlapping between the two. Thus, despite arising from the same cells of origin, the three experimental models used in this study have unique metabolic features which are likely to be a reflection of their growth phenotype and microenvironment.

The metabolic characteristics of acquired EGFR TKI resistance were assessed with PCA of the 1H NMR data derived from CALS and CALR cells within each model. The separate clustering of the data points corresponding to CALS and CALR on the score scatter plots in Figure 1B indicates a distinct metabolic profile for the sensitive and the EGFR TKI-resistant cells in every model.

The clearest separation was obtained in the tumours which showed that variability in the data could be described according to three main principal components, PC1, PC2 and PC3 (Figure 1B and 2A), that between them explain ~ 68% of the total variance (PC1: 34.8%, PC2: 18.4%, PC3: 15.1%). The resonances that appeared to be key in the separation between the CALS and CALR profiles include lactate, branched-chain amino acids (BCAAs), choline metabolites, acetate, myo-inositol, glutamine/glutamate and creatine (Cr) + phosphocreatine (PCr), as shown in Figure 2B.

To validate the metabolite changes identified in the PCA, we performed a targeted analysis of the data by integrating the individual peaks in the 1H NMR spectra. As shown in Table 1, and in agreement with the PCA method, univariate 1H NMR revealed a number of metabolic alterations in CALR xenograft tumours compared with their CALS counterpart. Specifically, the levels of GPC, lactate, BCAAs, alanine and aspartate were significantly elevated in CALR relative to CALS tumours. Total choline, which is predominantly comprised of GPC, phosphocholine (PC) and free choline, was also increased in CALR compared with CALS. The levels of Cr/PCr, acetate and glutamate showed a trend towards an increase, while myo-inositol showed a trend towards a decrease in CALR compared with CALS tumours but these effects did not reach statistical significance.

**31P NMR analysis of CALS and CALR tumour extracts corroborated the elevation in GPC and PCr levels and additionally revealed a rise in glycerophosphoethanolamine (GPE) and a fall in phosphoethanolamine (PE) levels in CALR relative to CALS tumours (Table 1 and Figure 2C). NTP levels were not reproducibly detectable in the 31P NMR spectra of tumour extracts, but the consistent and significant increase in CALR PCr levels suggests that the drug-resistant tumours have improved bioenergetics.

**Relationship between metabolism, drug resistance, growth environment and phenotype.** To investigate the significance of the metabolic changes observed in relation to the acquired drug...
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Figure 2. NMR profiling of CALS and CALR tumours. (A) Three-dimensional PCA score scatter plot showing separate clustering for 1H NMR data from CALS and CALR. (B) Score contribution plot showing changes in the 1H NMR peaks (and related metabolites) accounting for the differences between CALR and CALS tumours (plot obtained using the group-to-group comparison option in SIMCA). Positive scores represent increased PCr levels, while negative scores indicate decreased levels in CALR relative to CALS. (C) Representative 31P NMR spectra showing the differences in 31P-containing metabolites between CALS and CALR tumours. Abbreviations: Asp = aspartate; BCAA = branched-chain amino acids; Cr = creatine; PCr = phosphocreatine; PC = phosphocholine; PE = phosphoethanolamine; GPC = glycerophosphocholine; GPE = glycerophosphoethanolamine; Pi = inorganic phosphate; Glut = glutamine; Glutamate; Glx = glutathione; Myo-Ins = myo-inositol; ? = unidentified peak.

resistance phenotype and how this is impacted on by the microenvironment and the differential growth rates of the tumours, we assessed whether the metabolic signature of drug resistance identified in the xenograft tumours could also be present in cells grown in vitro. For these experiments we compared cells cultured as (a) 2D monolayers with comparable CALS/CALR growth rates and (b) 3D spheroids with a faster CALR growth rate, reminiscent of the in vivo xenograft model.

Univariate 1H NMR spectroscopy analysis of 2D cell extracts showed that, consistent with the tumour data, CALR cells exhibited higher levels of GPC relative to CALS cells while BCAAs lactate, Cr + PCr, aspartate and alanine, which were increased in the CALR tumours, were not significantly changed in the 2D cell cultures (Table 2).

For the analysis of 3D spheroids, as it was not possible to obtain an accurate estimate of cell number, we normalised the metabolite content relative to the PC signal since this was found to be comparable in the tumours as well as the 2D cultured cells.

The data showed that, similar to the tumour profiles, the ratios of GPC/PC, PCr + Cr/PC, alanine/PC and aspartate/PC were significantly elevated. However, in contrast to the observations made in solid tumours, the ratio of BCAA/PC appeared to decrease in CALR compared with CALS spheroids, although this was not statistically significant, while lactate/PC remained constant within experimental uncertainty (Table 2).

The increased lactate observed in tumours but not 2D or 3D cultured cells could suggest a shift towards glycolytic metabolism (also known as the Warburg effect) in vivo. To investigate this possibility we assessed the protein expression levels of the glucose transporter GLUT-1. Our data indicate that CALR have upregulated GLUT-1 expression compared with CALS tumours, consistent with an increased Warburg effect. Interestingly, this difference was not observed in cells grown as 2D monolayers or 3D spheroids (Figure 3), where lactate levels were unchanged.

Comparison of the data obtained from 2D cultures, 3D spheroids and xenograft tumours indicate that, relative to CALS, CALR cells displayed increased levels of lactate, BCAA and decreased PE in vivo only, elevated PCr, alanine and aspartate levels in 3D spheroids and tumours, and increased GPC in all three experimental models (Figure 4A and B). Thus the rises in lactate and GLUT-1 expression, together with increased BCAA and decreased PE are associated with the in vivo growth environment while the increase in alanine, aspartate and Cr + PCr is associated with the aggressive growth phenotype seen in 3D and in vivo. The build up of GPC is associated with EGFR TKI resistance independently of differences in growth rates or environment.

To assess the dynamics of the change in GPC, we monitored the levels of this metabolite following acute exposure to drug. 1H NMR spectroscopy of cells treated with 1μM gefitinib (EGFR TKI) for 24 h showed that GPC levels increased in CALS (to 157 ± 21% relative to controls, P = 0.04) but not in CALR cells (95 ± 13% relative to controls, P = 0.36), which were unresponsive to drug as determined by the effects of treatment on cell counts: 94 ± 5% of controls (P = 0.25) in CALR compared with 70 ± 5% (P = 0.001) in CALS (Figure 4C). Thus, the rise in GPC appears to be an early metabolic consequence of exposure to gefitinib, which is sustained with chronic exposure when cells acquire long-term drug resistance.
Acquired EGFR TKI resistance in the CALS/CALR model is associated with inhibition of GPC-pd. GPC is produced from the breakdown of the membrane phospholipid phosphatidylcholine (PtdCho) in a 2-step reaction mediated via phospholipase A and lysophospholipase, which concomitantly leads to the formation of free fatty acids (FFAs). GPC is further metabolised to glycerol-3 phosphate and choline in a reaction catalysed by GPC phosphodiesterase (GPC-pd).

To determine the metabolic processes underpinning the elevation in GPC levels, we initially assessed the levels of FFA breakdown with inhibition of GPC-pd.

The breakdown of GPC via GPC-pd was next investigated using a 1H NMR-based assay that monitored the formation of choline over time as a measure of GPC-pd activity (Figure 5A and B). This showed that the rate of choline formation as a result of GPC hydrolysis was 97 ± 19 pmol per cell per min in CALS monolayers, decreasing to 45 ± 13 pmol per cell per min in CALR monolayers (P = 0.03, n = 4), consistent with lower GPC-pd activity in this cell line (Figure 5C). Taken together these findings show that the increase in GPC content in the EGFR TKI-resistant CALR cells is likely to be due to a reduction of its metabolism by GPC-pd.

The metabolic signatures of acquired EGFR TKI resistance may be mechanism-dependent. Finally, to test the generalisability of our observations we assessed a second sensitive/resistant HNSCC model that we have previously described: the PJR and PJS cell lines (Box et al, 2013a). DNA staining and flow cytometry showed polyploidy in PJR cells, a phenomenon that was not observed in PJS, CALS or CALR cell lines (Supplementary Figure S1). Polyploidy has previously been linked to drug resistance (Puig et al, 2008; Shen et al, 2008), suggesting a potential difference in the underlying mechanisms of acquired resistance to EGFR TKIs between PJR and CALR models. Our 1H NMR data show that, unlike the case for CALR vs PJS cells with no significant changes in the levels of lactate, alanine, glutamate or BCAA (Table 3). Thus, acquired resistance to EGFR TKIs in the PJS/PJR model is associated with a differential metabolic response compared with the CALR/CALS model, which is likely to be related to the differences in accompanying cellular processes and potentially the underlying drug resistance mechanisms.

**DISCUSSION**

Acquired resistance to TKIs is a major challenge in personalised cancer medicine and the identification of mechanisms, as well as early biomarkers of patient relapse, will enable a timely switch to alternative therapies prior to further disease progression.

In this study we used TKI sensitive (CALS) and resistant (CALR) human HNSCC cells grown as standard 2D monolayers, 3D spheroids or tumour xenografts alongside NMR spectroscopy to explore metabolic characteristics of acquired resistance to EGFR TKIs which, if independently validated, could have potential as minimally invasive biomarkers, and to assess the influence of the microenvironment on the metabolic readouts.

Figure 3. GLUT-1 expression in CALS and CALR tumour models. Western blots showing an elevated GLUT-1 protein expression in CALR relative to CALS xenograft tumours but not in 2D monolayers or 3D spheroids. The individual lanes in the tumour blot represent samples from individual xenografts.

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### Table 1. Metabolite content of CALS and CALR HNSCC human tumour xenografts as detected by 1H and 31P NMR spectroscopy analysis of excised tumour tissue

| Metabolite | CALS (mg·g⁻¹ wet tissue) | CALR (mg·g⁻¹ wet tissue) | P*  |
|-----------|--------------------------|--------------------------|-----|
| GPC       | 27 ± 0.02                | 92 ± 0.16                | 0.07|
| PC        | 45 ± 0.5                 | 48 ± 0.5                 | 0.96|
| Aspartate | 0.33 ± 0.03              | 0.34 ± 0.03              | 0.84|
| PE        | 0.55 ± 0.07              | 0.31 ± 0.04              | 0.02|

**Table 2. 1H NMR spectroscopy-detectable metabolite levels in CALS and CALR cells grown as 2D monolayers or as 3D spheroids**

| Metabolite | 2D cell metabolites (au per cell volume) | 3D spheroid metabolites (ratio to PC) |
|-----------|-------------------------------------------|-------------------------------------|
| BCAA      | 13 ± 1                                    | 7.30 ± 1.30                         |
| Lactate   | 5.20 ± 0.60                               | 4.50 ± 0.40                         |
| Alanine   | 9.50 ± 1.20                               | 1.34 ± 0.28                         |
| Glutamate | 5.50 ± 0.50                               | 2.70 ± 0.50                         |
| Aspartate | 0.49 ± 0.06                               | 0.40 ± 0.04                         |
| Cr = PCr  | 2.20 ± 0.20                               | 1.90 ± 0.3                          |
| PC        | 10 ± 2                                    | 1.0 ± 2                             |
| GPC       | 1.90 ± 0.3                                | 3.90 ± 0.6                          |
| PE        | 0.61 ± 0.06                               | 0.54 ± 0.09                         |

**Table 3. 31P Metabolites (mmol·g⁻¹ wet tissue)**

| Metabolite | CALS (mmol·g⁻¹ wet tissue) | CALR (mmol·g⁻¹ wet tissue) | P*  |
|-----------|-----------------------------|-----------------------------|-----|
| PCr       | 46 ± 0.07                   | 8 ± 0.1                     | 0.04|
| Pi        | 2.14 ± 0.44                 | 2.44 ± 0.22                 | 0.57|
| GPC       | 0.11 ± 0.03                 | 0.53 ± 0.06                 | 0.001|
| GPE       | 0.05 ± 0.02                 | 0.19 ± 0.03                 | 0.009|
| PC        | 0.33 ± 0.03                 | 0.34 ± 0.03                 | 0.84|
| PE        | 0.55 ± 0.07                 | 0.31 ± 0.04                 | 0.02|

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*Two-tailed unpaired Student’s t-test, n = 5. Bold denotes statistically significant P values.

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EGFR TKI resistance in the CALS/CALR model is associated with inhibition of GPC-pd. GPC is produced from the breakdown of the membrane phospholipid phosphatidylcholine (PtdCho) in a 2-step reaction mediated via phospholipase A and lysophospholipase, which concomitantly leads to the formation of free fatty acids (FFAs). GPC is further metabolised to glycerol-3 phosphate and choline in a reaction catalysed by GPC phosphodiesterase (GPC-pd).

To determine the metabolic processes underpinning the elevation in GPC levels, we initially assessed the levels of FFA signals in the 1H NMR spectra of the organic phase of cells extracts. No significant differences were recorded between the profiles of CALS and CALR cells indicating that the rates of PtdCho breakdown were comparable in the two cell lines (Supplementary Table S1).
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Differences in metabolism between CALR and CALS tumour cells since the extent of stromal enrichment, as revealed by haematoxylin and eosin staining (Supplementary Figure S2), was comparable between the CALS and CALR tumours, indicating that any contributions from non-tumour cells to the metabolic readouts should be similar between the two models.

Assessment of this metabolic signature in in vitro models indicated that in 3D spheroids, where CALR had a more aggressive growth phenotype relative to CALS, increased GPC, Cr/PCr, aspartate as well as alanine were also observed alongside drug resistance. On the other hand, in 2D cultures, where CALR did not exhibit a faster growth rate than CALS cells, only an increase in GPC % change was observed.

Figure 4. Comparison of metabolite changes induced in EGFR TKI resistance models. (A) Diagram summarising the metabolite signature observed in CALR relative to CALS tumours and its presence in 2D and 3D in vitro cultures. (B) 1H NMR spectra of the choline region showing an increased GPC in CALR relative to CALS models. (C) Changes in GPC following an acute exposure to gefitinib (24 h, 1 μM) in CALS and CALR cell lines grown in 2D. *: not measured in spheroids or 2D monolayers, *P = 0.04, NS: P = 0.58, n = 3 at least.

Table 3. 1H NMR spectroscopy-detectable metabolite levels in PJR and PJR cells grown as 2D monolayers

| 2D cell metabolites (au/cell volume) | PJR | PJR | P* |
|------------------------------------|-----|-----|----|
| BCAA                               | 4.80 ± 0.25 | 5.4 ± 0.35 | 0.23 |
| Lactate                            | 6.90 ± 0.80 | 7.50 ± 1.00 | 0.66 |
| Alanine                            | 2.20 ± 0.30 | 2.20 ± 0.38 | 0.96 |
| Glutamate                          | 2.10 ± 0.20 | 2.40 ± 0.30 | 0.46 |
| PC                                 | 17 ± 2 | 11 ± 1 | 0.05 |
| GPC                                | 1.50 ± 0.10 | 0.54 ± 0.03 | 0.01 |
| PE                                 | 0.50 ± 0.14 | 0.20 ± 0.05 | 0.17 |

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*Unpaired 2-tailed Student's t-test, n = 3. Bold denotes statistically significant P values.

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Figure 5. GPC-pd activity assay in CALS and CALR monolayers. (A) Sequential 1H NMR spectra from the GPC-pd assay showing the changes in choline and GPC resonances over time. (B) Build up of choline in 2D CALS and CALR cells over time (total of 35 min) following addition of GPC. (C) Quantification of GPC-pd activity shows a decrease in CALR relative to CALS cells. *P = 0.03, n = 4.
High levels of cellular bioenergy metabolite levels including the phosphagen metabolite PCr have been reported to be linked to chemoresistance (Kaplan et al., 1991; Zhou et al., 2012) and could indicate an enhancement in energy metabolism required for sustaining increased cell proliferation (Cairns et al., 2011). We also observed increased aspartate and alanine in CALR relative to CALS tumours and spheroids. This is of interest as aspartate constitutes a pivotal component of the malate-aspartate shuttle, a process that enables efficient energy production in the cell (Wallace, 2012). A key role has recently emerged for glutamine-derived aspartate in the processes leading to the rise in GPC are triggered by drug resistance (Kaplan et al., 1991), in agreement with our findings. Clearly, analysis of a larger number of isogenic sensitive/resistant cell line pairs is required to assess the general applicability of the metabolic signatures observed here, and unravel the mechanisms underlying them.

A key objective of our study was to identify metabolic characteristics of acquired resistance to EGFR TKIs that could be developed as non-invasive imaging biomarkers. The glycolytic phenotype observed in the tumours can be monitored with 18F-fluorodeoxyglucose (FDG)-PET, the gold-standard method for clinical metabolic imaging, as well as lactate-selective 1H NMR spectroscopy techniques as shown in previous patient studies (including HNSCC) (King et al., 2010; Kim et al., 2011; Germuska et al., 2012; Jansen et al., 2012). Ex vivo analysis of tumour tissue may be particularly useful for monitoring metabolites not measurable by currently available imaging techniques, for example, aspartate and alanine as previously documented in clinical HNSCC samples (Somashkar et al., 2011). Longitudinal studies assessing the metabolic characteristics of human HNSCC and correlating them with patient outcome are, however, necessary to assess the utility of metabolic biomarkers for patient follow-up.

In summary, this study has revealed metabolic signatures associated with acquired EGFR TKI resistance that may be linked to the underlying drug resistance mechanisms as well as aggressive growth patterns in HNSCC models. The changes reported here can be monitored through in vivo as well as ex vivo examinations of patient tumours, highlighting their potential for translation to the clinic.

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CONFLICT OF INTEREST

The author declares no conflict of interest.

REFERENCES

Agulnik M (2012) New approaches to EGFR inhibition for locally advanced or metastatic squamous cell carcinoma of the head and neck (SCCHN). Mod Oncol 29(4): 2481–2491.

Ang KK, Berkey BA, Tu X, Zhang HZ, Katz R, Hammond EH, Fu KK, Milas L (2002) Impact of epidermal growth factor receptor expression on survival and pattern of relapse in patients with advanced head and neck carcinoma. Cancer Res 62(24): 7350–7356.

Bailey KM, Wojtkowiak JW, Hashim AI, Gillies RJ (2012) Targeting the metabolic microenvironment of tumors. Adv Pharmacol 65: 63–107.

Beloueche-Babari M, Arunan V, Troy H, te Poel RH, te Fong AC, Jackson LE, Payne GS, Griffiths JR, Judson IR, Workman P, Leach MO, Chung YL (2012) Histone deacetylase inhibition increases levels of choline kinase alpha and phosphocholine facilitating noninvasive imaging in human cancers. Cancer Res 72(4): 990–1000.

Beloueche-Babari M, Chung YL, Al-Saffar NM, Felk-Miniotis M, Leach MO (2010) Metabolic assessment of the action of targeted cancer therapeutics using magnetic resonance spectroscopy. Br J Cancer 102(1): 1–7.

Beloueche-Babari M, Jamin Y, Arunan V, Walker-Samuel S, Revill M, Smith PD, Halliday J, Waterton JC, Jariat H, Workman P, Leach MO, Robinson SP (2013) Acute tumour response to the MEK1/2 inhibitor selumetinib (AZD6244, ARRY-142886) evaluated by non-invasive diffusion-weighted MRI. Br J Cancer 109(6): 1562–1569.

Beloueche-Babari M, Workman P, Leach MO (2011) Exploiting tumor metabolism for non-invasive imaging of the therapeutic activity of molecularly targeted anticancer agents. Cell Cycle 10: 17.

Box C, Mendiola M, Gowan S, Box GM, Valenti M, Brandon AD, Al-Lazikani B, Rogers SJ, Wilkins A, Harrington KJ, Eccles SA (2013a) A novel serum protein signature associated with resistance to epidermal growth factor receptor tyrosine kinase inhibitors in head and neck squamous cell carcinoma. Eur J Cancer 49(11): 2512–2521.

Box C, Zimmermann M, Eccles S (2013b) Molecular markers of response and resistance to EGFR inhibitors in head and neck cancers. Front Biosci 18: 520–542.

Brahimi-Horn MC, Bellot G, Pouyssegur J (2011) Hypoxia and energetic tumour metabolism. Curr Opin Genet Dev 21(1): 67–72.

Cairns RA, Harris IS, Mak TW (2011) Regulation of cancer cell metabolism. Nat Rev Cancer 11(2): 85–95.

Deberardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: metabolic reprogramming fuels cancer growth. Cell Metab 7(1): 11–20.

Gee HE, Camps C, Buffa FM, Patel S, Winter SC, Betts G, Homer J, Deberardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008) The biology of cancer: the next generation. Cell Metab 8(3): 17.

Hanahan D, Weinberg RA (2011) Hallmarks of cancer: the next generation. Cell 144(5): 646–674.

Holohan C, Van Schaeybroeck S, Longley DB, Johnston PG (2013) Cancer drug resistance: an evolving paradigm. Nat Rev Cancer 13(10): 714–726.

Iorio E, Ricci A, Bagnoli M, Pisanu ME, Castellano G, Di VM, Venturini E, Glunde K, Bhuwalia ZM, Mezzanzanica D, Canevari S, Podo F (2010) Activation of phosphatidylcholine cycle enzymes in human epithelial ovarian cancer cells. Cancer Res 70(5): 2126–2135.

Janssen JF, Schober H, Lee NT, Stambuk HE, Wang Y, Furry MG, Patel SG, Pfister DG, Shah JP, Koutcher JA, Shukla-Dave A (2012) Tumor metabolism and perfusion in head and neck squamous cell carcinoma: pretreatment multimodality imaging with 1H magnetic resonance spectroscopy, dynamic contrast-enhanced MRI, and [18F]FDG-PET. Int J Radiat Oncol Biol Phys 82(1): 299–307.

Kaplan O, Jaroszewski JW, Clarke R, Fairchild CR, Schoenlein P, Goldenberg S, Gottesman MM, Cohen JS (1991) The multidrug resistance phenotype: 31P nuclear magnetic resonance characterization and 2-deoxyglucose toxicity. Cancer Res 51(6): 1638–1644.

Kim H, Catana C, Ratai EM, Andronesi OC, Jennings DL, Batchelor TT, Jain RK, Sorensen AG (2011) Serial magnetic resonance spectroscopy reveals a direct metabolic effect of cediranib in glioblastoma. Cancer Res 71(11): 3745–3752.

King AD, Yeung DK, Yu KH, Mo FK, Hu CW, Bhatai KS, Tse GM, Vlantis AC, Wong JK, Ahuja AT (2010) Monitoring of treatment response after chemoradiotherapy for head and neck cancer using in vivo 1H MR spectroscopy. EurRadiol 20(1): 165–172.

Koukourakis MI, Bentzen SM, Giannomanolaki A, Wilson GD, Daley FM, Saunders MI, Dische S, Sivridis E, Harris AL (2006) Endogenous markers of two separate hypoxia response pathways (hypoxia inducible factor 2 and alpha and carbonic anhydrase 9) are associated with radiotherapy failure in head and neck cancer patients recruited in the CHART randomized trial. J Clin Oncol 24(5): 727–735.

Le QT, Koong A, Lieskovsky Y, Narasimhan B, Graves E, Pinto H, Brown JM, Spiegelman D (2008) In vivo 1H magnetic resonance spectroscopy of lactate in patients with stage IV head and neck squamous cell carcinoma. Int J Radiat Oncol Biol Phys 71(4): 1151–1157.

Lovly CM, Shaw AT (2014) Molecular pathways: resistance to kinase inhibitors and implications for therapeutic strategies. Clin Cancer Res 20(9): 2249–2256.

Puig PE, Guilly MN, Bouchot A, Driou N, Cathelin D, Bouyer F, Favier L, Ghringhelli F, Kroemer G, Solary E, Martin F, Chauvet B (2008) Tumor cells can escape DNA-damaging cisplatin through DNA endoreduplication and reversible polyploidy. Cell Biol Int 32(9): 1031–1043.

Rogers SJ, Box C, Chambers P, Barbachano Y, Nutting CM, Rhys-Evans P, Workman P, Harrington KJ, Eccles SA (2009) Determinants of response to epidermal growth factor receptor tyrosine kinase inhibition in squamous cell carcinoma of the head and neck. J Pathol 218(1): 122–130.

Rogers SJ, Box C, Chambers P, Barbachano Y, Nutting CM, Rhys-Evans P, Workman P, Harrington KJ, Eccles SA (2009) Determinants of response to epidermal growth factor receptor tyrosine kinase inhibition in squamous cell carcinoma of the head and neck. J Pathol 218(1): 122–130.

Shen H, Moran DM, Maki CG (2008) Transient nutlin-3a treatment promotes endoreduplication and the generation of therapy-resistant tetraploid cells. Cancer Res 68(20): 8260–8268.

Somasekhar BS, Kamarajan P, Danciu T, Kapila YL, Chinnaiyan AM, Rajendiran TM, Ramamoorthy A (2011) Magic angle spinning NMR-based metabolic profiling of head and neck squamous cell carcinoma tissues. J proteome Res 10(11): 5232–5241.

Son J, Lysyiotis CA, Ying H, Wang X, Hua S, Ligorio M, Perera RM, Ferrone CR, Mullarky E, Shyh-Chang N, Kang Y, Fleming JB, Bardeesy N, Asara JM, Haigis MC, DePinho RA, Cantley LC, Kimmelman AC (2013) Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. Nature 496(7443): 101–105.

Vander Heiden MG, Cantley LC, Thompson CB (2009) Understanding the Warburg effect: the metabolic requirements of cancer cell proliferation. Science 324(5930): 1029–1033.

Vercschor ML, Wilson LA, Singh G (2010) Mechanisms associated with mitochondrial-generated reactive oxygen species in cancer. Curr J Pharmacol Pharmacol 88(3): 204–219.

Vinci M, Box C, Zimmermann M, Eccles SA (2013) Tumor sphere-based migration assays for evaluation of therapeutic agents. Methods Mol Biol 988: 253–266.

Vinci M, Gowan S, Boxall F, Patterson L, Zimmermann M, Court W, Lomas C, Mendiola M, Hardisson D, Eccles SA (2012) Advances in establishment and analysis of three-dimensional tumor sphere-based functional assays for target validation and drug evaluation. BMC Biol 10: 29.

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Vousden KH, Ryan KM (2009) p53 and metabolism. *Nat Rev Cancer* **9**(10): 691–700.

Wagle N, Emery C, Berger MF, Davis MJ, Sawyer A, Pochanard P, Kehoe SM, Johannessen CM, Macconaille LE, Hahn WC, Meyerson M, Garraway LA (2011) Dissecting therapeutic resistance to RAF inhibition in melanoma by tumor genomic profiling. *J Clin Oncol* **29**(22): 3085–3096.

Wallace DC (2012) Mitochondria and cancer. *Nat Rev Cancer* **12**(10): 685–698.

Ward PS, Thompson CB (2012) Metabolic reprogramming: a cancer hallmark even Warburg did not anticipate. *Cancer Cell* **21**(3): 297–308.

Workman P, Aboagye EO, Ballwill F, Balmain A, Bruder G, Chaplin DJ, Double JA, Everitt J, Farningham DA, Glennie MJ, Kelland LR, Robinson V, Stratford JJ, Tozer GM, Watson S, Wedge SR, Eccles SA (2010) Guidelines for the welfare and use of animals in cancer research. *Br J Cancer* **102**(11): 1555–1577.

Zhou Y, Tozzi F, Chen J, Fan F, Xia L, Wang J, Gao G, Zhang A, Xia X, Brasher H, Widger W, Ellis LM, Weihaa Z (2012) Intracellular ATP levels are a pivotal determinant of chemoresistance in colon cancer cells. *Cancer Res* **72**(1): 304–314.

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