Short Report

Non-invasive in vivo imaging of early metabolic tumor response to therapies targeting choline metabolism

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The cholinic phenotype, characterized by elevated phosphocholine and a high production of total-choline (tCho)-containing metabolites, is a metabolic hallmark of cancer. It can be exploited for targeted therapy. Non-invasive imaging biomarkers are required to evaluate an individual’s response to targeted anticancer agents that usually do not rapidly cause tumor shrinkage. Because metabolic changes can manifest at earlier stages of therapy than changes in tumor size, the aim of the current study was to evaluate 1H-MRS and diffusion-weighted MRI (DW-MRI) as markers of tumor response to the modulation of the choline pathway in mammary tumor xenografts. Inhibition of choline kinase activity was achieved with the direct pharmacological inhibitor H-89, indirect inhibitor sorafenib and down-regulation of choline-kinase α (ChKA) expression using specific short-hairpin RNA (shRNA). While all three strategies significantly decreased tCho tumor content in vivo, only sorafenib and anti-ChKA shRNA significantly repressed tumor growth. The increase of apparent-diffusion-coefficient of water (ADCw) measured by DW-MRI, was predictive of the induced necrosis and inhibition of the tumor growth in sorafenib treated mice, while the absence of change in ADC values in H89 treated mice predicted the absence of effect in terms of tumor necrosis and tumor growth. In conclusion, 1H-choline spectroscopy can be useful as a pharmacodynamic biomarker for choline targeted agents, while DW-MRI can be used as an early marker of effective tumor response to choline targeted therapies. DW-MRI combined to choline spectroscopy may provide a useful non-invasive marker for the early clinical assessment of tumor response to therapies targeting choline signaling.

Choline phospholipid metabolism is significantly increased in cancer cells. This specific cholinic phenotype is a new metabolic hallmark of cancer associated to elevated phosphocholine (PC) and increased production of total choline (tCho)-containing metabolites.1,2 Choline kinase (ChK) is the first cytosolic enzyme of the choline pathway.3 This enzyme catalyzes the ATP-dependent phosphorylation of choline to form PC, initiating the choline pathway for phosphatidylcholine (PtCho) biosynthesis.3 In cancer, accumulation of intracellular PC is believed to reflect increased ChK activity. Chk induction is a general cellular response to growth factor stimulation, and is essential for cell growth and viability. Malignant transformation,

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Abbreviations: ADCw: apparent-diffusion-coefficient of water; ChKA: choline-kinase α; DW-MRI: diffusion-weighted MRI; ERK: extracellular signal-regulated kinase; HLSVD: Hankel Lanczos Singular Value Decomposition; MAPK: mitogen-activated protein kinase; PC: phosphocholine; RECIST: response evaluation criteria in solid tumors; ROI: regions of interest; shRNA: short-hairpin RNA; tCho: total-choline; TE: echo time; TR: repetition time

Additional Supporting Information may be found in the online version of this article.

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facilitated by both ChK activity and PC production, is therefore characterized by abnormal choline metabolism. At least three ChK isoforms exist in mammalian cells, which are encoded by two genes: choline kinase α (ChKA) and choline kinase β (ChKB). At least one isoform, probably ChKA, is involved in the promotion of cell growth during carcinogenesis. Therefore, inhibition of ChK activity may provide a novel strategy for the development of new families of targeted anticancer drugs.

In animal models, various pharmacological anticancer agents have an effect on phospholipid metabolism. In particular, ChK inhibition by RSM-932A and MN58b significantly decreases PC levels vascular disrupting agents such as 5,6-dimethyl-xanthene-4-acetic acid induce a decrease in tCho gene therapy as a treatment of glioma induces early apoptosis with a significant accumulation of glycerophosphocholine (GPC) and PC, and radiation therapy can decrease tCho levels, as observed 24 hr after a 20 Gy X-ray dose. Effective methods to characterize an individual’s response to treatment are mandatory to assess the therapeutic benefits of novel targeted therapies. Conventional, anatomically based endpoints are deemed inadequate to monitor a tumor response to targeted agents that usually do not affect tumor size when used as a monotherapy. As an alternative, in vivo proton MR spectroscopy (1H-MRS) is a non-invasive technique that can provide information on tumor metabolism for experimental investigation and, potentially, for tumor diagnosis and monitoring. Metabolic changes can manifest at earlier times during therapy with respect to changes in tumor size. Hence, a rapid reduction in tCho levels (comprising mixed signals from choline, GPC and PC) has been proposed as a non-invasively marker of the tumor status and of inhibition of cellular proliferation. In this context, 1H-MRS imaging of tCho levels in many cancers, including breast cancer, have been performed and linked to tumor evolution and diagnostic performance.

Besides 1H-MRS, diffusion-weighted MRI (DW-MRI) is another non-invasive method than can be used to monitor the evolution of a tumor and its response to treatment. DW-MRI primarily reports on loss of cellularity, which is the ultimate outcome not only of extensive necrosis but also of other types of cell death, including apoptosis and mitotic catastrophe. Changes in the Apparent-Diffusion-Coefficient of water (ADCw) of tumors often precedes any measurable change in tumor size or volume. Hence, determination of ADCw changes may influence clinical practice by allowing much earlier adjustments in therapy. In many cancers, an increase in ADCw has been reported within the first two weeks after the start of chemotherapy or radiotherapy and was correlated with tumor response to treatment.

The aim of the current study is to evaluate tCho (detected with 1H-MRS) and ADCw (detected with DW-MRI) as markers of the response of mammary tumor xenografts to the modulation of the choline pathway using direct and indirect ChK inhibitors in mice.

**Material and Methods**

**Targeting of ChK activity**

MDA-MB-231 tumor-bearing mice (tumor volume: 0.2 ± 0.1 cm³, see Supporting Information for tumor induction protocol) were intraperitoneally treated daily with a direct inhibitor of ChK activity (H89: 20 mg/kg dissolved in 35 µl of DMSO) for 5 days, an indirect inhibitor of ChK activity (sorafenib: 40 mg/kg dissolved in 35 µl of DMSO) for 5 days, or vehicle (DMSO, 35 µl). H89 is used in this study for his direct and independent inhibition of ChK activity, whereas sorafenib is a well-documented multi-kinase inhibitor. For ChKA silencing, we used short hairpin (sh)RNA (clone ID TRCN0000006050) from ABgene (Epsom, Surrey, United Kingdom). Scramble shRNA (shSCR) was used as a negative control. Cells were transfected as reported previously (see Supporting Information). Reduction in choline kinase expression was assessed by western blotting analysis (see Supporting Information).

**1H-MR choline spectroscopy**

Animal experiments were performed with an 11.7-Tesla, 16-cm inner diameter bore system (Bruker, Biospec, Ettlingen, Germany) equipped with a quadrature volume coil (40-mm inner diameter). Animals (tumor volume: 0.2 ± 0.1 cm³) were anesthetized by isoflurane inhalation (3% in air for initiation and 1–2% in air for maintenance). They were laid on a warm water blanket connected to a circulating water bath to maintain body temperature (checked using a rectal temperature probe). A pressure cushion was used to monitor breathing.

For single voxel spectroscopic data acquisition, volumes of interest were placed inside tumors according to T2-weighted reference images. Optimization of magnetic field homogeneity (localized shimming) was performed until achieving a linewidth of water resonance below 50 Hz. Automatic shimming and manual water suppression (VAPOR) were used. 1H-MR spectra were acquired using a point-resolved spectroscopy (PRESS) localization technique. Typical acquisition parameters were repetition time (TR) = 2.5 sec, echo time...
520 ms, averages 5256, voxel size 4434 mm3 and total acquisition time 510 min50s. MR spectra were analyzed using jMRUI software version 5.0. Metabolite model signals used in quantitation based on quantum estimation (QUEST) were simulated in NMR-SCOPE (NMR spectra calculation using operators; jMRUI). Signals were imported in jMRUI, pretreated by Hankel Lanczos Singular Value Decomposition (HLSVD) to eliminate any residual water peak, and rephased. Model fitting was performed using the QUEST routine of jMRUI. Peak areas were measured for tCho peak (δ = 3.21 ppm) and normalized with the water peak area (δ = 4.7 ppm) from the non-water suppressed scans using a same volume of interest and geometry.

For DW-MRI, a transverse echo planar imaging sequence was used with the following acquisition parameters: TR/TE = 3,000/27 ms, duration of diffusion gradients (d) = 7 ms, separation of diffusion gradients (Δ) = 14 ms, slice number = 12; slice thickness = 1 mm, interslice distance = 1.2 mm, acquisition time = 5min24sec. DW images were acquired using b-values of 0–100–200–400–600–800–1000–1200–1500 sec/mm 2. The b-value is equal to \( \gamma^2 G^2 \delta^2 (\Delta - (\delta/3)) \), where Gd is the strength of the diffusion-weighting gradient, and \( \gamma \) is the gyromagnetic ratio for protons. Mean apparent diffusion coefficients (ADCw) were calculated from DW images and averaged for every slice where the tumor was found using a homemade program in Matlab software (The MathWorks Inc., Natick, MA) to define regions of interest (ROI). The exponential decay of the signal as a function of the b-value was measured according to the Stejskal–Tanner equation. ADC maps were generated by non-linear least squares regression of a mono-exponential to the experimental signal intensity for all b values.

**Results**

**Direct (H89) and indirect (sorafenib) targeting of ChKA decreases tumor choline content**

In mice bearing a MDA-MB-231 human breast cancer, we first quantified tumor tCho content non-invasively using...
single voxel $^1$H-MR choline spectroscopy. A significant decrease in the tCho to water ratio was observed after 48 hr of treatment with H89 ($p_{\text{ANOVA}} = 0.02$, CI 0.06–0.72; $n = 6$) and with sorafenib ($p_{\text{ANOVA}} = 0.006$, CI 0.095–0.5, $n = 7$) (Fig. 1a). Decreases were still significant after 5 days (measurement at 108 hr) of treatment. In the control group ($n = 8$), there was no significant change of the tCho peak during the 5 days of monitoring (Fig. 1a). Corroborating these in vivo data, ex vivo quantification of PC by mass spectrometry (HPLC-ESI-MS, see Supporting Information methods) of four tumors of each group did confirm the significant difference between control tumors and tumors treated with sorafenib or H89 ($p = 0.05$, CI 0.17–2.51; $p = 0.007$, CI 0.67–3.00 respectively) (Fig. 1b). The decrease in tCho and PC demonstrate a modulation of the choline cycle. In this regard, ChKA expression was shown to be significantly decreased to 49.7% after treatment with sorafenib ($p = 0.003$; $n = 4$, independent experiments), and to 71.9% after treatment with H89 (*, $n = 4$, independent experiments) respectively, with respect to control cells (Supporting Information Fig. 1).

In mice bearing shRNA-transfected MDA-MB-231 tumors, a significant decrease ($p = 0.003$, $n = 9$) was also observed in the tCho to water ratio when comparing anti-ChKA versus SCR shRNA-expressing tumors ($n = 10$) (Fig. 1c). Again, ex vivo quantification of PC by mass spectrometry of 4 tumors of each group did confirm the significant difference between shSCR and shChKA tumors ($p = 0.0001$, CI −4.52/−1.80) (Fig. 1b). ChKA expression was efficiently silenced by the specific shRNA, as shown by Western blot analysis in vitro on MDA-MB-231 cells (Fig. 1c). Typical $^1$H- MRS spectra from in vivo shSCR and shChKA tumors are shown in Figure 1d. Thus, both direct and indirect pharmacological inhibition of the choline cycle activity, as well as genetic targeting of ChKA expression, all significantly reduced the tCho content of MDA-MB-231 tumor xenografts in mice. Relative tCho tumor content was decreased by 54.4% after 48 hr using H89, 49.3% after 48 hr using sorafenib, with respect to Day 0 and by 57.2% using anti-ChKA shRNA, with respect to shSCR. Importantly, the results also illustrate the high heterogeneity in the basal tCho/water ratios from one tumor to another (large standard errors of the mean).

**Sorafenib, but not direct ChK targeting, decreases intratumoral cellularity**

In MDA-MB-231 tumor-bearing mice, intratumoral cellularity was assessed using DW-MRI and quantified from ADCw.
No significant change in ADCw was observed following treatment with H89 (n = 6), whereas sorafenib induced a progressive increase in ADCw that was significant after 5 days of treatment ($p_{\text{ANOVA}} = 0.01, CI -0.54$–$-0.07, n = 9$) (Fig. 2a), which is generally described to reflect a decrease in cellularity. Typical ADCw maps pre and post-sorafenib treatment are shown in Figure 2b. Similarly to H89 ChK inhibition, MDA-MB-231 tumor expressing an anti-ChKA shRNA had similar ADCw compared to those expressing SCR shRNA (data not shown). Thus, only sorafenib increased ADCw in MDA-MB-231 xenografts.

**Direct and indirect ChK targeting can induce tumor necrosis**

Tumors were harvested after 5 days of treatment. Hematoxylin and eosin staining of tumor slices revealed a significant increase in necrosis with sorafenib treatment ($p = 0.048, n = 4$; Fig. 2c) with respect to control tumors ($n = 6$). Anti-ChKA shRNA also caused a significant tumor necrosis ($p = 0.015, n = 4$), but not H89 ($n = 6$) after 5 days of treatment (only a non-significant trend towards increased necrosis was seen). Typical H&E stained tumor sections with or without treatment with sorafenib are shown on Figure 2d.

**Discussion**

Most targeted therapies for cancer cause tumor stabilization rather than shrinkage, thus reducing the sensitivity or rendering inappropriate the standard metrics of response, including response evaluation criteria in solid tumors (RECIST). There is therefore a need for alternative quantitative biomarkers of response. Among these, our study shows that tCho (measured using $^1$H-MRS) and ADCw (measured using DW-MRI) can be used to non-invasively assess metabolic tumor responses to treatments in vivo.

Our data document that intracellular tCho levels can be determined non-invasively in tumors with $^1$H-MRS to evidence ChK target inhibition. This pharmacodynamic parameter was suitable to demonstrate that treatments were acting on their targets. However, it was not suited to predict the response of a tumor to choline-targeted treatment, and additional imaging markers should be considered to predict the response of a tumor to treatment. This is illustrated in our study by the fact that, when targeting ChK activity with a direct inhibitor (H89), a significant decrease of tCho and PC was observed that was not associated with a drop of cellularity and a stabilization of tumor growth. Comparatively, we used sorafenib, a well-documented clinical multi-kinase inhibitor that primarily decreases the phosphorylation of extracellular signal-regulated kinase (ERK) in the mitogen-activated protein kinase (MAPK) pathway, and also targets ChK activity. Daily injections of sorafenib at high dose induced a rapid (48-hr) decrease in the tumor content of tCho and PC, which preceded decreased cellularity (measured with DW-MRI), necrosis and, ultimately, a reduction of tumor size (which became significant only 5 days post-treatment). In line with our findings, inhibition of MAPK signaling with the U0126 MEK inhibitor has been previously reported to cause a drop in intratumoral tCho and PC levels in tumors. Finally, targeting ChKA with a specific shRNA decreased tCho and PC levels in tumors. We found that the knock-down of the ChKA induces tumor necrosis and tumor growth retardation. We did not observe any significant
difference in basal ADCw values between MDA-MB-231 tumor expressing an anti-ChKA shRNA compared to those expressing SCR shRNA. Indeed, Diffusion-Weighted MRI is adapted to a longitudinal follow up of individual tumors before and after treatment, which is not possible with this approach. Of note, our DW-MRI results were interpreted as a drop of cellularity, while other factors can contribute to ADCw changes, including tissue disorganization, extracellular space tortuosity and integrity of cellular membranes that can also impact on motion of water molecules.23

Publically available 1H-MRS data support variable independent conclusions regarding the usefulness of in vivo spectroscopy for monitoring the response of breast cancer to therapy. One source of variability is that choline transport rates and ChK activity are increased in breast cancer cells that express elevated levels of PC.24 Another source of variability could be attributed to the significant induction of ChK expression with the histological tumor grade.25 Consistently, the association between ChK overexpression and tumor aggressiveness has been reported for ChK inhibition by small interfering RNAs26 and by the specific inhibitor MN58b.6 These studies nevertheless concluded that ChK inhibition significantly reduces PC and tCho levels in breast cancer cells, and proposed MRS to be used as a potential non-invasive marker of ChK inhibition and of tumor response to treatment. However, authors also pointed out limitations to the use of the composite choline signal as a marker of response, which notably included a large inter-subject variability in the level of metabolites detected using 1H-MRS.14 Here, we show that tCho level quantification in vivo with 1H-MRS is a sensitive pharmacodynamic marker of a tumor to choline targeted treatment. However, this measurement was not per se predictive of the tumor response to a treatment as tumors were responsive to sorafenib and not to H89. Longitudinal pre- and post-treatment measurements of ADC values were more consistent in terms of response as there was a close agreement between ADC values, tumor growth and tumor necrosis for both H89 and sorafenib treatments.

Of note, besides the multiple kinases inhibited by Sorafenib, the drug has also shown significant anti-angiogenic properties that could also be responsible for changes in tumor ADCw. In this context, we previously showed that, while the anti-angiogenic effect of sorafenib can be quantified as soon as 48 hr post-treatment using anti-CD105 antibody, it is not reflected in terms of ADCw at this time point, and only becomes significant at day 5.19,27 Moreover, tumor cell proliferation assessed in vivo using 18F-FLT with a similar protocol does significantly increase at day 2. All these data do suggest that the anti-angiogenic property of the drug is not the major factor responsible for the change in ADCw. Finally, regardless the major factor influencing the change in ADCw in response to treatment with sorafenib, the current results do still demonstrate that tCho can be modified with no consecutive effect in terms of tumor growth.

In conclusion, DW-MRI combined with choline spectroscopy may provide a useful non-invasive marker of response for choline signaling-targeted therapies, with the ultimate goal of improving individualized drug therapy.

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5. Aoyama C, Liao H, Ishidate K. Structure and properties that could also be responsible for changes in tumor ADCw. In this context, we previously showed that, while the anti-angiogenic effect of sorafenib can be quantified as soon as 48 hr post-treatment using anti-CD105 antibody, it is not reflected in terms of ADCw at this time point, and only becomes significant at day 5.19,27 Moreover, tumor cell proliferation assessed in vivo using 18F-FLT with a similar protocol does significantly increase at day 2. All these data do suggest that the anti-angiogenic property of the drug is not the major factor responsible for the change in ADCw. Finally, regardless the major factor influencing the change in ADCw in response to treatment with sorafenib, the current results do still demonstrate that tCho can be modified with no consecutive effect in terms of tumor growth.

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