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Mycology

Diagnosis of cerebral cryptococcoma using a computerized analysis of ^{1}H NMR spectra in an animal model

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Abstract

Viable cryptococci load in biopsy material from an animal model of cerebral cryptococcoma were correlated with ^{1}H NMR spectra and metabolite profiles. A statistical classification strategy was applied to distinguish among high-resolution ^{1}H NMR spectra acquired from cryptococcomas, glioblastomas, and normal brain tissue. The overall classification accuracy was 100% when a genetic-algorithm-based optimal region selection preceded the development of linear discriminant analysis-based classifiers. The method remained robust despite differences in the microbial load of the cryptococcoma group when harvested at different time points. These results indicate the feasibility of the method for diagnosis without isolation of the pathogenic microorganism and its potential for in vivo diagnosis based on computerized analysis of magnetic resonance spectra.

Keywords: Proton NMR; Statistical classification strategy; Cryptococcoma; Glioblastoma; Animal model; Rapid methods

1. Introduction

Potentially life-threatening neurological infections caused by the yeast *Cryptoccoccus neoformans* most commonly present as meningitis, but in up to 14% of cases, clinical manifestations result from circumscribed lesions known as cryptococcomas, in the brain parenchyma (Casadevall and Perfect, 1998; Chen et al., 2000). Brain biopsy is required for diagnosis when lesions are confined to the brain or in the absence of other diagnostic material (Mitchell et al., 1995), because the pathological characteristics of infective lesions cannot be reliably distinguished by modalities such as computed tomography or magnetic resonance imaging (Andreula et al., 1993; Garg et al., 2004). Nuclear magnetic resonance (NMR) spectroscopy has been applied successfully to the noninvasive clinical diagnosis of some acute bacterial abscesses based on its ability to detect low molecular weight molecules from microbial cells and/or cells recruited during the host response to infection (Garg et al., 2004; Lai et al., 2002; Himmelreich et al., 2005). Interpretation of clinical spectra is subjective and is problematic in cases where typical marker metabolites are not detectable.

We identified 2 marker metabolites of *C. neoformans*, α,α-trehalose and mannitol, in NMR spectra of biopsy samples from an animal model of acute cerebral and pulmonary cryptococcoma (Himmelreich et al., 2001; Himmelreich et al., 2003a). Resonances of these compounds were readily observed in 1-dimensional (1D) ^{1}H NMR spectra. However, the resonances from trehalose and mannitol were reduced or not clearly distinguished in spectra from some animals at a later stage of infection.

The application of computerized data analysis using pattern recognition techniques to determine the pathological status of tissue based on NMR spectra would provide an

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objective and robust means for diagnosis. Pattern recognition techniques have been applied to in vivo NMR spectra for tumor diagnosis and grading using cluster analysis (Hagberg, 1998), artificial neural networks (Somorjai et al., 1996; Poptani et al., 1999), or linear discriminant analysis (LDA) (Somorjai et al., 1996; Preul et al., 1996). A statistical classification strategy (SCS) was specifically developed for the analysis of spectroscopic data from biofluids and tissue biopsies, where data sets contain many fewer spectra than data points (attributes) present in each spectrum (Somorjai et al., 2002; Nikulin et al., 1998). This method has resulted in the development of highly accurate and reliable classifiers for a variety of clinical and diagnostic problems, including the identification of pathogenic yeasts in cell suspensions (Himmelreich et al., 2003b; Baumgartner et al., 2004).

It was our aim to develop a robust and objective method to distinguish between tumors and cryptococcoma, collected at different stages of evolution, and to correlate the load of viable cryptococci with trehalose content. This approach was used to allow for variations in the time between natural acquisition of human infection and clinical presentation and thus provide a robust, rapid, and objective method to distinguish between cryptococcomas and the major differential diagnosis, namely, cerebral tumors.

2. Materials and methods

2.1. Animal model

A virulent strain of *C. gattii* serotype B (McBride), isolated from a cat, was obtained from the Westmead Hospital culture collection and used to establish cerebral cryptococcomas as described previously (Himmelreich et al., 2001). In brief, male Wistar-Furth rats (body weight 200–250 g) were anesthetized and the head immobilized in a stereotactic head frame. Coordinates for cerebral microinjections were 2.2 mm below dura; lateral: 3 mm; anterior-posterior: +2.4 mm relative to ear bar zero. Five microliters of phosphate-buffered saline (PBS) containing 5×10^4 CFU *C. neoformans* ($n = 64$), 5 μL containing 1×10^6 CFU C6 tumor cells ($n = 50$), or 5 μL of PBS ($n = 45$) were injected slowly. Infected and control animals were killed 7, 14, 21, and 28 days after injection or when they developed signs of illness. All other parameters were as stated in the work of Himmelreich et al. (2001).

Pathology was confirmed by microscopy of tissue sections stained with hematoxylin-eosin or periodic acid-Schiff reagent. Brain samples (maximum diameter of 5 mm) obtained from each animal were suspended in PBS-containing deuterated water (PBS/D_2O, Australian Nuclear Science and Technology Organization, Lucas Heights, Australia), snap-frozen in liquid nitrogen, and stored at −70 °C for up to 4 months before NMR spectroscopy. Animal experimentation was carried out according to the Australian National Health and Medical Research Council Guidelines and with ethical approval from the University of Sydney Animal Ethics Committee. Viable cryptococci in the cryptococcomas were quantified for 12 animals.

2.2. NMR spectroscopy

^{1}H NMR spectra were obtained at 37 °C on a Bruker Avance 360 MHz NMR spectrometer using a 5-mm [^{1}H, ^{13}C] inverse-detection dual-frequency probe. ^{1}H NMR spectra were acquired with acquisition parameters as follows: frequency 360.13 MHz, pulse angle 90° (6–7 μs), repetition time 2.3 s, 4096 data points, 128 transients, spectral width 3600 Hz. Deuterium lock was used to optimize magnetic homogeneity. Water suppression was performed by selective excitation field gradients. Spectra were processed using Bruker XWINNMR 3.1 software. Chemical shift calibration was performed by setting the center of the spectrum to 4.65 ppm (the nominal position of the water resonance with respect to tetramethylsilane in PBS/D_2O at 37 °C).

Two-dimensional (2D) homo- and heteronuclear correlation spectra were acquired from cryptococcomas harvested at selected time points to assign ^{1}H NMR resonances to specific compounds according to Himmelreich et al. (2001). The trehalose concentration was estimated using calibrated cross peak volumes from ^{1}H, ^{1}H COSY spectra compared with *p*-amino benzoic acid as a concentration standard in 12 animals with cerebral cryptococcoma (Himmelreich et al., 2001).

2.3. Microbiology

Cryptococcomas from 12 animals (3 at each time point) were weighed, dispersed, and spread on plates made from serial dilutions of cryptococcal suspensions. The number of colony-forming units per gram was determined after 48 h of incubation at 35 °C. Cryptococci were biotyped and serotyped (Crypto Check agglutination test, Iatron Labs, Chiba, Japan).

2.4. Classification of NMR spectra

An SCS approach was used for data analysis (Somorjai et al., 2002; Nikulin et al., 1998). NMR data were prepared using software developed in house (Xprep, IBD, NRC, Winnipeg, Canada). Magnitude spectra, consisting of 4096 data points over a spectral width of 10 ppm, were reduced to 1500 points between 0.35 and 4.05 ppm. The spectra were normalized to unit area in this region. The correct alignment of the NMR spectra was verified by simultaneous and sequential display of all NMR spectra using the lipid/lactate resonance at 1.3 ppm.

The magnitude NMR spectra were analyzed by a genetic-algorithm-based optimal region selection process (GA-ORS) to reduce the number of attributes and hence eliminate redundant information (Nikulin et al., 1998). Two maximally discriminatory subregions in the 1D NMR spectra of each class (pathology) were selected for development of LDA-based classifiers. The averages of these subregions