Short Communication

A case study of primary malignancy of buccal mucosa using proton HR-MAS NMR spectroscopy on tissue specimens

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ABSTRACT

The diagnosis and confirmation of oral SCC (squamous cell carcinoma) is still dependent on histopathology report in spite of development of radiological investigations. It is, thus important to understand the underlying molecular mechanisms and how the alterations in metabolic pathways effect the tumor development and progression. The simultaneous and comprehensive information about the presence and absence of small molecule metabolites and their relative concentrations has been provided by ¹H HR-MAS NMR spectroscopy on tissue specimens. In this paper a unique case study was presented in order to correlate histological and NMR spectroscopic findings. The patient's initially lesion was found to be non-malignant in nature based on histological findings but its periodic localized recurrence even after laser ablation prompted us to perform HR-MAS based analysis and its role in identifying the metabolic alterations in known pathways occurring during its progressions. Thus it was confirmed after analysis that HR-MAS NMR can also be used as an analytical tool which is reliable in order to distinguish between malignant and non-malignant tissues, in combination with histopathology.

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1. Introduction

Cancer is a major cause of mortality and morbidity with approximately 6 million deaths each year worldwide.¹ The oral cavity cancer comprises of the seven sub-sites, which comprises of mucosal lips, oral tongue, buccal mucosa, floor of the mouth, retromolar trigone lower and upper alveolar ridges and hard palate.²

It is observed that patients who are having greater stage and size of tumours and whose metastatic potential is high their survival rate is 30% while patients who are having lower stage and size of tumor or cancer are having 80–90% rate of survival.³

With the development and usage of certain diagnostic aids the difficulty of delayed appearance of oral lesions could be overcome. For diagnosis of oral SCC (squamous cell carcinoma) in spite of using PET, CT or MRI as radiological investigations⁴ still gold standard technique which is confirmation given by histopathology report is being used. Therefore, it is necessary to understand the underlying molecular mechanisms and how the alterations in

metabolic pathways effect the tumor development and progression.

Numerous cellular and molecular markers at genetic and protein level have been identified to probe the complex metabolic processes occurring during the phenomenon of cancer. The simultaneous and comprehensive information about the presence and absence of small metabolites and their relative concentrations has been provided by the newly emerging field of ¹H NMR based metabolomics/metabonomics (both the terms are used interchangeably nowadays). The metabolic analyses provide an overview of the lexicon of alterations which occur at genetic and protein level in terms of metabolic perturbations occurring during the pathogenesis, correlating well with the clinical outcomes. The identification of molecular biomarkers for a specific molecular aberration will play an important role in the prognosis and improved patient management.

The ¹H NMR spectroscopy plays a considerable role in various studies of toxic effects, cancer and inborn error of metabolism. In HR-MAS NMR spectroscopy, intact tissues are used for data acquisition in such a way that minimizes the effect of line broadening caused due to sample heterogeneity and residual anisotropic NMR parameters. The technique significantly provides relevant biochemical information regarding small molecule metabolites present in the tissues, which can be mapped and

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quantitated from a single spectral window. Furthermore, wherever applicable, the technique proves to be non-invasive; in case of tissues, the same specimen could be further used for histopathological examination. We, therefore, wish to present a unique case study where blinded prediction of the tissue type has been performed using metabolomics followed by its histological correlation. The patient's initially lesion was found to be non-malignant in nature based on histological findings but its periodic localized recurrence even after laser ablation prompted us to perform HR-MAS based metabolomic analysis and its role in identifying the metabolic alterations in known pathways occurring during its progressions.

2. Materials and methods

2.1. Clinical methods

Two tissue specimens (25–30 mg) were obtained from the patient during biopsy and comprising of the lesion from buccal mucosa were obtained. In order to prevent metabolic and enzymatic activities these tissue samples were kept in plastic vials of good quality and then in liquid Nitrogen, and were then on the same day was subjected for NMR experiments.

2.2. Tissue samples

Before NMR analysis the stored tissues were thawed and in order to remove blood, contamination or impurities they were washed using D₂O. The tissues were then carried for HR-MAS NMR experiments. The samples were then loaded into ZrO₂ rotor of 4 mm having 50 μ l capacity and D₂O having a volume of 20 μ l with 0.03% TSP which acts as a reference of chemical shift. After this the rotor was transmitted into the HR-MAS NMR probe.

2.3. Magnetic resonance spectroscopy

The recording of proton HR-MAS NMR spectra was done on a Bruker Advance 400 MHz spectrometer, operable at a proton frequency of 400.13 MHz. It is also having a 4 mm HR-MAS ¹H-¹³C dual probe head with magic-angle gradient.

To remove the signals arising because of rotation side-bands the spinning of samples were done at 4.0 kHz. Proton NMR spectra having water suppression were obtained when one-dimensional NOESY pulse-sequence was used. Experimental parameters that were used were as follows- spectral width of 8250.8 Hz, total relaxation time of 3.99 s, mixing time τ_m of 100 ms, 128 transients having 9 min as total recording time. For each tissue sample recording of one-dimensional CPMG (Carr-Purcell-Meiboom-Gill)

Fig. 1. The histopathological results along with their corresponding ¹H NMR spectra.

experiment having water suppression was done which has an echo-time of 160 ms.⁵ When the acquisition of spectra was done the temperature of the samples were maintained at 283 K by usage of cooled nitrogen gas. Even after exposing the tissue samples at 283 K for about 1 hr long NMR measurements there were hardly any differences seen in the spectral metabolite pattern.⁶

2.4. Histopathology

After fixing the tissue samples in 10% formalin they were further fixed by using paraffin. At the interval of 100 μ m 5 mm sections were taken which comprises the whole tissue specimen and using eosin and haematoxylin were stained. Assessment of sections were done to prove malignancy of the tissue. Fig. 1 shows the spectra which is obtained from tissue specimens along with their respective histopathology.

3. Results

The ¹H HR-MAS NMR CPMG spectra (Fig. 2) provides well-resolved resonances for nearly all of the frequently seen metabolites which can be attributed on the ground of some earlier reported values accessible in other studies.^{7–9}

At 3.03 ppm singlet signal arises because of the methyl group of creatine which was used as a reference for an internal chemical shift. Other than lipid resonances which was prominently seen,

other metabolites which were identified were valine (Val), leucine (Leu), isoleucine (Ileu), lysine (Lys), lactate (Lac), acetate (Ace), alanine (Ala), threonine, methionine, glutamate (Glu), glutamine, aspartic acid, asparagine, citrate, tyrosine, choline (Cho), ethanolamine, phenylalanine, phosphocholine (PCho), myo-inositol, α - and β -glucose, phosphocholine, glycerol, taurine, triglyceride, creatine (Cr) and glycine (Gly).

4. Discussion

The HR-MAS NMR spectroscopy method seems to be quite promising in, firstly identifying the end-point biomarkers of a particular pathology and the underlying biochemistry and secondly in distinguishing between the two states with high sensitivity and specificity. As shown in Fig. 2, the variations in resonances of acetate, glutamate, lactate, choline, phosphocholine, glycine, taurine, leucine, isoleucine, valine, lysine, alanine, creatine and polyunsaturated fatty acids (PUFA) were quite significant. Previous biochemical studies on expeditiously developing hepatoma cells revealed the changes in membrane bilayer fluidity including alterations in enzymatic activities of microsomal and mitochondrial membranes. These are the consequences of modifications in lipid composition of membrane which includes the decrease in PUFA content,^{10,11} similar findings are significantly observable in the spectra provided.

Fig 2. The CPMG proton HR/[HYPHEN]MAS NMR spectra of the same tissue specimens. The chemical shift region from 0.6 ppm to 4.4 ppm is represented. The lipid resonances at 0.90 ppm, 1.25 and 2.05 ppm masking the signals of lactate, alanine and other branch chain amino acids in benign tissue specimen.

Similar prominent variation in spectral intensity ratios of mobile lipids (1.3/0.9 ppm) was observed in a previous ex vivo MRS study in head- and neck cancer on the prediction of treatment response.¹² In 1956, Otto Warburg reported that cancer cells depicts elevation in glycolytic rates and reduction in mitochondrial oxidation as compared to normal cells. Both these metabolic activities favor the survival of cell under tumor's hypoxic micro-environment.¹³ Also, Mcfate reported that when glucose is converted to lactate it protects the cancer cell from oxidative stress induced by reactive oxygen species.¹⁴ Thus, higher lactate accumulation was observed as a pronounced consequence. Not only lactate but also alanine a biomarker of hypoxic conditions occurred in tumor bearing tissue. Transamination of pyruvate under hypoxic conditions in order to stop the further increase in lactate concentration give rise to Alanine.¹⁵

The energy requirement of rapidly proliferating cancer cells is accomplished through creatine phosphate of high-energy. This in return is created from creatine by the intervention of creatine kinase an enzyme.¹⁶ After the formation of phosphates of high energy it is found the creatine deficiency in the cells which is evident in the spectrum in form of low intensity of creatine peak. There is a significant rise in the Cho/Cr ratio during in vitro proton MRS study of extracranial head and neck SCC.¹⁷

Choline (3.20 ppm) performs active cell proliferation with elevated membrane phospholipid turnover and is also believed to have a crucial aspect in cell membranes' phospholipid metabolism. PCho is formed after the phosphorylation of choline then series of biochemical reactions take place to form phosphatidylcholine. So, elevated Cho and PCho constituents in the cancer cells are considered as notable markers of elevated cell proliferation. When there is an increase in lipid biosynthesis other than Cho and PCho, it was also found that acetate exhibits higher level. These three metabolites thus shows immensely active tumor cells' proliferation. In yet another study it was shown that there is migration and proliferation of tumor cells which is facilitated by glutamate and thus it helps in tumor progression.¹⁸

In some previous studies on cell lines of breast tumor it has been reported that glycine an amino acid acts as a precursor during de-novo synthesis (a pathway for biosynthesis of nucleotides) of Purine (Adenine and Guanine).¹⁹ Thus when its concentration is increased in malignant tumors it is often used as a marker for upgraded nucleotide synthesis. Apart from these functions along with some different amino acids glycine, alanine and glutamine participate in protein synthesis as well, which increases manifold in malignant tissues²⁰ for their appropriate proliferation and progression. The NMR spectra of the malignant tissue reveals this fact that there is a prominent enhancement of the metabolites' relative intensities.

During unhindered cancer proliferation lipidogenesis, nucleic acid formation and enhanced protein synthesis takes place along with osmoregulation and volume regulation. It is found that higher concentration of Taurine which acts as an osmolyte helps in regulating cell swelling induced by hypoxia,^{19,21,22} thus also helps in maintaining the cell volume. Because of their uninterrupted proliferation of cells Taurine is also known as a free-radical scavenger and is the one which reduces the oxidative stress of the cells.²³ As reported by previous studies Taurine concentration was found to be higher in several tumors. These metabolites have crucial role in distinguishing malignant tissues from normal ones thus they act as a biomarker for aggravation or suppression of several cellular processes which takes place inside cancer cells.

The variations of these metabolites can be evaluated with supervised and unsupervised pattern recognition techniques and a model can be prepared for identification of the pathological status of the tissues.^{6,24} A cut-off value can be defined on the basis of the PLS scores and thus the assumption of class-membership of

unidentified tissue specimens can be performed. ¹H HR-MAS NMR can also be used as an analytical tool which is reliable in order to distinguish between malignant and non-malignant tissues, in combination with histopathology. In most kind of tumors histopathological assessment has become so crucial because of its prognosis, staging and therapeutic intervention more so the analysis of HR-MAS NMR spectroscopy might have added significant implications.

In this unique case study the patient's lesion initially was found to be benign and was confirmed histopathologically. But a recurrence was observed even after laser ablation. After performing HR-MAS based analysis, after six months the metabolic alterations were identified which played role in malignancy. These findings were then confirmed by histopathological reports. This case report thus illustrates the importance of HR-MAS spectroscopy in confirming the malignancy of an oral tissue.

5. Conclusion

In this study, the malignant tissues has a specific signature of varied cellular processes due to which it became convenient to distinguish from non-malignant tissues. In this research there was an observation of cancerous processes that takes place in tumor tissues.

This technique has another benefit and that is it maintains the absolute condition of tissues because there are no derivatization or extraction processes involvement as observed in other analytical techniques like HPLC and GC–MS. In combination with histopathology this study also displays that HR-MAS may extricate the doubt on range of tumor penetration as per the clinical scenario. It may play an important role in optimizing the clinical management strategy i.e. providing adjuvant treatment and keeping patients under strict follow-up and may contribute in understanding oral carcinogenesis.

Molecular studies of OSCC using MR spectroscopy and chemical shift imaging integrated into clinical findings, seems to have an immense prospective ahead. Such integration seems to be fruitful and will provide oncologists an opportunity to design a more concrete post-surgical patient management and/or chemotherapy.

Conflict of interest

None to declare.

References

- Petersen PE. Oral cancer prevention and control—The approach of the World Health Organization. *Oral oncol.* 2009;45(4 (May 31)):454–460.
- Law CP, Chandra RV, Hoang JK, Phal PM. Imaging the oral cavity: key concepts for the radiologist. *Br J Radiol.* 2014;(January 28).
- Tiziani S, Lopes V, Günther UL. Early stage diagnosis of oral cancer using 1 H NMR-based metabolomics. *Neoplasia.* 2009;11(3 (March 31)): 269–IN10.
- Chawla S, Kim S, Wang S, Poptani H. Diffusion-weighted imaging in head and neck cancers. *Future Oncol.* 2009 Sep 30;5(7):959–975.
- Wang Y, Bollard ME, Nicholso JK, Holmes E. Exploration of the direct metabolic effects of mercury II chloride on the kidney of Sprague–Dawley rats using high-resolution magic angle spinning 1 H NMR spectroscopy of intact tissue and pattern recognition. *J Pharm. Biomed. Anal.* 2006;40(2 (February 13)):375–381.
- Srivastava S, Roy R, Gupta V, Tiwari A, Srivastava AN, Sonkar AA. Proton HR-MAS MR spectroscopy of oral squamous cell carcinoma tissues: an ex vivo study to identify malignancy induced metabolic fingerprints. *Metabolomics.* 2011;7(2 (June 1)):278–288.
- Chae EY, Shin HJ, Kim S, Baek HM, Yoon D, Kim S, Shim YE, Kim HH, Cha JH, Choi WJ, Lee JH. The Role of High-Resolution Magic Angle Spinning 1H Nuclear Magnetic Resonance Spectroscopy for Predicting the Invasive Component in Patients with Ductal Carcinoma In Situ Diagnosed on Preoperative Biopsy. *PLoS One.* 2016;11(8 (August 25)):e0161038.
- Cheng LL, Chang IW, Louis DN, Gonzalez RG. Correlation of high-resolution magic angle spinning proton magnetic resonance spectroscopy with histopathology of intact human brain tumor specimens. *Cancer Res.* 1998;58(9 (May 1)):1825–1832.

9. Tugnoli V, Mucci A, Schenetti L, Calabrese C, Di Febo G, Rossi MC, Tosi MR. Molecular characterization of human gastric mucosa by HR-MAS magnetic resonance spectroscopy. *Int J Mol Med*. 2004;1(14 (December)):1065–1072.
10. Mansilla MC, Cybulski LE, Albanesi D, de Mendoza D. Control of membrane lipid fluidity by molecular thermosensors. *J Bacteriol*. 2004;186(20 (Oct 15)):6681–6688.
11. Sanders CR, Mittendorf KF. Tolerance to changes in membrane lipid composition as a selected trait of membrane proteins. *Biochemistry*. 2011;50(37 (Sep 20)):7858.
12. Bezabeh T, Odum O, Nason R, Kerr P, Sutherland D, Patel R, Smith IC. Prediction of treatment response in head and neck cancer by magnetic resonance spectroscopy. *Am. J. Neuroradiol*. 2005 Sep 1;26(8):2108–2113.
13. Warburg O. On the origin of cancer cells. *Science*. 1956;123(3191 (February 24)):309–314.
14. McFate T, Mohyeldin A, Lu H, Thakar J, Henriques J, Halim ND, Wu H, Schell MJ, Tsang TM, Teahan O, Zhou S. Pyruvate dehydrogenase complex activity controls metabolic and malignant phenotype in cancer cells. *J Biol Chem*. 2008;283(33 (August 15)):22700–22708.
15. Ben-Yoseph O, Badar-Goffer RS, Morris PG, Bachelard HS. Glycerol 3-phosphate and lactate as indicators of the cerebral cytoplasmic redox state in severe and mild hypoxia respectively: a ¹³C- and ³¹P-nmr study. *Biochem J*. 1993;291(3 (May 1)):915–919.
16. Podo F. Tumour phospholipid metabolism. *NMR Biomed*. 1999;12(7 (November 1)):413–439.
17. Mukherji SK, Schiro S, Castillo M, Kwock L, Muller KE, Blackstock W. Proton MR spectroscopy of squamous cell carcinoma of the extracranial head and neck: in vitro and in vivo studies. *Am J Neuroradiol*. 1997;18(6 (June 1)):1057–1072.
18. Rzeski W, Turski L, Ikonomidou C. Glutamate antagonists limit tumor growth. *Proc Nat Acad Sci*. 2001;98(11 (May 22)):6372–6377.
19. Griffin JL, Shockcor JP. Metabolic profiles of cancer cells. *Nat Rev cancer*. 2004;4(7 (July 1)):551–561.
20. Tessem MB, Swanson MG, Keshari KR, Albers MJ, Joun D, Tabatabai ZL, Simko JP, Shinohara K, Nelson SJ, Vigneron DB, Gribbestad IS. Evaluation of lactate and alanine as metabolic biomarkers of prostate cancer using ¹H HR-MAS spectroscopy of biopsy tissues. *Magn Reson Med*. 2008;60(3 (September 1)):510–516.
21. Shen MR, Chou CY, Ellory JC. Swelling-activated taurine and K⁺ transport in human cervical cancer cells: association with cell cycle progression. *Pflügers Archiv Eur J Physiol*. 2001;441(6 (March 5)):787–795.
22. Srivastava S, Roy R, Singh S, Kumar P, Dalela D, Sankhwar SN, Goel A, Sonkar AA. Taurine—a possible fingerprint biomarker in non-muscle invasive bladder cancer: a pilot study by ¹H NMR spectroscopy. *Cancer Biomarkers*. 2010;6(1 (January 1)):11–20.
23. Rosado JO, Salvador M, Bonatto D. Importance of the trans-sulfuration pathway in cancer prevention and promotion. *Mol Cell Biochem*. 2007;301(1–2 (July 1)):1–2.
24. Brindle JT, Antti H, Holmes E, Tranter G, Nicholson JK, Bethell HW, Clarke S, Schofield PM, McKilligin E, Mosedale DE, Grainger DJ. Rapid and noninvasive diagnosis of the presence and severity of coronary heart disease using ¹H-NMR-based metabolomics. *Nat Med*. 2002;8(12 (December 1)):1439–1445.