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NMR (^{1}H and ^{13}C) based signatures of abnormal choline metabolism in oral squamous cell carcinoma with no prominent Warburg effect

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ABSTRACT

At functional levels, besides genes and proteins, changes in metabolome profiles are instructive for a biological system in health and disease including malignancy. It is understood that metabolomic alterations in association with proteomic and transcriptomic aberrations are very fundamental to unravel malignant micro-ambient criticality and oral cancer is no exception. Hence deciphering intricate dimensions of oral cancer metabolism may be contributory both for integrated appreciation of its pathogenesis and to identify any critical but yet unexplored dimension of this malignancy with high mortality rate. Although several methods do exist, NMR provides higher analytical precision in identification of cancer metabolomic signature. Present study explored abnormal signatures in choline metabolism in oral squamous cell carcinoma (OSCC) using ^{1}H and ^{13}C NMR analysis of serum. It has demonstrated down-regulation of choline with concomitant up-regulation of its break-down product in the form of trimethylamine N-oxide in OSCC compared to normal counterpart. Further, no significant change in lactate profile in OSCC possibly indicated that well-known Warburg effect was not a prominent phenomenon in such malignancy. Amongst other important metabolites, malonate has shown up-regulation but D-glucose, saturated fatty acids, acetate and threonine did not show any significant change. Analyzing these metabolomic findings present study proposed trimethyl amine N-oxide and malonate as important metabolic signature for oral cancer with no prominent Warburg effect.

1. Introduction

Apart from proteomic and genomic observation, metabolomic studies play crucial role in addressing theragnostic ambiguities and deregulated cellular machinery in malignancy [1]. It gives a deeper understanding of metabolic derangement in malignancy. The functional level of biological system is controlled by genome, transcriptome, proteome and metabolome. However it is the latter which needs higher analytical precision in cancer diagnosis, as it heavily suffers from imprecision in the identification of definite biomarker. The oral cancer is also no exception [2]. Recent metabolite approaches have revealed many biomarkers for different type of cancer. However, the need for a reliable biomarker that is reproducible with high precision is still lacking. Mortality rate of oral cancer could not be reduced significantly through early detection, although there is a reasonable advancement in the relevant research activities [2].

Metabonomics is defined as "the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modifications" [3]. In this connection, studying 'Warburg effect' the well known metabolomic deregulation in different types of cancer has diagnostic significance [4]. The up-regulation in lactic acid pathway of glucose metabolism due to enhanced aerobic glycolysis is known to happen in such condition [4].

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Amongst other types of metabolomic alterations, choline metabolism is remarkably changed in many epithelial malignancy *viz.* that in breast, prostate etc. [5]. The tumor having abnormal physiological microenvironments such as hypoxia, acidic extracellular pH is known to influence choline metabolism [6]. In this context it may be noted that sarcosine, a choline break-down product remarkably increase in prostate cancer [7].

Activated choline metabolism is characterized by increased phosphocholine (PCho) and total choline-containing compounds (tCho) which are referred to as the cholinic phenotypes, is a fairly new metabolic hallmark [5]. Furthermore, elevated glycerophosphocholine (GPC) levels are found in acidic extracellular pH [8]. It has been also reported that in hypoxic situation tCho and PCho levels increase remarkably [9]. Phosphocholine, a precursor as well as a breakdown product of phosphatidylcholine (PtdCho), together with other phospholipids such as phosphatedylethanolamine (PtdEtn) forms the characteristic bi-layer structure of cellular membranes and regulates membrane integrity [10]. Moreover, the high-energy intermediates cytidine diphosphate (CDP), choline and CDP-ethanolamine are required to synthesize PtdCho and PtdEtn. Indeed, choline metabolism, a network of transporter systems and enzymes involved in choline phospholipid metabolism are deregulated in cancer cells [5]. Hence, choline metabolism including expression of its precursors and break-down products are remarkably disturbed in malignant conditions [5,7,8].

Amongst the 'Omics' studies, metabolomics employs analytical instrumentation in conjunction with pattern recognition techniques to monitor the molecular changes. Newer hallmarks in this regard are discovered mostly using the magnetic resonance spectroscopy (MRS) studies of tumors [1]. Metabolic characterization of tumor was previously reported using high-resolution magic angle spinning proton (^{1}H) magnetic resonance spectroscopy [MRS] technique [11]. Abnormal choline metabolism in rectal adenocarcinoma was observed through MRS study [11].

Our study demonstrated the abnormal choline metabolism through documenting significant down-regulation of choline and concomitant up-regulation of its break-down product trimethylamine N-oxide [TMAO] in oral squamous cell carcinoma [OSCC] by ^{1}H and ^{13}C NMR studies. Interestingly, present study also speculated that 'Warburg effect' was not supportive for OSCC as lactate status remained unchanged in such cancer compared to normal. Present study has also revealed an abnormal glucose-energy metabolism in rapidly proliferating cancerous cell by indicating the up-regulation of malonic acid in association with altered choline metabolism.

2. Material and methods

2.1. Sample collection

A total of 23 (<40 years) incision oral biopsy specimens were collected for histopathological gradation by expert pathologists. Among these, 18 specimens were confirmed as OSCC. Further for constituting a control study group, 12 (<40 years) tissue specimens were surgically excised as superfluous tissues during trans-alveolar and intra-alveolar root-canal extractions. From the same patients and normal volunteers, intravenous blood was collected and centrifuged at 1500 × g for 15 min. Serum aliquots (250) μl were then transferred into −80 °C for long term storage. Blood samples and biopsy specimens were obtained from Guru Nanak Institute of Dental Science and Research (GNIDSR), Kolkata, India. It was ensured that the specimens were collected under informed consent of patients and adhering to the ethical clearance of GNIDSR (GNIDSR/IEC/07/15).

2.2. Sample preparation

2.2.1. Hematoxylin and eosin staining

The excised tissue samples were fixed in 10% phosphate buffered formalin and processed for obtaining 4 μm thick paraffin sections and subsequently placed on albumin-coated (chicken-egg) glass slide. Following this, the slides were de-paraffinated by 10–20 min of xylene treatment. These sections were stained with Harris' Hematoxylin (Cat. No. AG2AF62372, Merck, Mumbai, India) and counter stained with eosin (Cat. No. MI7M572117, Merck, Mumbai, India).

2.2.2. NMR spectroscopy

NMR spectra were recorded at 298 K using a Bruker 600 MHz (for ^{1}H) and 150 MHz (for ^{13}C) AVANCE AVIII spectrometer. Serum samples were thawed by gradual temperature gradation and homogenized using a vortex mixer. 200 μl of serum was mixed with 400 μl deuterium oxide (D_2O) in 5 mm NMR tube. Solvent residual peak of D_2O was considered as standard NMR experiments. Water peak were suppressed for clarity of NMR spectrum.

2.3. Data processing and analysis

Data processing and analysis were performed through Bruker TOPSPIN 2.1 and Mest ReNOVA 7 software.

3. Result

3.1. Confirmation of sample conditions by gold standard hematoxylin and eosin staining

Pathological confirmation of the biopsy samples were done by histopathological staining. As per expert onco-pathologist, Fig. 1A and B represented the features of normal oral mucosa (NOM) and oral squamous cell carcinoma (OSCC) respectively.

3.2. NMR (^{1}H and ^{13}C) spectroscopic evaluation

NMR spectroscopy of the OSCC and control samples revealed that choline has been down-regulated [pick no. 1; 3.20 ppm, 3.63 ppm and 4.05 ppm for ^{1}H (Fig. 2 and S1, Table 1); 56.2 ppm, 62.5 ppm and 72.6 ppm for ^{13}C (Fig. 3 and S2, Table 1)] in OSCC compared to control. All of its NMR peaks (^{1}H and ^{13}C) present in control-NMR spectra, but absent in cancer −NMR spectra (Figs. 2 and 3; Table 1). On the other hand, trimethylamine N-oxide (TMAO), a break down product of choline remarkably increased (pick no. 3; 3.23 ppm for ^{1}H; 61.7 ppm for ^{13}C) in OSCC compared to control sample both in ^{1}H and ^{13}C spectra (Figs. 2, S1, 3 and S2; Table 1). Malonic acid also increased (pick no. 2; 3.14 ppm in ^{1}H; 51.6 ppm and 180.2 ppm in ^{13}C) in OSCC (Figs. 2, S1, 3 and S2; Table 1). Surprisingly, There were no significant changes in intensities of lactate profile in OSCC compared to normal (Figs. 2 and 3; Table 1). Other metabolites like glucose, satd. Fatty acids, acetate, threonine in OSCC did not show significant changes compared to control (Fig. 2).

4. Discussion

Recent advancement in cancer diagnosis highly appreciates spectroscopic evaluation of metabolic alterations having correlation with proteomic and genomic expressional attributes [1,12-15]. The identification of metabolomic signatures related to the systemic metabolic cycles in malignant condition deciphers insight for cancer prognosis and relevant biomarker discovery. Hence this

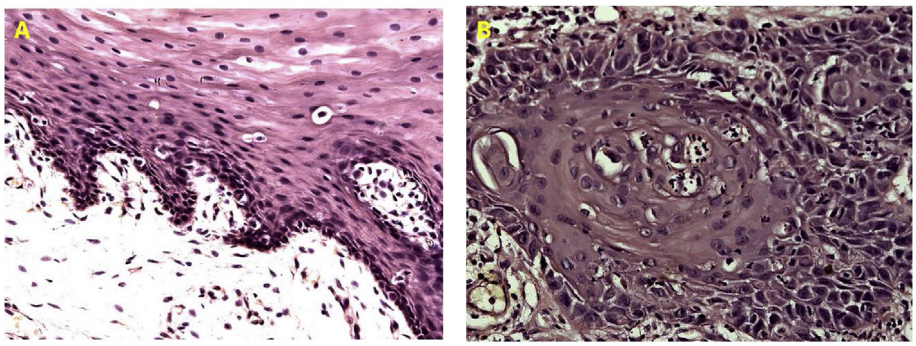

Fig. 1. Microphotographs (200×) of H and E staining (A) Normal Oral Mucosa (NOM). (B) Oral Squamous cell Carcinoma (OSCC).

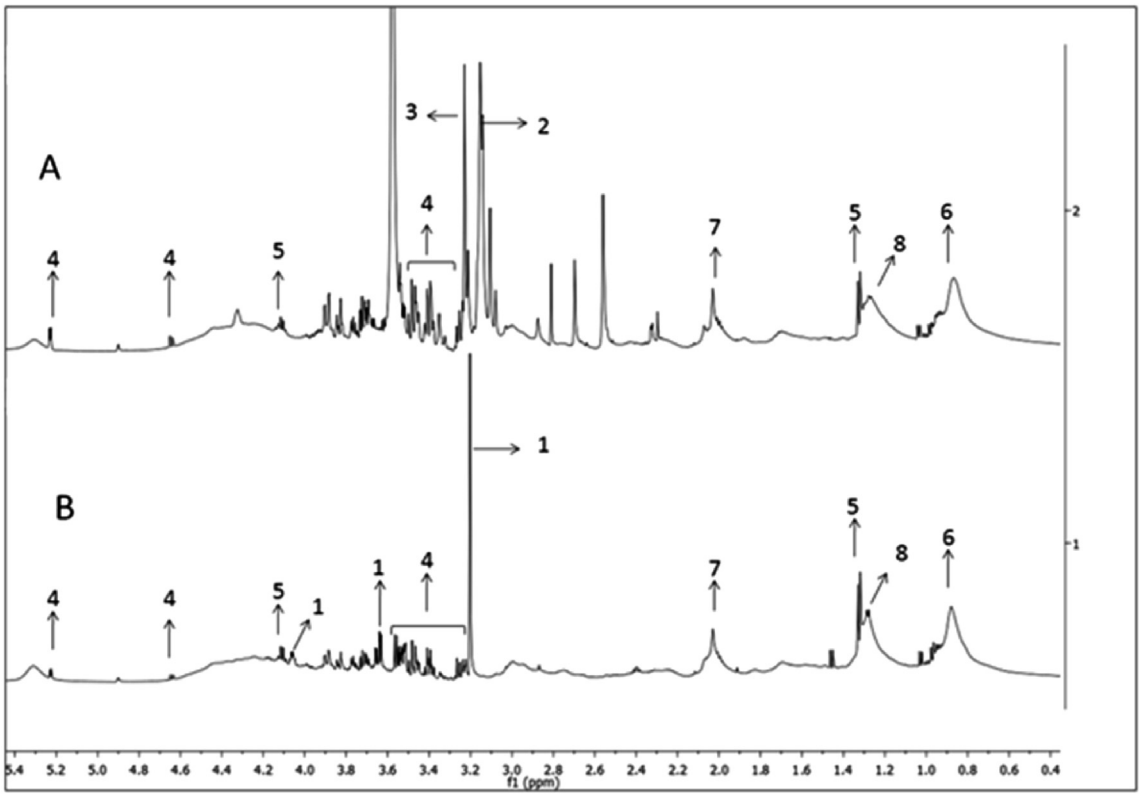

Fig. 2. Stacked ^{1}H NMR spectra of A. Cancer sample; B. Normal sample. 1. Choline; 2. Malonic acid; 3. Trimethylamine N-oxide; 4. D-glucose; 5. Lactate; 6. Saturated fatty acids; 7. Acetate; 8. Threonine.

study demonstrated the deregulated metabolomic status in OSCC using both ^{1}H and ^{13}C NMR (Figs. 2 and 3; Table 1).

Amongst different metabolic attributes in cancer pathobiology, choline metabolism significantly altered in varied epithelial malignancies (e.g. breast, prostate) [5]. In epithelial malignancies there are evidences of abnormal choline metabolism in both anabolic and catabolic pathways that lead to increase amounts of choline-containing precursors or choline break-down products [5]. Choline precursors and breakdown products containing quaternary ammonium core can be easily detected using non-invasive magnetic resonance spectroscopy (MRS). Therefore, they have been used as biomarkers to detect the cancer progression [5]. Besides, the features of choline and its derivatives contribute to the essential components of lipoproteins, blood, membrane lipids and as precursor of the neurotransmitter acetylcholine [16]. Generally, choline and betaine are metabolically related quaternary ammonium compounds [17]. They are metabolically linked to both lipid and folate-dependent one-carbon metabolism, and studies in animals and humans have provided results suggesting their involvement in neurodevelopment and the pathogenesis of various chronic diseases and points to a role in risk assessment and disease prevention [17].

In this context, up-regulation of choline break-down product like sarcosine was reported in prostate cancer [7]. Interestingly for the first time, present study elucidates the elevated level of another choline break-down product, timethylamine N-oxide, (Table 1, Figs. 2, S1, 3 and S2) in serum of OSCC patients by corroborating with other relevant work in cancer metabolomics [5]. Crosstalk between the oncogenic pathway [such as COX2, ERBB2, RAS, MAOK, HIF1] and choline metabolism is deregulated in malignant cells [4,5] due to increase demand of choline in rapidly proliferative cancerous cells. Here it may be noted that, choline metabolism is

Table 1
Chemical shifts of major metabolites identified through comparison with HMDB database values.

Metabolites	^{1}H NMR chemical shifts (ppm)		^{13}C NMR chemical shifts (ppm)		Altered status in OSCC
	Found	HMDB database	Found	HMDB database	
Choline	3.20 (s)	3.20 (s)	56.2	56.5	Down regulated
	3.63 (m)	3.51 (m)	62.5	58.6	
	4.05 (m)	4.06 (m)	72.6	70.1	
Trimethylamine N-oxide	3.23 (s)	3.25 (s)	61.7	62.3	Up regulated
Malonic acid	3.14 (s)	3.11 (s)	51.6	50.3	Up regulated
			180.2	180.0	
Lactate	1.32 (d)	1.32 (d)	70.3	71.8	Unchanged
	4.11 (q)	4.10 (q)			
D-glucose	3.20–3.5(m)	3.20–3.55 (m)	96.5	98.6	Unchanged
	4.64 (d)	4.63 (d)	92.6	94.8	
	5.23 (d)	5.22 (d)	76.5	78.6	
			76.3	78.5	

Spectra in HMDB http://www.hmdb.ca/ databases are recorded at 25 °C, pH = 7. But the sample pH is 6.2 and spectra are recorded at 30 °C. Hence, small shifts in ppm value were observed. s = singlet, d = doublet, q = quadrate, m = multiplet.

associated with deregulated membrane phosphlipid homeostasis during malignant transformation [18]. In this regard, it is also reported that oncogenic RAS pathway induces phosphatidylcholine-specific phospholipase D [PC-PLD] enzyme which increases the synthesis of choline [5]. However, our study demonstrated the down-regulation of choline expression (Figs. S1 and S2) in serum of OSCC patients compared to normal counter-part. It logically supports the notion that more utilization of choline by cancerous cell in OSCC generates more break-down product [TMAO] of it. However, intracellular enzymatic conversion of choline to TMAO is yet to be explored. It will be interesting study to find participating enzymes and use them for therapeutic purpose.

Interestingly, from our study it has also been revealed that unlike most of the cancers [1], 'Warburg effect' linked to lactic acidosis has no significant contribution in case of OSCC as there is no significant change in intensities of lactate profile in cancer sample compared to normal (Table 1, Figs. 2 and 3). It should be mentioned that dual metabolic nature of cancer cells and reverse Warburg effect have been reported recently by Xie et al., 2014 [19]. Besides, we have observed increased amount of malonate in cancer sample as compared to normal sample (Table 1, Figs. 2, S1, 3, S2). It may be mentioned that malonate can be produced directly from pyruvate (glycolysis product) through acetate [20]. The elevated level of malonate is understandable as malonate is a crucial component for fatty acid synthesis and related membrane biogenesis necessary for highly proliferating cells in OSCC [21]. Thus, in addition to abnormal choline metabolism, we have also found abnormal glucose metabolism in oral cancer cell compared to both normal cell and general cancer cell (Fig. 4).

Hence, present study has demonstrated abnormal choline metabolism in systemic circulation of oral cancer. At the same time the Warburg effect (up-regulated lactate production), the hallmark

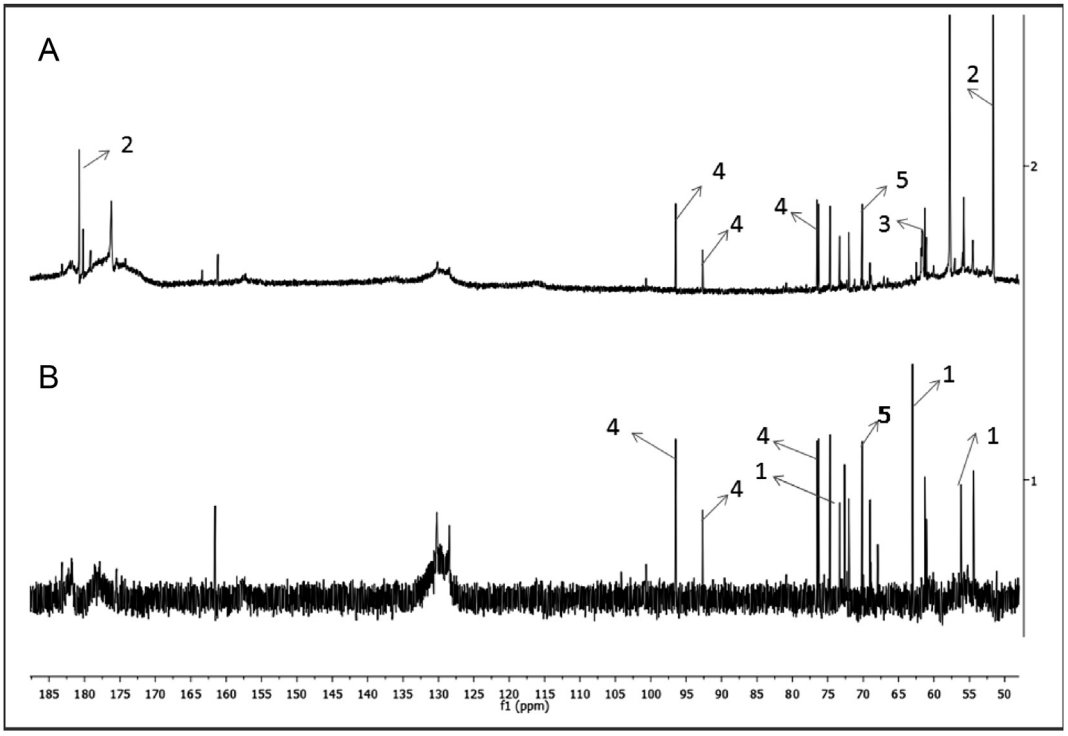

Fig. 3. Stacked ^{13}C NMR spectra of A. Cancer sample; B. Normal sample; 1. Choline; 2. Malonic acid; 3. Trimethylamine N-oxide; 4. D-glucose; 5. Lactate.

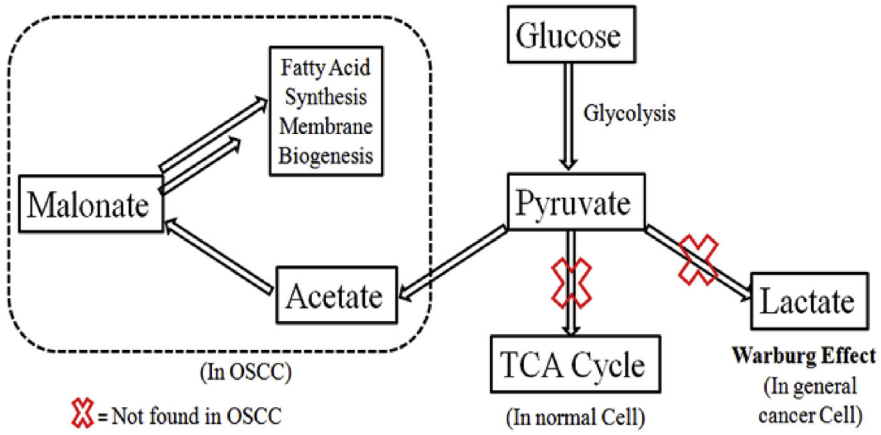

Fig. 4. Proposed abnormal glucose metabolism in OSCC compared to both normal cell and general cancer cell.

of most cancers, is absent in oral squamous cell carcinoma. Trimethylamine N-oxide, the choline breakdown product and malonate could be important biomarker in oral cancer. This study will also help the future researchers to understand the malignant micro-ambience as well as to identify up-regulation of key enzymes in the light of altered metabolomic conditions.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.02.149.

Transparency document

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