Review Paper

Oral Squamous Cell Carcinoma: Insights with metabonomics

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Abstract: Metabonomics has shown substantial potential as a diagnostic technique in cancer research including cancer recurrence. The minimal acquisition time and maximum information gained justifies its impact in identifying novel cancer biomarkers and developing cancer therapeutics. We discuss the potential application of NMR based metabonomics for biomarker identification in different biofluids and tissue biopsies from patients suffering from oral squamous cell carcinoma. In this review, we broadly emphasise in vitro, ex vivo, in vivo NMR spectroscopy and MRI as diagnostic and prognostic methods.

Introduction

Cancer is a major cause of mortality and morbidity with approximately 6 million deaths each year worldwide and squamous cell carcinoma of head and neck (HNSCC) is fifth most common cancer in the world [1]. It is commonly prevalent in males and is more prevalent in developing countries as compared to the developed ones. The incidence rate varies in men from 1 to 10 cases per hundred thousand populations in many countries. According to the World Health Organization report, cancer of oral cavity ranks among three most common types in South Central Asia [2]. Squamous cells of epithelium constitute a superficial layer in different parts of body like skin, lungs and oral cavity. Thus, these cells are more exposed to the environmental stresses including those from carcinogens (X-ray, smoke, dyes etc). This exposure to carcinogens along with other risk factors like tobacco chewing, human papilloma virus and alcohol consumption causes genetic abnormalities in cells which result into uncontrolled proliferation of the cells [3, 4]. These cells evade the immuno-surveillance of body and produce angiogenic factors resulting in clinical carcinomas [5]. Lips, buccal mucosa, upper and lower alveolar ridges, floor of the mouth, anterior two-thirds of tongue, retromolar trigone with hard palate are seven sub-sites of oral cavity for carcinoma [6], which are vastly originated from squamous cells.
The five-year relative survival rate of cancer of oral cavity and pharynx in US, by stage, at the time of diagnosis is represented in Fig.1. Even aggressive combinations of surgery, radiotherapy and chemotherapy, could not much improve the five-year survival rates for all stages combined [5]. Patients with high-stage tumors of larger size and higher metastatic potential have only a 30% chance of survival at five years and patients with low-stage cancer have a higher probability of survival [7]. This poor survival rate is probably due to the development of multiple primary tumors and advanced extent of disease at the time of diagnosis [8]. The problem of late presentation of disease could be overcome by the development and use of diagnostic aids that could readily assess the oral lesions. The higher incidence and poor survival rate attracted numerous radiological investigations like PET, CT and MRI [9, 10] for detection and diagnosis of oral SCC but final confirmation comes from histopathology of tumor tissues, still the gold standard technique. Whereas, PET/CT and MRI contribute towards presurgical assessment of the aggressiveness of tumor and presence of mandibular invasion, other ‘-omics’ techniques like proteomics, genomics and metabonomics play a crucial role in identifying biomarkers, understanding the underlying molecular mechanisms and how the alterations in metabolic pathways effect the tumor development and progression. As the former aspect affects the surgical strategies and removes the very cause of discomfort in patients, the other facet improves the post-surgical management of patients and thereby, heralding increased survival and decreased recurrence rates.

Various proteins and gene-expression studies have attempted to explore the presence of disease and its stage in patients suffering with head and neck squamous cell carcinoma. Reportedly, fibroblast growth factor family members [11, 12], p53 over-expression [13], VEGF (Vascular-endothelial Growth Factor) [14] play an important role in induction of angiogenesis and cell proliferation in OSCC. Cytokines including IL-4, IL-6 and IL-8 have also been reported to be expressed in OSCC [15]. High levels of nitric oxide synthase have been found in solid tumors, which reportedly play an important role in angiogenesis and neo-vascularization in OSCC [16]. Numerous such cellular and molecular markers at genetic and protein level have been identified to probe the complex metabolic processes occurring during the phenomenon of cancer. Major contributions have been made to the field by the genomics, transcriptomics and proteomics which explained the multiple gene expression changes at RNA level and its consequent effect on protein expression which in turn induce changes at small molecule metabolite levels. This 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 \(^1\)H NMR based metabolomics/metabonomics (both the terms are used interchangeably nowadays). The metabolic analyses provide an overview of all the 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 study of all biomarkers is too vast as per the scope of this review. Therefore, some of the protein and genetic biomarkers present in biofluids and tissues with specific emphasis on metabolic biomarkers identified with MRI/MRS and high resolution \(^1\)H NMR spectroscopy for evaluating metabolites biomarker will be discussed with the future perspectives of
Chemometrics

The metabolites are indicators of particular metabolic pathway occurring during normal cell procedures. The measurement of these metabolites, analysis of the extent of deviation and its significance under pathological conditions play an important role in biomarker validation under clinical settings. Since metabolomics involves large sample size, these deviations and their significance levels with the identification of outliers in a population are analysed with chemometric statistical methods. The most important and commonly used approaches of chemometric methods towards metabolomic data are unsupervised principal component analysis (PCA) and supervised orthogonally corrected partial least square discriminant analysis (O-PLSDA) for generation of a robust model to be used for evaluating higher predictability of unknown samples. The PCA is used for reducing the dimensionality of the huge metabolic data to only few correlated metabolites and based upon those metabolites an overview of the data is represented. During PCA, the original data matrix gets reduced to two new matrices corresponding to score plot and loading plot. A PCA graphical plot of principal components (score plots) and its corresponding loadings provide two major informations about the relationship between the two groups (whether similar or dissimilar) and the metabolites involved for their differentiation respectively. For example, Figure 2 shows the score plot between first three principal components with explained variance of 66% between diseased and normal groups of samples. The corresponding loading plot depicts the significant resonances of metabolites responsible for the differentiation among two groups. PCA is performed on a dataset for the detection of any existing trends and patterns among different groups by many variables.

The detection of trends in a data matrix by PCA calls for a model preparation which should robust enough for classification as well as for prediction purposes. The classification among the different groups and prediction of group of an unknown tissue biopsy, based upon the classification model. Such multivariate statistical methods are known as the supervised approaches. The model preparation is based upon the specific prior knowledge (Y-matrix, i.e. diagnosis obtained from gold standard methods) of group membership of samples for maximizing the group separation. The O-PLSDA is one such supervised technique of pattern recognition. The robustness of this supervised model should be tested on unknown samples, validated with the gold standard methods so that it can be routinely used for differentiating malignancy. Other chemometric methods include hierarchical cluster analysis (HCA), fuzzy k-means cluster analysis, artificial neural networks (ANN) and support vector machines (SVM).

High Resolution $^1$H NMR spectroscopy in OSCC:

In vitro studies in biofluids:

Although, changes in enzyme concentrations and activities (‘the proteome’) have a small impact on metabolic fluxes (the rate at which material passes through a given metabolic pathway) but changes in flux have a significant impact on metabolite concentrations. This is because the metabolic flux of a pathway depends upon enzymes present in the pathway [17, 18]. Furthermore, it is not necessary that a reliable quantitative relation exists between mRNA concentrations and enzyme function [17], but as metabolites are end-products of both transcription and translation, they are potentially a better
indicator of enzyme activity. These shortcomings led to the emergence of field of “metabolomics”. The basic idea behind the $^1$H NMR based metabonomics study was to identify those metabolites which get deranged during pathology or due to some toxic insult for their quantitative measurement [19]. The extension of technique to in vitro, ex vivo and in vivo conditions is unique which allows unraveling the complexity of metabolic information present in pathological conditions. The in vivo and ex vivo $^1$H NMR studies present a wide range of metabolites in a single-go which in turn provide information about the cellular metabolism. $^1$H NMR spectroscopy plays a considerable role in various studies of toxic effects [17], cancer [20] and inborn error of metabolism [21-23]. The in vitro NMR analyses of bio-fluids and tissue extracts provide tissue biochemistry and also help in analyzing the cancer-related biomarkers present in tissues.

The in vitro studies of bio-fluids provide a bigger picture for small molecule composition during patho-physiological diseased state by reflecting changes in metabolism. The study of these bio-fluids fulfills the need of a diagnostic medium which is inexpensive, non-invasive and readily accessible. Among saliva, blood serum/plasma and urine, saliva ranks first for biomarker discovery for being in closest proximity with tumor and being totally non-invasive in nature. The closer proximity increases the probability of observing the changes in normal saliva composition with respect to pathology. This resulted in numerous studies involving proteomics [24], transcriptomics [25] and genomics and successfully defined the variations in metabolome of saliva. A study on circulatory epithelial tumor markers of saliva: CA125, TPS, Cyfra 21-1, CA19-9, CEA, SCC showed that these markers are found to increase from 1.4 to 4.2 times in OSCC patients [26]. About 3000 different human mRNA have been identified in saliva of normal human being [25] which reinforces the candidature of saliva for monitoring human health. Coming downstream towards the endogenous metabolites, a UPLC-QTOF-MS analysis was conducted for identification of salivary metabolic signatures in OSCC and oral Leukoplakia (OLK). Oral leukoplakia is considered to be a pre-cancerous malignant lesion with a malignant transformation rate of 1.58-27.27% [27, 28]. It showed variations in forty-one different small metabolites among control and OSCC patients and sixty-one metabolites varied among OSCC and leukoplakia patients. The most discriminatory metabolites among three groups were gamma aminobutyric acid, phenylalanine, valine, n-eicosanoic acid and lactic acid [24]. Such studies bridge the gap between altered biochemistry and progression of malignant transformation. A urinary metabonomics study had been performed to completely separate patients with OSCC from the OLK and healthy controls and to obtain data-rich information for oral cancer diagnoses. The study was planned for identifying urinary metabolites, by GC-MS, as potential biomarkers to diagnose and stratify OSCC and precancerous lesions. These biomarkers can be used as a complementary diagnosis method by using saliva and blood [29]. The minimal sample pre-processing in NMR studies make it more appropriate technique for biomarker identification but such investigations are yet to come for urinary and salivary signatures in oral squamous cell carcinoma.

Blood serum is equally non-invasive but a bit-distant bio-fluid as compared to saliva. However, malignant tumors are characterized by the growth of new blood vessels from the existing ones (angiogenesis), therefore, serum/plasma may also help in unscrambling the biochemical processes and related biomarkers. The presence of tumor
markers in serum indicated towards the alterations in total metabolic pool of the body, which may be easily probed-in with the help of different bio-markers. A pilot study with the objective of detecting biomarkers in early stage tumors (size < 2cm; 0.005% of body mass) was performed on serum samples of patients suffering with oral squamous cell carcinoma. The alteration in concentration of glucose, lactate, valine, alanine, ethanol, acetate, phenylalanine, tyrosine, methanol, formaldehyde, formic acid, pyruvate and other small metabolites indicated towards the metabolic perturbations occurring during the pathological state and its different stages. These metabolites are involved in different pathways and the perturbations in its concentration indirectly indicate the rate of corresponding pathway. The further evaluation of blood serum metabolite profile of benign pathologies like leukoplakia (a pre-cancerous lesion) with respect to OSCC will provide an insight of the physiological disturbances occurring during the course of progression of malignancy and will provide a more robust screening technique too. However, care must be taken while analyzing bio-fluid for the tumor of size less than 0.005% of body weight as the metabolic perturbations might mirror other pathologies as well and also mask the effect of actual alterations. Therefore, analysis of body fluid for identifying tumor metabolism should be done with caution and screening tests for confirming the proper functioning of other organs must also be considered to rule out variations occurring due to other pathologies.

The alterations in these metabolites represent the perturbations in the metabolic pathways occurring in the body for maintaining the homeostasis. Decreased levels of lactate and alanine in serum indirectly indicate the rapid glycolysis in tumor cells. Lactate is a marker for malignancy and in most of the cancers, it is found to be increased when compared with healthy controls as the Warburg effect (aerobic glycolysis) dominates during malignancy. Lactate accumulation indicates the high glycolytic rates and reduced mitochondrial oxidation which favors the cell-survival in hypoxic micro-environment of tumor[30]. However, its decreasing concentration in serum samples of oral cancer patients requires more attention as the situation is vice-versa in other cancers. Decreased concentration of alanine, which is another end-product of glycolysis, is another source of tumor proliferation and growth[31].

Glucose is an integral part of energy metabolism of cancer tissues as it acts as a substrate for glycolysis which is responsible for ATP (adenosine triphosphate) production. The higher glucose concentration in serum not only indicates towards the higher glycolytic rates but also towards increased energy production for cellular proliferation. In HNSCC, GLUT1- and GLUT3-mediated glucose transporters facilitate glucose entry into the cell which participates in increased glucose metabolism [32]. As suggested by Stefano Tiziani et al. that the higher levels of glucose in serum may be linked to the unique behavior of oral cancer, which interferes with the ability of insulin to modulate the uptake of glucose thus regulates consequent energy metabolism favoring the accumulation of carbohydrates and the process of ketogenesis [33]. The increase in acetoacetate and acetate marks the enhanced process of lipolysis.

Choline and choline containing compounds (glycerophosphocholine and phosphatidylcholine) increase during the pathological condition due to increased phospholipid metabolism which aids in cell proliferation processes during malignant transformations. These malignant transformations involve choline
kinase mediated phosphorylation into PCho, following the Kennedy pathway, which further gets converted into PtdCho [34]. The activation of enzyme choline kinase has been recently found to play a relevant role in the regulation of cell proliferation, oncogenic transformation and human carcinogenesis [35]. Also, the activity of enzymes phosphatidylincholine-specific phospholipase C (PLC) and phospholipase D (PLD) is responsible for the degradation of phosphatidylincholine and was higher in human mammary epithelial and epithelial ovarian carcinoma cell lines [36]. These findings suggest that both biosynthetic and catabolic pathways of the phosphatidylincholine cycle contribute to the accumulation of Choline containing compounds in tumor tissues. Due to a strong association of these phospholipases (choline kinase, PLC, PLD) in malignant transformation of most of the human cancers, these enzymes may provide molecular targets for anticancer chemotherapies[34]. The increase in phosphatidylincholine and total choline content are currently interpreted as biomarkers for tumor progression. The metabolism of these small metabolites is unique in saliva, serum and urine which is differentially affected by the pathophysiological stimulus which has been briefly described in Table 1, based on the literature available.

**In vivo and ex vivo studies of tissue biopsies:**

Whereas the bio-fluids play an important role for screening of disease, the tumor tissues themselves contains a plethora of information regarding its nature, aggressiveness and the extent of penetration. The *in vitro* NMR analyses of tissue extracts provide an insight in tissue biochemistry and also help in analyzing the cancer-related biomarkers present in tissues. The perchloric acid method of extraction from tissues extracts all the water soluble metabolites like lactate, alanine, choline, glycerophosphocholine and other small metabolites, while the lipid extracts give an idea about the lipid composition of the tissues. However, tissue extraction procedures on cancer biopsies provided an insight of its biochemical processes, but at the cost of tissue destruction and modifications in composition of actual metabolite pool and also consume more time. Keeping these limitations in mind earlier studies on oral OSCC was designed on whole tissues and not on their extractions. Such studies have shown the utility of *ex vivo* MRS in identifying tumor biomarkers and explored tissue biochemistry in pathological conditions. An *ex vivo* study conducted by Mukherji et al had shown that total choline/creatine (Cho/Cr) peak intensity ratio can be used to differentiate OSCC of upper aerodigestive tract from surrounded uninvolved muscle [39]. Another study on OSCC has demonstrated the diagnostic potential of *ex vivo* proton MRS, wherein higher total Cho/Cr ratio along with taurine, glutamic acid and lipids of histopathologically confirmed malignant tissue were observed as compared to normal tissue [40]. The total choline signal (3.21-3.24 ppm), being broad in nature, includes the signals arising due to phosphocholine and glycerophosphocholine and more often the effect of malignancy on these particular metabolites along with other small metabolites like alanine, lactate etc. could not be clearly ascertained. This limitation of MR spectroscopy was overcome by a technique HR-MAS NMR spectroscopy, introduced independently by Andrew et al. [41] and Lowe [42]. 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. Rapid spinning of the sample, speed (in kHz) depending upon the field strength, at an angle of 54.7° relative to the axis of magnetic field minimizes the line-
broadening effects and so the loss of resolution and information contained in a tissue. Earlier applications of HR-MAS NMR were confined to the solid-state investigations [43] but soon after the development of MAS probes with a deuterium channel for lock signal detection, the HR-MAS NMR based metabonomic studies came into realization. Since then a number of research articles and reviews were published regarding the metabolite identification and differentiation of benign and malignant human tissue specimens [44, 45] by $^1$H NMR HR-MAS spectroscopy. It has already demonstrated real insights into the mechanisms of toxicity and pathology at molecular level [46-49]. The technique greatly enhances the spectral resolution and provides relevant biochemical information regarding small metabolites present in tissues, which can be mapped and 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. A recently published study demonstrates that histopathologic and genetic microarray analysis can be successfully performed on prostate surgical and biopsy tissues following HR-MAS analysis but biopsies become more fragile than surgical tissues [50]. Considering these applications of MRS in disease diagnosis, the role of HR-MAS technique has been evaluated in various cancers.

At this point, the utility of $^1$H HR-MAS MR spectroscopy in disease diagnosis seemed to be quite convincing in terms of information it provides. In case of malignant tumors, sampling must be done at different points, including the tumor itself and the peri-tumoral region which consisted of adjacent ‘margins’ of tumor and area underlying tumor or tumor ‘bed’ left after tumor resection. Multiple sampling not only ensured the minimum probability of leaving any cancerous cell but also defined the extent of disease penetration. The margins and bed biopsies, in some cases, include few histologically proven malignant cells.

The sample preparation, however, does not require any pre-processing procedures like extraction processes for its analysis but tissue handling under different conditions leads to different results. Thus, metabolic stability of tissue while performing an experiment is of foremost concern in pathological studies. Earlier studies have shown the effect of tissue sample size, temperature, storage [51] and sample spinning [52] on the data acquired. These studies suggested that symmetrical spherical insert in a 4-mm rotor improves the resolution of spectrum by narrowing the line-widths and improving the peak heights, even with the lesser amount of tissue. This is due to more homogeneity in the sample and so it indicates that biopsies weighing as low as 10mg can also provide the spectral information with improved sensitivity and resolution.

A study on human lung cancer tissues [53] showed that even at low temperature (277K), glycerophosphocholine and phosphocholine conversion to choline, and amino acids variations were observable after 2 hrs of acquisition. In another study on renal cortex of rats, where the samples were stored on ice for 4 hrs, similar results were obtained along with decrease in the triglyceride and trimethylamine-N-oxide resonances. On the contrary such changes were not observed in case of liver specimens, even after 5 hrs of excision [51]. This shows that different tissue specimens have different rates of enzymatic reactions of different kinds. Temperature is a crucial physiological parameter that might affect the tissue stability by effecting its enzymatic reactions. Hence, temperature must be below the optimum range of enzymatic
reactions so that the metabolite degradation could be minimized. Therefore, it is recommended to perform experiments at a probe head temperature of ≤10°C, which can be achieved on most spectrometers using cooled nitrogen gas. In oral cancer tissue biopsies, no significant differences were observed in the spectral metabolite pattern even after subjecting the tissue specimens for approximately one hour long NMR measurements, at 283K [38]. Earlier HR-MAS NMR study has been performed on tissues obtained from patients suffering with oral SCC in order to identify the metabolic perturbations of malignant tumor from non-malignant bed and margins tissue specimens, which may be helpful in understanding the extent of tumor penetration in the neighbouring tissues [38]. The study was focussed on identifying the biomarkers present in tumor tissues as compared to the healthy neighboring tissues and also statistically defined that total contributions from these metabolites play an important role in segregating malignant tissues from the benign ones. Another extension of the same $^1$H HR-MAS NMR study was performed on patients suffering with head and neck cancer and lymph node metastasis. They demonstrated that multiple metabolic alterations occur in tumor tissues which include highly active glycolysis, increased influx of amino acids (anaplerosis) into the tricarboxylic acid cycle, membrane choline phospholipid metabolism, oxidative and osmotic stress mechanisms. Moreover, decreased levels of TG may indicate lipolysis of TG and concomitant β-oxidation of fatty acids which may exist to deliver bioenergy for rapid tumor cells proliferation and growth [32]. An interesting extension of ex vivo HR-MAS NMR spectroscopy based metabolic phenotyping has been proposed for monitoring surgical patients. This will provide phenotypic information about the tumor status of patient and provide real time biomarker information for surgeons for assessment of the extent of tumor penetration and according resection [54].

The ex vivo studies seemed to have a wide potential in disease diagnosis but in vivo studies being totally non-invasive play a fundamental role evaluating patient suffering from cancer or a neoplasm. MRI may assist in the staging processes by detecting local extension of disease which is not apparent during a physical examination. Imaging biomarkers e.g. Apparent Diffusion Coefficient (ADC) play an important role in detection, characterization and monitoring the response to therapy. In-vivo DCE-MRI (Dynamic Contrast-Enhanced Magnetic Resonance Imaging) using Gd-DTPA (Gadolinium Diethylene Triamine Pentaaacetic acid) has recently been utilized for the pre-operative assessment of mandibular invasion in SCC (adjacent- or fixed to the mandible), which can discriminate SCC-with-medullary-invasion from SCC-without-medullary-invasion [55]. Not only the extent of local penetration of disease but the nodal metastasis can also be detected simultaneously along with primary tumors by imaging techniques. A study on bone marrow invasion of the mandible or maxilla in patients with oral cavity squamous cell carcinoma (OCSCC) involving PET/CT and MRI showed that MRI has higher specificity than PET/CT (97% vs 78%) [56]. Recently, the current understanding of diffusion weighted (DW) MRI and its far-reaching implications in cancer research make it a cancer imaging biomarker. DW MRI depends on the microscopic mobility (Brownian motion) of water which is due to the thermal agitation and is highly influenced by the cellular environment of water. The measurement of the extent of diffusion of molecules in a human body is called as apparent diffusion coefficient (ADC). The contrast obtained in a diffusion weighted image is due to the variable diffusion rates of the water in the tissues, depending upon
their micro-environment. The DW MRI was a good challenge due to the susceptibility artifacts, until 2001. Jichen Wang et al. published their work on characterization of cancerous and benign lesions of head and neck. They reported that mean ADC value of malignant lymphomas is significantly lower than carcinomas which is lower than mean ADC values of benign solid tumors. ADC values were used to predict malignancy with 86% accuracy, 84% sensitivity and 95% specificity achieved [57]. Recent studies on DW MRI have shown high accuracy in differentiating between persistent or recurrent head and neck SCC from non-tumoral post radiotherapeutic alterations. ADC values were significantly lower for cancerous tissues that for non-tumoral tissues (p< 0.0001), resulting in 94.6% sensitivity, 95.9% specificity and 95.5% accuracy [58]. DWI is a feasible clinical technique, improving the assessment of metastatic spread in routine magnetic resonance imaging examinations.

However, MRI provides an excellent soft tissue contrast but still its sensitivity and specificity is limited due to its limitation in identifying tumor from edema and secondly due to the existence of Gd-enhanced necrosis commonly mistaken as tumor. These limitations indicate a more non-invasive imaging technique with an additional asset of metabolite profiling. MRS seems to be one such promising non-invasive imaging technique for evaluation of metabolic information arising from normal and abnormal tissue morphologies. Numerous single voxel studies [59], [20, 60-62] involving both proton and phosphorous MRS have shown its potential role in evaluation and management of patients suffering with head and neck cancer. Whereas, proton MRS majorly evaluates the total choline content for assessing the membrane turnover during the malignant progression and lactate concentration, which is a marker for anaerobic glycolysis, phosphorus MRS concentrates on bioenergetics of the tumor. Moreover, recent advances in post-processing techniques of MRS data have led to improved water and lipid suppression and thereby in better results. Single voxel MRS provides metabolic information present in a tumor but does not provide any information regarding spatial heterogeneity and therefore, the spatial penetration of a lesion cannot be defined [63]. Keeping these aspects in mind, multi-voxel CSI may provide metabolic information about the tumor heterogeneity and its extent of penetration simultaneously. Nevertheless, the studies on brain tumor and prostate cancer [64] have shown its utility in disease diagnosis. In case of head and neck cancers, multi-voxel CSI studies impose a challenge during shimming, due to large susceptibility differences between air and tissues, as poor shimming means spectral peak broadening, frequency shifts and poor signal-to-noise ratio [59]. Thus reduction in susceptibility differences will result in better shimming and thereby, in better spectra. This limitation could be overcome by surrounding the organ of interest with a layer of susceptibility matched material, Perfluorocarbon-inflated probe (PFC) as suggested by a study on prostate, which effectively reduces field inhomogeneity effects and produce better quality images [65]. Recently, a study on CSI of head and neck at 3 Tesla MRI scanner has proposed an optimum shimming technique and a simple anti-susceptibility device PFC (perfluorocarbon) for reduced susceptibility differences and better spectral quality of head and neck region. Application of second-order shimming strategy and PFC pads around the neck resulted in success rate of 70% [59]. This study opened a possibility for further studies on primary tumors and cancer of head and neck for tumor penetration, its aggressiveness and metastasis.
Conclusion:

The increased understanding about different metabolic perturbations during a malignant transformation not only provide an overview of underlying biochemistry but also indicate an opportunity for development of appropriate therapeutic agents. The detected biomarkers by various spectroscopic techniques integrated with gene expression and biochemical analyses of cancer cells may help in monitoring the effectiveness of the treatment and post-surgical management of the patients. The studies can further be extended to the peri-neural invasion (PNI: neoplastic invasion of nerves) during malignant progression, as it is still suggested to be the main cause behind recurrence, metastasis and poor survival rate. An extensive sampling of tissues pertaining to nerve, vessels and arteries for evaluating peri-neural invasions using HR-MAS NMR [66] study as well as real time HR-MAS MAS analyses during surgery is required Fig.3. As the nerves involvement in tumors may act as a channel for tumor progression to other parts of the body including the brain. Since the PNI status greatly affects the surgical planning and adjuvant therapies to the patients for improved survival and better quality of life, newer and robust biomarkers are required for its identification. MRI is considered to be a superior imaging modality, with reported sensitivity upto 95%, for diagnosing peri-neural spread because of better soft tissue contrast. The future studies with the proper use of imaging and spectroscopic techniques on this aspect of cancer of oral cavity may provide an edifice for better therapeutic developments.

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Fig. 1: 5-year survival rate (in %) of cancer of oral cavity and pharynx by stage of diagnosis as by courtesy of US National Cancer Institute, SEERS (Surveillance Epidemiology And End Results) from 2001-2007.
Figure 2: The NMR spectroscopy based PCA score plot between two groups of tissue biopsies (Voilet squares= Diseased and Green circles= Normal tissue). The Loading plots indicate the resonances responsible for grouping of two groups.

Table 1: Metabolic signatures [7, 32, 37, 38] of oral squamous cell carcinoma present in different biofluids:

<table>
<thead>
<tr>
<th>S. no.</th>
<th>Metabolite</th>
<th>Biofluid</th>
<th>Metabolism</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Lactate</td>
<td>Serum, Saliva</td>
<td>Hypoxic condition, altered glycolysis</td>
</tr>
<tr>
<td>2</td>
<td>Alanine</td>
<td>Serum, Urine</td>
<td>Reduced pyruvate oxidation, altered glycolysis</td>
</tr>
<tr>
<td>3</td>
<td>GABA</td>
<td>Saliva</td>
<td>altered glutamate oxidation, signaling pathways</td>
</tr>
<tr>
<td>4</td>
<td>PA</td>
<td>Saliva, Serum, Urine</td>
<td>Enters in TCA cycle through fumarate, fatty acid synthesis</td>
</tr>
<tr>
<td>5</td>
<td>Tyrosine</td>
<td>Serum</td>
<td>Kreb’s cycle affected</td>
</tr>
<tr>
<td>6</td>
<td>Citrate</td>
<td>Serum</td>
<td>Lipolysis</td>
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<tr>
<td>7</td>
<td>Acetoacetate</td>
<td>Serum</td>
<td>Lipolysis</td>
</tr>
<tr>
<td>8</td>
<td>Acetone</td>
<td>Serum</td>
<td>Altered glycolytic rates, interferes with the ability of insulin, regulates consequential energy metabolism favouring the accumulation of carbohydrates.</td>
</tr>
<tr>
<td>9</td>
<td>Glucose</td>
<td>Serum</td>
<td>Phospholipid metabolism, cellular proliferation</td>
</tr>
<tr>
<td>10</td>
<td>Choline, choline containing compounds</td>
<td>Serum</td>
<td>Phospholipid metabolism, cellular proliferation</td>
</tr>
<tr>
<td>11</td>
<td>Acetate</td>
<td>Serum</td>
<td>Lipolysis</td>
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<tr>
<td>12</td>
<td>Formate</td>
<td>Serum</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>Formaldehyde</td>
<td>Serum</td>
<td>Protein biosynthesis</td>
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<td>14</td>
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<td>15</td>
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<td></td>
<td>Isoleucine</td>
<td>Leucine</td>
<td>Glycine</td>
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<tr>
<td>16</td>
<td>Serum</td>
<td>Serum, Urine</td>
<td>Serum</td>
</tr>
</tbody>
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Figure 3: The overall representation of the spectroscopic and imaging modalities for identifying metabolic biomarkers in malignant and benign tissue biopsies along with suspected neighboring nerves, arteries and veins and their correlation with histopathological studies. The encircled area in MRI images represents the location of sampling while surgical procedures in patients. The study is planned for identifying the status of perineural invasion in oral SCC patients.

References


