Exploring photoacoustic spectroscopy-based machine learning together with metabolomics to assess breast tumor progression in a xenograft model ex vivo

Jackson Rodrigues1 · Ashwini Amin2 · Chandavalli Ramappa Raghushaker1 · Subhash Chandra1 · Manjunath B. Joshi3 · Keerthana Prasad4 · Sharada Rai5 · Subramanya G. Nayak2 · Satadru Ray6 · Krishna Kishore Mahato1

Received: 9 October 2020 / Revised: 6 March 2021 / Accepted: 6 March 2021 / Published online: 19 April 2021
© The Author(s) 2021. This article is published with open access

Abstract
In the current study, a breast tumor xenograft was established in athymic nude mice by subcutaneous injection of the MCF-7 cell line and assessed the tumor progression by photoacoustic spectroscopy combined with machine learning tools. The advancement of breast tumors in nude mice was validated by tumor volume kinetics and histopathology and corresponding image analysis by TissueQuant software compared to controls. The ex vivo tumors in progressive conditions belonging to time points, day 5th, 10th, 15th & 20th, were excited with 281 nm pulsed laser light and recorded the corresponding photoacoustic spectra in time domain. The spectra were then pre-processed, augmented for a 10-fold increase in the data strength, and subjected to wavelet packet transformation for feature extraction and selection using MATLAB software. In the present study, the top 10 features from all the time point groups under study were selected based on their prediction ranking values using the mRMR algorithm. The chosen features of all the time-point groups were then subjected to multi-class Support Vector Machine (SVM) algorithms for learning and classifying into respective time point groups under study. The analysis demonstrated accuracy values of 95.2%, 99.5%, and 80.3% with SVM- Radial Basis Function (SVM-RBF), SVM-Polynomial & SVM-Linear, respectively. The serum metabolomic levels during tumor progression complemented photoacoustic patterns of tumor progression, depicting breast cancer pathophysiology.

Introduction
The most common malignancy among women worldwide is breast cancer. According to GLOBOCAN 2018, breast cancer accounts for ~2.1 million new cases and 0.6 million deaths in women worldwide due to breast cancer [1]. Breast cancer in an advanced stage with distant metastases is almost incurable with the existing therapeutic methods. Therefore, detecting breast cancer before time is the best possible treatment planning option, thereby eliminating the disease’s apparent consequence [2]. Histopathology is currently considered the gold standard for diagnosing various cancers, including breast cancer, by assessing structural changes in tissues [3]. Various researchers worldwide have made continuous attempts to
find appropriate methods for diagnosing breast cancer, and many of the techniques currently being used are extensively being tested. Mammography, which uses ionizing radiation, demonstrates low sensitivity and specificity upon the increase of tissue density. Ultrasound being a low-resolution technique, sometimes cannot confirm malignancy without a biopsy. Magnetic Resonance Imaging (MRI), because of its restricted specificity, often gives false-positive results and is not suitable for in situ carcinoma. The use of Computed tomography (CT) has radiation risks, expensive as well as time-consuming. In the case of Positron Emission Tomography - Computed Tomography (PET/CT), skilled personnel to handle radioactive tracer injection is required and is not suitable for young women [4–6]. Microwave biosensor-based methods for early-stage breast cancer detection have shown the potentiality of Microwave Imaging (MI) as an alternative or additional tool to mammography. However, there are quite a few limitations to the practical application of this technique, primarily being computationally expensive. Other constraints, like penetration of microwaves in breast tissues, are dielectric property dependent, vary with frequency and temperatures. Also, a significant attenuation of the electromagnetic waves within the tissues with higher conductivity, limitation in spatial resolution, phase distortion inside and around the biological objects, etc. are other drawbacks of MI. Therefore, developing a high-sensitive and quick diagnostic technique for early-stage breast cancer is crucial [7, 8].

There are reports of using optical techniques in diagnosing malignant breast tissues [9, 10]. Optical techniques like fluorescence imaging and fluorescence lifetime imaging provide a non-destructive probing of tissue structures and composition, making them safe for intraoperative use [11]. Photoacoustics, on the other hand, is an emergent imaging modality in the biomedical field and has demonstrated its application in a variety of tissue types involving pre-clinical and clinical models [12]. The technique has been tested preclinically and clinically for the imaging of the brain, thyroid, breast as well as for dermatologic imaging [13–16]. Intraoperative imaging, Lymphatic system imaging, Gynecological, and urologic imaging, etc. [17]. The technique provides high image contrast and, with the help of endogenous and exogenous contrasting agents, adequate depth of imaging. In recent times, there has been tremendous progress achieved in the instrumentation and imaging applications by the technique [18]. The photoacoustic spectroscopy's ability to detect biochemical alterations upon the onset of breast tumor development has been demonstrated [19]. In combination with machine learning algorithms, photoacoustic spectroscopy has shown tremendous improvement in the classification of abnormal tissues from healthy, signifying its diagnostic capability and possible alternative to currently available tools for disease diagnosis [20].

The experimental data obtained from biological specimens are the multivariate types, which arise mostly from the samples' heterogeneity nature. Various statistical approaches have been used in the past and present to extract meaningful information from such data, uncovering obscure patterns within datasets and revealing the underlying pathology and pathogenesis [21]. Among the tested statistical approaches, Machine learning is gaining more popularity because of its performance and sensitivity to data classification. Machine learning (ML) algorithms involve mathematical models applied to train data sets by adapting their parameters to predict. Machine Learning can identify distinct features from the data to be used for predicting them. The Machine Learning algorithms has broadly been used in the prognosis and monitoring of cancer by extracting the molecular features from cell-free DNA in the blood [22], also used in the classification of melanoma [23] and for detecting lymph node metastases in breast cancers from histological images [24]. However, training a machine learning model for optimal data classification typically requires large training dataset which poses a practical challenge to obtain from biological samples due to non-availability and ethical issues. Data augmentation techniques can be implemented to generate synthetic training data to address this issue of data scarcity. Data augmentation enhances the available limited dataset by transforming existing samples to create new ones [25]. This approach of augmenting data is a familiar methodology in computer-vision domain but has not been fully explored in addressing time-series classification, which is attempted in the present study on time-domain photoacoustic spectra.

For the spectral signals of various sample types, the hidden features can be extracted through spectral transformations using “Fast Fourier Transform” - FFT and wavelet transform – WT tools. Further, since an appropriate spectral feature is needed to classify data through Machine learning accurately, selecting the right spectral transformation tool for feature extraction is the key to data classification. In most real-time signals, including photoacoustic signals, WT demonstrates a better performance than FFT. This is because “FFT” does not significantly extract minor differences in the input signals compared to WT, making WT a better data transformation tool for feature extraction [26, 27]. There are many “wavelet transform” (WT) types, and they decompose signal waves onto a set of essential functions known as wavelets, obtained from a single model wavelet, called mother wavelet [28]. In WT, “Wavelet Packet Decomposition” (WPD) is used to generate both “approximations” and “details” coefficients from the input signals understudy in the low and high-frequency regions, respectively. WPD is an iterative process carried out until the desired levels are achieved. The spectral features thus extracted from various pathological samples can be used for discrimination analysis applying different machine learning
tools, including Support Vector Machine (SVM) analysis [29]. SVM is an effective supervised learning method applied to data classification of different sample types used in high-throughput technologies generating a large volume of data. In the present study, to classify photoacoustic spectral data of tumor progression in the xenograft mice model, SVM learning has been used.

In the present study, upon breast tumor induction using MCF-7 cells injection to athymic nude mice evaluated for tumor progression by photoacoustic spectroscopy- a novel approach combined with machine learning tools. The tumor progression was further validated using histopathological evidence of tumor growth and corresponding quantitative image analysis by TissueQuant software compared to control. The serum metabolites analysis of the control and progressive stages of the tumor was also performed in the study, drawing a correlation of the biochemical changes with corresponding photoacoustic signatures upon tumor progression.

Materials and methods

Tumorigenesis and sample collection

The current study was carried out as per the guidelines and approval of the Institutional Animal Ethics Committee (IAEC), MAHE, Manipal, India. The breast tumor xenograft was developed in BALB/c, athymic nude mice (n = 5) using the MCF-7 cell line. According to established protocol, ~5 million of 75–85% confluent MCF-7 cells suspended in 100 μl of 100% Matrigel were injected subcutaneously on the animals’ flank region for inducing tumor xenograft model and 100 μl of 100% Matrigel alone to the control group. The animals were regularly checked for tumor development, and the tumor progression was measured for length and breadth using a digital Vernier caliper. The tumor volume kinetics was calculated using the formula mentioned below [19]. Based on the allowed tumor volume limit after MCF 7 cells injection, the entire tumor growth period was divided into 4 assessment time points (day 5, 10, 15, and 20) and conducted the study considering day 0 as control.

Tumor Volume = (width² × length)/2 mm³

On reaching the defined time points, the animals were anesthetized, injecting 50 mg/kg Sodium Thiopentone intraperitoneally and collecting 2 ml of blood from each through the retro-orbital puncture. Immediately after the withdrawal of blood samples, the secondary physical method of euthanasia was followed to ensure animals’ death, and tumor masses were harvested. A small piece of the tumor mass was fixed immediately in 10% Neutral buffered formalin (NBF) for histopathological study. The remaining tumor tissues were snap-frozen and stored at −80°C for further studies. The frozen tissues were thawed to room temperature, and photoacoustic spectra were recorded.

Photoacoustic spectral recordings

The photoacoustic spectra from the tumor tissues were recorded ex vivo using the experimental setup consisting of an Nd-YAG laser (LITRON Lasers, United Kingdom) pumped frequency-doubled dye laser (PULSARE Pro, FINE ADJUSTMENT, Germany) as an excitation source. The second harmonic (532 nm) of the Nd-YAG laser was used to pump the dye laser containing Rhodamine 6G dye to obtain 281 nm laser light for the samples’ excitation. The laser light (energy = 100 μJ per pulse) was focused onto the tissue samples placed in a quartz cuvette of dimension: 35 mm (Height) x 10 mm (Width) x 10 mm (Depth) using a 6 mm focusing fused silica ball lens (Edmund Optics), held in contact with the PZT detector (PI Ceramics, Germany), maintaining a constant pressure throughout. As a result of 281 nm pulsed laser excitations, the induced photoacoustic signals in the tumor tissues were detected by the PZT detector, and after amplification using a preamplifier, they were recorded in the time domain on an oscilloscope (Tektronix, TDS 5034B) as shown in Fig. 1. The photoacoustic spectra were recorded from each tumor tissue in 4 different positions and 5 spectra from each location. Hence, a total of 500 photoacoustic spectra (5 spectra × 4 positions × 5 tissues × 5 groups) were recorded. The photoacoustic spectra were then processed using MATLAB R2019b (MathWorks, USA) software. The sampling frequency was set to 2.6 MHz during the photoacoustic signal acquisition.

Data analysis

The typical raw photoacoustic spectra of different time point groups under study recorded in time-domain in the region 0–2 ms are shown in Fig. 2A. The raw spectra were processed for further analysis using MATLAB R2019b software (MathWorks, USA).

Pre-processing

Initially, we pre-processed the spectra from each sample of all the experimental groups under study. The pre-processing step involved detrending, baseline correction, background subtraction, and normalization of the spectra. After pre-processing, we selected a common ‘region of interest’ (ROI) with a maximum spectral variation for all the photoacoustic spectra under study as shown in Fig. 2B. In the present study, ROI between 0.27
and 0.6 ms along the time axis showed maximum spectral variations and was selected for further analysis.

**Augmentation**

In the present study we followed Time-series Data Augmentation. We have adopted rescaling as a data augmentation technique, which changes the raw data’s magnitude, preserving the labels. Scaling is implemented by multiplying the original data by a random scalar [25]. In the current study we have employed rescaling as data augmentation technique using 0.5–4.5 variants in an increment of 0.5 on the original photoacoustic spectra to increase 10-fold data strength as compared to original data. A typical augmented photoacoustic data obtained from a day 20th photoacoustic spectrum is shown in Fig. 3. The augmented and original data were then combined together and used for further analysis.

**Wavelet transformation**

The pre-processed ROI selected photoacoustic spectra (Fig. 2B, typical spectra) along with augmented spectra were subjected to wavelet transformation using a Wavelet Packet Transform (WPT)/Wavelet Packet Decomposition (WPD) to extract the hidden spectral features from them.
WPT offers optimal signal analysis given by three parameters: scale/level, position, and frequency. A pictorial representation of the WPT workflow (Binary tree) is shown in Supplementary Fig. S1. In the present study under WPT, we have used the sub-band filtering on the input spectral signals of equal widths for each decomposition level to capture the right information from the data and approximation and detail information from the spectra decomposed. Each decomposed level preserved the global/total energy and can reconstruct the exact/original spectral patterns. In the present study, the mother wavelet db6 was used as a predictive model because it demonstrated better results than others. The energy distribution of the wavelet coefficients for the level 2 decomposition for the photoacoustic signals of day 0th, 5th, 10th, 15th, and 20th groups under study found to be ~99.2%, 99.8%, 99.8%, 99.9%, 99.9% respectively for approximate coefficient ‘AA2’ as shown in Supplementary Fig. S2. As depicted in Figs. S1 and S2, the optimal signal strength was observed in decomposition level 2 under frequency level (2,0) i.e., AA2 and hence was considered the ideal decomposition level. Since the frequency of interest has maximum energy falling under AA2, it was used for further analysis.

**Feature selection**

In the present study, ‘minimal Redundancy Maximal Relevance’ (mRMR) feature selection algorithm was used to eliminate redundant features confined in the WPT coefficient (2,0). The algorithm selects the best among the features by eliminating the noise and discriminates the class better [30]. The mRMR uses an empirical method in choosing the features that are mutual and having maximum dissimilarities. This method was followed by the feature ranking based on prediction importance score. In the present study, 10 features were found optimal for the discrimination of the spectra from different groups under study and were used as an input feature matrix for the machine learning algorithms for training and testing [31].

**Classification**

The features selected using the mRMR algorithm from the combined original and augmented photoacoustic spectral data of different time point groups (day 0th, 5th, 10th, 15th & 20th) under study were fed to the SVM learning algorithm for training and classification to their respective time points groups. The feature matrix contained 10 features belonging to 5000 photoacoustic spectra, 500 original spectra, 100 belonging to each of 5 time points groups (day 0th, 5th, 10th, 15th & 20th) plus 4500 augmented spectra obtained with 9 augmentations on each spectrum. A multi-class SVM learning model (RBF, Polynomial and Linear) trained using 80% of the feature matrix data belonging to each group under study and 20% used for testing of the model.

These SVM algorithms work on both linear and nonlinear modes of classification. It separates the classes using hyperplanes, created by forming support vectors and margins. The support vectors are the data points that are closer to the hyperplanes. Margin is the maximum value of the separation of the hyperplanes of different classes. The hyperplane position depends on the locus of the support vectors. There are different kinds of kernel functions; ‘Radial Basis function’ (RBF), ‘Polynomial’, ‘Linear’, and ‘Sigmoid’ used in SVM, ‘RBF’ and ‘Polynomial’ are used for nonlinear classifications. In the present study, we have used SVM-RBF, SVM-Polynomial, and SVM-linear models to train and test the data. The models’ performance was evaluated by ‘Specificity’, ‘Sensitivity’ & ‘Accuracy’. For SVM, the classification score for a sample value (x) is the signed distance from x to the decision boundary, ranging from $-\infty$ to $+\infty$. The highest score value indicates that it belongs to the target class and a negative score indicates that it belongs to another category. The predicted score is
given by the function, as below,

\[ \hat{f}(x) = \sum_{j=1}^{n} a_j y_j G(x, x_j) + b \]

where \((a_1, a_2, \ldots, a_n, b)\) is the estimated SVM parameters which are obtained by the trained model, \(G(x, x_j)\), known as the dot product between \(x\) and the support vectors in the predictor region for the training set of the sum of observations [29].

**Histology**

The formalin-fixed tumor tissues were dehydrated with graded ethanol (50–100%), further defatted in xylene, and embedded in paraffin. Subsequently, a 5 µm thick section of the paraffin-embedded tissues were used for Hematoxylin and Eosin (H&E) staining. The paraffin sections were deparaffinized and were rehydrated and stained with hematoxylin solution for 5 min. This step was followed by 1 dip in 1% acid alcohol solution (1% HCl in 70% ethanol), the excess stain was washed in tap water. Afterward, the sections were stained using eosin solution for 3 min and dehydrated with graded alcohol and cleared in xylene [32].

**Liquid chromatography-mass spectrometry (LCMS)**

The metabolomic analysis was performed using Agilent 6520 Accurate-Mass Q-TOF LC/MS System with Agilent 1200 Series HPLC unit (Agilent Technologies, Santa Clara, California, USA). The blood serum samples obtained from the experimental animals of various time-point groups were used for the metabolomic analysis. The metabolites were extracted by mixing the serum and ice-cold methanol in 1:2 ratio, followed by vortexing the mixture for 15 min. This was centrifuged at about 12,000 rpm, 12 min. The supernatant collected was lyophilized, and the residue was dissolved with 0.1% formic acid in 30 µl of 5 % acetonitrile (95:5). An 8 µl of the processed serum samples from different experimental groups under study were injected into the reversed-phase column (ZORBAX Eclipse Plus C18, 4.6 mm × 150 mm, 5 µm; Agilent Technologies). Metabolites were separated using a gradient system in LC maintained by Solvent A (0.1% formic acid in water) and B (0.1% formic acid in 90% acetonitrile). Basic and neutral metabolites were eluted in positive mode using a gradient flow rate of 400 µl/min in mobile phase A and mobile phase B (92% A & 8% B in 25 min, 8% A & 92% B for 10 min and equilibrated to 92%A & 8% B for 10 min) [33].

The raw data obtained from each run were processed using the Qualitative Mass Hunter Analysis Software B.07.00 (Agilent Technologies, Santa Clara, California, USA), a molecular feature extraction tool. The data files containing monoisotopic mass, respective abundance, and retention time were used for data alignment and filtering under Mass Profiler Plus software (MPP) (Agilent Technologies, version B.12.5). Features that were present in at least 60% of the sample numbers (3 out of 5) in each group were considered for further analysis. Compounds were identified in METLIN and HMDB database based on isotopic pattern distribution and accurate mass within a specified tolerance (15 ppm error). Fragmentation patterns of metabolites were matched with in-house developed MS/MS spectral library. The intensity values of the identified metabolites from the control and test groups were log10 transformed and used to plot a circular bar graph using CIRCOS. Metabolites were then subjected to one-way ANOVA for multiple groups in the study. Subsequently, Metabolomic Pathway Analysis (MetPA) was carried out using MetaboAnalyst software 4.0.

**Statistical analysis**

The results are expressed as the mean ± standard error (SEM), and Student’s t test and one-way ANOVA performed for statistical significance. A p value < 0.05 (n = 5) was considered significant. The statistical analyses were carried out using GraphPad Prism 8.0 software (San Diego, CA, USA).

**Results**

**Tumorigenesis**

In the study, the tumor xenograft in athymic nude mice was established using MCF-7 cell lines. In the 5th day of post-MCF-7 cells injection, a small palpable tumor was observed, and the tumor volume showed an exponential growth in subsequent days till day 20, reaching the maximum permissible tumor volume limit of 1000 mm³. The control group has shown no growth at the site of injection. By considering the tumor volume of the control group as 0 mm³, the statistical analysis, as mentioned before, was performed. The data obtained from tumor volume kinetics were plotted, demonstrating the progressive tumor condition shown in Fig. 4A.

**Histology**

The histological findings in the study showed different tumor development stages, as shown in Fig. 4, depicting the picture of intact tumor in nude mice (Fig. 4B-top row) and H&E stained images in 100x and 400x (Fig. 4B – middle & bottom row), respectively. On day 5th, approximately one-fifth of the
tumor cells were found viable. But as the tumor progressed, a better establishment of the tumor was seen on day 10th, showing more viable cells with few blood capillaries. Day 15th showed well-developed blood capillaries compared to the former stage and viable tumor cells. The day 20th histological finding showed well-developed blood capillaries, and, in some regions, extensive necrosis was also observed.

**Photoacoustic study**

A differential photoacoustic spectral pattern was observed for different experimental groups under study. The mean pre-processed photoacoustic spectra of each group after WPT demonstrated varied amplitude along the time axis. The wavelet transformed spectra subsequently subjected to the mRMR algorithm and the top 10 features necessary for the discrimination of all the spectra belonging to different groups based on their prediction ranking values were obtained by the analysis, as shown in Fig. 5. As can be seen from the figure, the best feature is the feature 1 with a maximum prediction score value of 0.093 at wavelet coefficient level 15. The rest of the features were of lesser prediction scores. These were ranked from all groups as follows; feature 2 – coefficient level 5, prediction score 0.081; feature 3 – coefficient level 16, prediction score 0.056; feature 4 – coefficient level 14, prediction score 0.051; feature 5 – coefficient level 17, prediction score 0.041; feature 6 – coefficient level 9, prediction score 0.036; feature 7 – coefficient level 18, prediction score 0.034; feature 8 – coefficient level 1, prediction score 0.026; feature 9 – coefficient level 52, prediction score 0.024; feature 10 – coefficient level 10, prediction score 0.022, as shown in Fig. 5. The variations in
the features for all the time point groups under study were visualized by plotting them using GraphPad prism 8.0, which is shown in Fig. 6 along with their prediction scores. These top 10 features from all the time points groups were fed to the machine learning algorithm as an input feature matrix for classification by using multi-class SVM-RBF, SVM-Polynomial, and SVM-Linear algorithms with 80% data for training and 20% for testing. The random training and testing of the classification models were done by repeating the process 10 times and selecting best classification model for the analysis as shown in Supplementary Fig. S3. The sensitivity, specificity, and accuracy for the best classification by the models were also calculated, as listed in Table 1. The sensitivity values for classification using SVM-RBF, SVM-polynomial and SVM-linear for the 5th, 10th, 15th and 20th day groups are found to be 98%, 98%, 90.5%, 92.5%; 100%, 99.5%, 100%, 98% and 98%, 64.5%, 73.5%, 83.5% respectively. Similarly, the specificity and accuracy values for the analysis using SVM-RBF, SVM-Polynomial, and SVM-Linear are found to be 97%, 100%, 92% and 95.2%, 99.5%, 80.3% respectively.

**Metabolomics**

LC-MS data analysis of serum samples obtained from the control and test groups under study revealed the presence of a total of 3997 spectral features/metabolites. The data was filtered to their intensity, present in a minimum of 3 out of 5 animals (60%) per group. These metabolites were annotated using the m/z ratio in METLIN and HMDB databases.
within 15 ppm tolerance. There were 114 compounds annotated, excluding xenobiotics and drugs. Further were the intensity of the identified compounds to visualize the global metabolome of control and test groups using Circos illustration, as shown in Fig. 7A. The Circos representation showed differential distribution patterns across the control and test groups. Intensity and presence of metabolites were considered in each sample of all the groups, and upon performing ‘Student’s t test’ and ‘One-way ANOVA’ on the annotated compounds, found that 19 metabolites present in all the experimental groups were altered as shown in Supplementary Fig. S4. The metabolites were subjected to MS/MS analysis to find the fragmentation patterns to validate the annotated compounds.

The significantly altered compounds were subjected to fold change analysis. The fold change was calculated for days 5th, 10th, 15th & 20th groups with respect to control, as shown in the Supplementary Fig. S5. The fold change analysis showed that the serum levels of most of the metabolites to be downregulated as the tumor progressed with respect to control. Further, the metabolites from all the time-points were subjected to ‘Pearson’s correlation analysis,’ as shown in the Supplementary Fig. S6. The differentially altered serum metabolites in control and test groups were detected in serum samples based on the most abundant and significantly altered intensities and were transformed to log10 and represented as Box-Whiskers plots. Statistically significant changes in metabolite intensity between the control and day 20th group are represented by asterisk (****p < 0.001, **p < 0.01, *p < 0.05). However, some metabolites showed no statistical significance compared to control, as shown in Supplementary Fig. S4.

Among 114 identified compounds, the amino acid levels, lipid levels, and other compounds showed significant variations in the current study. The student’s t test was performed for day 0 versus day 20th group, the amino acids, threonine, proline-betaine, L-valine, tryptophan, tyrosine, and other metabolites such as 5-methylcytidine, serotonin, piperidine and propionyl choline were found to be significantly decreased in progressive tumor condition compared to control. Further, carnitine and o-propanoyl-L-carnitine also showed similar intensity trend upon tumor progression to day 20th. However, metabolites such as acylcarnitine, valeryl carnitine, pantothenic acid, succinyl acetoacetate and 5-(3-Pyridyl)-2-hydroxytetrahydrofuran had altered intensities upon tumor progression, nevertheless, did not show significance in downregulation.

Pathway analysis

The annotated metabolites (114 compounds) were subjected to pathway analysis using MetaboAnalyst 4.0. It was observed that most of the pathways were affected. Among them, the tryptophan pathway was also impacted with 3 hits (tryptophan, serotonin & kynurenine) with a raw p value of 0.0443 and a pathway Impact of 0.34, respectively. The graphical representation of pathway analysis is shown in Fig. 7B with the list of pathways impacted.

Discussion

In the present study, breast tumor progression was assessed by photoacoustic spectroscopy combined with machine learning using support vector machine analysis. Initially, the tumor induced as mentioned in the materials and methods was validated by tumor volume kinetics (Fig. 4A) and histological study (Fig. 4B), also explained in the Supplementary Section 1. Tumor progression on day 0, day 5, day 10, day 15, and
day 20 post tumor induction, was also validated by histological image processing, as explained in Supplementary Section 4. Photoacoustic spectroscopy, which can detect minor biochemical changes in the biological samples, was then used to record spectral profiles of the progressive tumor conditions [34]. As mentioned in the materials and methods, there were 500 original photoacoustic spectra, 100 from each 5-time point group recorded. After pre-processing and selecting ROI containing maximum variation with tumor progression, the original spectra were augmented 10 folds (5000 spectra, 1000
from each group), the original and augmented spectral data analyzed using an SVM algorithm to predict functional outcomes of disease diagnoses [35]. Support vector machine has widely been used, replacing some of the commonly used logistic functions like SoftMax as a classifier in deep learning [36]. In cancer classification, SVM has been used as a classifier on high throughput microarray gene expression [37]. Moler et al. applied SVM in a colon cancer tissue classification using microarray gene expression data. When the performance of SVM was compared with the naïve Bayes classifier using different numbers of selected genes, the former was found to outperform the latter [38]. The SVM is thus most suitable for tissue classification on a wide range of data types [39].

In the present study, to increase the data diversity and size for limiting overfitting in machine learning, all 500 ROI-selected photoacoustic spectra were subjected to data augmentation. In a study, Rashid et al., 2019 have shown a machine learning-based framework for identifying construction equipment activities to assess productivity, safety, and environmental impact on construction sites using the time-series data [25]. The study showed the impact of augmentation on the machine learning model. In the present study, data augmentation was done by adopting the option rescaling to achieve 10-fold increase in the data strength. The combined original and augmented data were subjected to Wavelet Transformation (WT) to extract hidden spectral features from them. We selected WT over FFT for feature extraction because FFT does not significantly extract minor variations from the input signals like real-time photoacoustic signals compared to WT [27]. Further, there are many WT types, and they decompose signal waves onto a set of essential functions known as wavelets, obtained from a single model wavelet, called mother wavelet [28]. In this study, we have used db6 wavelet, as mother wavelet. In WT, Wavelet Packet Decomposition (WPD) generates both ‘approximations’ and ‘details’ coefficients from the input data understudy in the low and high-frequency regions, respectively, to create the full binary tree, as shown in Supplementary Fig. 1. WPD is an iterative process, repeats until a desired level is achieved. It provides an actual signal variation required for the analysis under a particular sub-band in the process. In the present study, AA2 (2,0) sub-band demonstrated optimal signal variation and was used to select the spectral features for the analysis. WPD has also been used to classify ECG signals, providing significantly detailed signal decomposition hence improving the time-frequency resolution of the signal [26].

After the transformation of the photoacoustic spectra, feature extraction, which plays a vital role in deciding the performance of machine learning by accurately classifying the data, selecting the right spectral transformation tool for feature extraction is the key to data classification. The reduced redundancy in data contributes to the reduced noise-based prediction and results in better classification accuracy [40]. Therefore, the feature selection algorithm, the minimal Redundancy Maximal Relevance (mRMR) algorithm, is faster and computationally feasible and does not involve learning was used. The mRMR minimizes the correlation between different features of a target class in the analysis, ensuring feature ranking based on mutual information and prediction ranking, as shown in Fig. 5. As mentioned earlier, 10 features were selected by mRMR and used for data classification. Previously, mRMR based feature selection was used in microarray data analysis for determining biologically relevant genes in cancer samples. Genes selected by mRMR algorithms showed a better characteristic of phenotypes and improved prediction [30].

In the present study, the spectral features selected by mRMR were subjected to Machine Learning using Support Vector Machine (SVM) analysis to classify the photoacoustic spectral data of progressive tumor stages. SVM is an effective supervised learning method applied for data classification of different sample types in high-throughput technologies generating a large volume of data [29]. In our study, the top 10 features selected by mRMR were used as an input feature matrix for classification using multi-class SVM learnings. The multi-class SVM models used were SVM-RBF, SVM-Polynomial, SVM- Linear algorithms, and varied classification accuracy of 95.2%, 99.5% & 80.3%, respectively, were obtained. Our analysis demonstrated the best results in SVM-Polynomial compared to the rest, as shown in Table 1 and Supplementary Fig. S3. The performance metrics of the machine learning models, the sensitivity values for days 5th, 10th, 15th, and 20th groups using SVM-RBF are 98%, 98%, 90.5%, 92.5%, respectively, using SVM-Polynomial are 100%, 99.5%, 100%, and respectively and using SVM-Linear are 98%, 64.5%, 73.5%, and 83.5% respectively. The specificity values of the analysis using SVM-RBF, SVM-Polynomial, and SVM-Linear are found to be 97%, 100%, and 92%, respectively. These outcomes are also listed in Table 1.

In the present study, photoacoustic signals were from the tissue samples of progressive tumor conditions based on tryptophan excitation. It was also observed that spectral features 2, 6, 8, and 10 extracted from photoacoustic patterns shown increased wavelet coefficients with the tumor progression. This may be due to the uptake of tryptophan by tumor tissues and contributing to corresponding photoacoustic spectra, as shown in Fig. 8A. Further, the metabolomic analysis conducted in the present study using MetaboAnalyst software revealed tryptophan metabolism, one of the most dysregulated pathways with tumor progression. The altered serum tryptophan levels showed a decreasing trend (Fig. 8B) as the tumor progressed compared to the control group, which has also been explained in the Supplementary Data (Section...
3). The decrease in serum tryptophan levels at progressive time points demonstrated an inverse correlation with spectral features 2, 6, 8, 10 in the study. This decrease in serum tryptophan levels may be due to higher uptake of tryptophan by the tumor. Further, photoacoustic spectral analysis of tumor tissues involving WPT and mRMR for classification applying multi-class SVM-RBF, SVM-Polynomial, and SVM-Linear algorithms demonstrated varied classification accuracy of 95.2%, 99.5%, and 80.3%, respectively. The SVM-polynomial showed the highest classification accuracy in the study indicating photoacoustic spectroscopy combined with machine learning could be a potential prognostic tool for assessing breast tumor progression. As per our knowledge, the present study is the first to use photoacoustic spectroscopy and machine learning for evaluating breast tumor progression supported by serum metabolomic analysis. This approach holds a very high potential for clinical translation; however, upon further modifications/improvements, the method can be brought into the clinical setting for early detection of breast cancer conditions.

**Data availability**

Data will be available upon request after the execution of a data use agreement.

**Acknowledgements** Authors would also like to thank Professor K. Satyamoorthy, Director, Manipal School of Life Sciences, for being a part of the project and his encouragement and support throughout the study. The authors would also like to thank Ms. Monica Shirley Mani, Ph.D. Scholar in the institution, for helping in metabolomic data analysis; Mr. Gangadhara Prabhu, Technical staff, for assisting in animal handling; and Ms. Nitufa J. Mulla, for helping as Technical Assistant during the initial days of the project.

**Author contributions** KKM designed the research and experiments; KKM, SC designed and developed photoacoustic instrumentation; JR performed all the experiments including animal handling; JR, AA and SGN analyzed photoacoustic data; JR, MBJ analyzed metabolomics data; JR, SRai analyzed histological data; JR, KP done Image analysis; KKM verified data and data analysis; JR wrote the manuscript; KKM, SRay, MBJ, CRR critically reviewed the manuscript.

**Funding** This work was financially supported by the Department of Biotechnology, Government of India, New Delhi (Sanction ID: BT/PR14776/MED/32/460/2015). The authors would like to thank Manipal Academy of Higher Education (MAHE), Manipal, India, and TIFAC-CORE, DST, Govt. of India, and BTFS, Govt. of Karnataka, and Manipal School of Life Sciences for infrastructure facilities. One of the authors, JR, would like to thank the Indian Council of Medical Research, Government of India, New Delhi, for granting Senior Research Fellowship (FileNo-5/38/45/ITR-F/2019-ITR-IRIS Cell No. 2019-5005) to him. Open access funding provided by Manipal Academy of Higher Education, Manipal.

---

**Fig. 8 Correlation between metabolomic analysis and photoacoustic measurements of tumor progression.** (A) Tryptophan intensity found in Day 0th, 5th, 10th, 15th, and 20th obtained from serum metabolomics data analysis and t-test performed between day 0th and day 20th predicted $p$ value as significant, $p = 0.0109$ (*). (B) Wavelet coefficients of the features extracted from the photoacoustic signals of tumor tissues belonging to Day 0th, 5th, 10th, 15th, and 20th. (i) Feature 2, (ii) Feature 6, (iii) Feature 8 and (iv) Feature 10. One-way analysis of variance test predicted $p$ value as significant, $p < 0.0001(***)$.
Compliance with ethical standards

Conflict of interest The authors declare no competing interests.

Ethical approval The current study was carried out as per the guidelines and approval of the Institutional Animal Ethics Committee (IAEC), MAHE, Manipal, India. Study number - IAEC/KMC/96/2015.

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/.

References

1. Bray F, Ferlay J, Soerjomataram I, Siegel RL, Torre LA, Jemal A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. 2018;68:394–424.

2. Harbeck N, Penault-Llorca F, Cortes J, Ganot M, Houssami N, Poortmans P, et al. Breast cancer. Nat. Rev. Dis. Prim. 2019;5:66.

3. Makki J. Diversity of breast carcinoma: histological subtypes and clinical relevance. Clin. Med. Insights Pathol. 2015;8:CPath. S31563.

4. Lobbes MBI, Smidt ML, Houwers J, Tjan-Heijnen VC, Wildberger JE. Contrast enhanced mammography: techniques, current results, and potential indications. Clin. Radiol. 2013;68:935–44.

5. Hooley RJ, Scoutt LM, Philpotts LE. Breast ultrasonography: state of the art. Radiology. 2013;268:642–59.

6. Schneble EJ, Graham LJ, Shupe MP, Flynt FL, Banks KP, Kirkpatrick AD, et al. Future directions for the early detection of recurrent breast cancer. J. Cancer. 2014;5:291–300.

7. Wang L. Microwave sensors for breast cancer detection. Sensors. 2018;18:655.

8. Islam MT, Mahmud MZ, Islam MT, Kibria S, Samsuzzaman M. A low cost and portable microwave imaging system for breast tumor detection using UWB directional antenna array. Sci. Rep. 2019;9:15491.

9. Godavarthy A, Rodriguez S, Jung YJ, Gonzalez S. Optical imaging for breast cancer prescreening. Breast Cancer Targets Ther. 2015;7:193.

10. Akers WJ, Edwards WB, Kim C, Xu B, Erpelding TN, Wang LV, et al. Multimodal sentinel lymph node mapping with single-photon emission computed tomography (SPECT)/computed tomography (CT) and photoacoustic tomography. Transl. Res. 2012;159:175–81.

11. Phipps JE, Gorparas D, Unger J, Darrow M, Bold RJ, Maruc LC. Automated detection of breast cancer in resected specimens with fluorescence lifetime imaging. Phys. Med. Biol. 2017;63:15003.
33. Joshi MB, Pai S, Balakrishnan A, Bhat M, Kotambail A, Sharma PSVN, et al. Evidence for perturbed metabolic patterns in bipolar disorder subjects associated with lithium responsiveness. Psychiatry Res. 2019;273:252–9.
34. Teo YH, Lim ICZY, Tseng FS, et al. Predicting clinical outcomes in acute ischemic stroke patients undergoing endovascular thrombectomy with machine learning. Clin. Neuroradiol. 2021. https://doi.org/10.1007/s00062-020-00990-3.
35. Tang Y. Deep learning using linear support vector machines. arXiv.org. 2013. Available at https://arxiv.org/abs/1306.0239.
36. Golub TR, Slonim DK, Tamayo P, Huard C, Gaasenbeek M, Mesirov JP, et al. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science. 1999;286:531–7.
37. Moler EJ, Chow ML, Mian IS. Analysis of molecular profile data using generative and discriminative methods. Physiol. Genom. 2000;4:109–26.
38. Huang S, et al. Applications of support vector machine (SVM) learning in cancer genomics. Cancer Genom Proteomics. 2018;15:41–51.
39. Li H, Yuan D, Ma X, Cui D, Cao L. Genetic algorithm for the optimization of features and neural networks in ECG signals classification. Sci. Rep. 2017;7:41011.
40. Hira ZM, Gillies DF. A review of feature selection and feature extraction methods applied on microarray data. Adv. Bioinform. 2015;2015:1–13.