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Review Article

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Integrative chemo-immune-radiological treatment of HER2 positive breast cancer combined with a review of potential microRNAs involved and analysis of experimental results from sub-THz vibrational spectroscopy

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Abstract

In this paper we present the results of a Breast Cancer study by integrative analysis of a NIH approved treatment for HER2 positive breast cancer. This study is combined with analysis of Micro-RNA involvement from application of sub-THz spectroscopy for visualization of molecules circulating in blood by measuring saliva. The combination of Taxol-Generic Name-Paclitaxel (PT-J9267) and Trastuzumab-ANNS 0/0 and KadcylaTM (Genetic Name Ado-Trastuzumab Emtansine) were used in 3-stages of combined chemotherapeutic and immune-treatments followed by Radiation treatment. The goal of using PT was to stop spread of the disease to other organs outside of the breast and under arm lymph nodes, as well as to shrink the size of the tumor to facilitate surgery and radiology in continuation of the treatment. Trastuzumab (TZ) was added to PT in the 2nd step to prevent the development of chemoresistance. Intravenous infusion of Kadcyla was used mainly to prevent metastasis. Integrative analysis of microRNA participation was conducted based on literature review and sub-Terahertz vibrational spectroscopy measurements of absorption spectra from samples taken weekly before and after each treatment, using Vibratess' spectrometer. The results from sub-THz spectroscopy in this work demonstrate dramatical modification of spectroscopic signatures from patient samples following disease development and the initial steps in the course of treatment. These changes reflect the deep global regulation (reduction) of the initially participating microRNAs amounts and changes in the microRNAs contributing to the spectra.

Introduction

About 1 in 8 U.S. women (~12%) will develop invasive breast cancer over the course of her lifetime. Worldwide breast cancer among females is the most commonly diagnosed cancer constituting approximately 30% of all cancer types and is the leading cause of cancer death [1-3]. It is a heterogeneous disease with complex clinical behavior and responses to therapeutic intervention and metastasis is the leading cause of death with the median survival of 2-4 years [4-6].

Patients with breast cancer are still grouped according to clinicopathological criteria to decide the appropriate therapy and predict their prognosis although a recent study has shown that breast cancer heterogeneity extends beyond the traditional histopathological classification (lymph nodes and distant metastases), to include in addition the status of the hormone receptors: estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) [7-10]. Although decreasing mortality rates over the last three decades, this classification failed to predict the outcome in different patients, as patients with the same tumor features may have completely different outcomes, while other patients suffer from significant toxic side effects from therapy [10]. Thus, accurate stratification of breast cancers into clinically related subtypes is of major importance for therapeutic decision making [11].

Parallel studies of gene expression through messenger RNA (mRNA) profiling have shown that tumor cells respond differently to treatment depending on intrinsic molecular features [12]. This discovery has allowed intrinsic classification of breast cancer into five main subtypes [7]: luminal A (ER+/PR+/HER2−) that are usually of low grade, luminal B (ER+/PR−/+/HER2+/−) that are generally of higher grade with higher proliferation rate, normal-like subtype that resembles normal breast tissue and is associated with good prognosis, triple-negative breast cancer (ER−/PR−/HER2−), and HER2 enriched (positive) subtype (ER−/PR−/HER2+) [4,6,7,9,13-15]. HER2 is a growth promoting protein on the outside of all breast cells. HER2-positive breast cancers have cells with higher than normal levels of HER2, and represents ~20% of breast cancer cases [7, 15-17]. This aggressive subtype has a poor prognosis, short survival, and high rates of recurrence [17], as these cancers tend to grow and spread faster than other breast cancers.

The intensive downstream signaling controlled through the HER2 pathways result in metastatic spread from the initial breast cancer site [15-18]. Recent treatment advances, particularly with therapies that target the HER2 protein, have made this subtype more curable in its early stages. They have also extended the survival period for patients whose cancer has spread beyond the breast and nearby lymph nodes [19].

Gene expression profiling can be used to define breast cancer subtypes, but it is an extensive process, and requires biopsy material of the tumor cells to test for expression. One of the major challenges in cancer research is the identification of stable biomarkers, which can be routinely measured in easily accessible samples. Existing biomarkers and diagnostic tools for breast cancer, such as carcinoembryonic antigen (CEA) and carbohydrate antigens (CA), are of too low sensitivity [7, 20] to be used as screening tools. Early detection of cancer as well as monitoring disease progression and response to treatment requires using good biomarkers. An ideal biomarker should be obtained noninvasively, should be highly sensitive to detect the tumor as early as possible [21], and should provide specificity to cancer subtypes. Such biomarkers would have a major impact on clinical management including breast cancer classification, prognosis, predicting therapy outcome, as well as allow follow-up after surgery, and could be monitored for prediction of metastasis, and tumor recurrence.

About 28 years ago, microRNAs (miRNAs) were discovered as a novel class of evolutionarily conserved, small (18-24 base pairs in length) non-coding RNA molecules, which are important regulators of gene expression, playing a major role in various cellular processes including cell growth and apoptosis, gene expression modulation and controlling major pathways [22,23]. Deregulations in miRNA expression are associated with multiple human diseases such as cancer, and miRNAs can act as promoters or suppressors of tumors [24]. miRNAs are known to be deregulated in breast cancer and other cancer types [25,26], to play roles in disease progression and they represent potential therapeutic targets for cancer [27,28]. These molecules are important for regulating cancer biology and can serve in tumor samples as biomarkers to predict the prognosis and treatment sensitivity in various tumor types [29]. miRNA's signatures can further sub-classify breast cancer [30], leading to the identification of new molecular subtypes [31].

In this paper we introduce and demonstrate highly resolved sub-THz spectroscopy of biological molecules in saliva as a fast, non-invasive method to fingerprint miRNA in ultra-small amounts of samples from patients. Use of this method could potentially begin to address the issues with identification of good biomarkers for both screening and clinical management of cancer patients.

Integrative Analysis of NIH Approved Treatment HER2 Positive Breast Cancer

A. Combined chemotherapeutic and immune-treatments (Dr. Erika Struble).
B. Surgery (Dr. Linda Sommers).
C. Radiation Therapy (Dr. Silvia Hendrix).

Combined chemotherapeutic and immune-treatments (Dr. Erika Struble)

In this study of a HER2 Positive Breast Cancer patient, Paclitaxel (brand name Taxol) was used in the first chemotherapy step through intravenous treatment. Paclitaxel is the most well-known natural-source cancer drug in the United States. It is derived from the bark of the Pacific yew tree and is used to treat various types of cancer. It is a cancer chemotherapy drug that works by slowing or stopping cancer cell growth [32].

Trastuzumab (brand name Herceptin) was used in the second step treatment. It is an example of an immune targeted therapy that works by attaching itself to the HER2 receptors on the surface of breast cancer cells and blocking them from receiving growth signals. As a result, Herceptin can slow or stop growth of the breast cancer cells. Trastuzumab, a recombinant humanized monoclonal antibody developed against the extracellular domain of the HER2 protein, is currently broadly used as a therapy for HER2-overexpressing breast cancer patients [33-36]. Approved

by FDA in 1998, Trastuzumab is the oldest and most prescribed selective estrogen receptor modulator (SERM) currently used as a therapy for HER2-overexpressing breast cancer patients. It reduces the risk of a new cancer developing, shrinks large hormone receptor positive breast cancer before surgery, and slows or stops metastasis of cells from the original cancer site. However, despite the availability of targeted therapy, almost 40% of patients with metastases develop resistance to trastuzumab, as approximately 60% of patients present with acquired resistance following one year of treatment [37].

The third step of Chemo/Immune therapy used an intravenous infusion and included KadcylaTM, (Ado-Trastuzumab Emtansine). This treatment combines an Anti- HER2+ monoclonal antibody with a microtubular inhibitor and is prescribed after surgical treatment and after using trastuzumab, and when there is a possibility of cancer remaining in the tissue not removed during surgery.

Surgery (Dr. Linda Sommers)

Surgery was conducted in the facilities at Martha Jefferson Hospital Cancer Center (Charlottesville, VA) by Dr. Linda Sommers MD, with several goals: to remove as much of the cancer as possible (breast conserving surgery-mastectomy on one side) and to remove the lymph nodes under the arm as necessary to stop cancer spread (identified using sentinel lymph node biopsy or axillary lymph node dissection) and relieve symptoms of advanced cancer if possible. The surgery was controlled using ultrasound imaging technology.

In the mastectomy of this patient, the entire breast was removed, including all of the breast tissue. Additionally, several close lymph nodes were removed, where the cancer would likely spread first, in an attempt to slow the spread of the cancer, to find out if the breast cancer had spread to underarm (axillary) lymph nodes, and to determine the cancer stage using the subsequent lab analysis. Removing only a few lymph nodes reduced the risk of side effects from the surgery, such as arm swelling (lymphedema).

Radiation Therapy (Dr. Silvia Hendrix)

Radiation therapy was performed in parallel with the third step of Chemo/Immune therapy. In this procedure, originally reported by the National Cancer Institute [38,39], high-energy radiation from a linear accelerator, including photons and electron beams, is used to kill cancer cells for tumors on the skin or near the surface and to prevent skin metastasis. Using images from CT scans, a 3 dimensional conformal radiation design is developed that conform to the shape of the tumor using beams from multiple directions. The 3D conformational design allows the radiation to affect cells only in the part of the body that is treated with the radiation. Used in breast cancer, radiation therapy may be employed to destroy any mutated cells that remain in the breast or armpit area after the surgery. In this patient case, the beam radiation therapy used a highly focused X-ray beam to target the cancerous area for two to three minutes. This treatment involved multiple appointments - as many as five days a week for eight weeks using reduced doses of radiation; this was required because of the patient's previous treatment with the high dose radiation.

MicroRNAs and Breast Cancer-Literature Review

Since the discovery of microRNAs, small RNA molecules (19-22 nucleotides) in 1993 [40], hundreds of different miRNA molecules have been found in various organisms, suggesting their potential roles in all biological events. MicroRNA (miRNA) molecules are highly conserved across different species, highly specific, and play crucial functions in the regulation of important processes in cell biology with a key role as regulators of gene expression. One microRNA can regulate multiple different genes, with some miRNAs suggested to have hundreds of different gene targets and ~30-60% of genes known to be regulated by at least one miRNA [7]. miRNAs are evolutionarily conserved and repress gene expression post-transcriptionally. In normal cells miRNA regulates formation of messenger RNA (mRNA) inhibiting transfer of information between DNAs and proteins [40-47].

Most miRNAs are transcribed in the nucleus from DNA by RNA polymerase, resulting in a stem loop RNA (pre-miRNA) with approximately 70-nucleotides. These pre-miRNAs are then exported to the cytoplasm via Exportin 5 and cleaved by Dicer to give approximately 22 nucleotide duplexes named miR/miR*. One of the strands of the duplex, miR, with decreased base-pairing at its 5' end later functions as a mature miRNA, while the other strand (a passenger strand) is degraded. Mature single stranded miRNA is further incorporated in the RNA-induced silencing complex known as miRISC, and as part of this complex can regulate gene expression by some degree of degradation of messenger RNA (mRNA) and translation inhibition to protein [46,47].

Cancer Biology

Numerous publications have presented evidence indicating the role of microRNAs as regulators that control every cellular process including disease development and progression. [42,47. These small molecules are present in tissue and are also found circulating in blood and other body fluids. They are very stable, and the presence of specific microRNAs and their amounts reflect the pathological conditions of patients. [48, 49]. In normal cells, microRNAs control normal rates of cellular growth, proliferation, differentiation and apoptosis [7,47].

Publications in the medical literature are also reporting that miRNAs play crucial roles in regulating cancer biology as well. Tumor cell lines have been reported to have widespread deregulation of miRNAs, and expression of some miRNAs correlates with diverse pathological parameters [50,43,46,47]. These alterations in miRNA-expression can affect crucial biological processes in cancer development and progression.

A decrease in expression of some miRNAs observed in human cancers compared to normal levels may indicate that these miRNAs act as tumor suppressors playing an important role in the development or progression of cancer. The global loss in expression of these miRNAs enhances tumorigenesis. [47,51].

Breast Cancer

A breast cancer tumor was one of the first tumors to be profiled for miRNA expression in 2005 [3,43,44], and work in this area continues [46]. As discussed above, it is a heterogeneous disease

with complex clinical behavior and responses to therapeutic intervention [8,9] and is classified based on gene expression profiling as luminal A or B, basal-like and presence of hormone receptors [I-4].

Deregulation of miRNAs in breast cancer has been shown by profiling breast tissues from healthy individuals and those from breast cancer patients see reviews [7,52] and references within, [53,54]. While miRNAs can be detected in tissues samples, they are also detectable in body fluids such as blood, serum, and urine from breast cancer patients, thus indicating miRNAs to be easily accessible without the need for invasive procedures see reviews [7,45,56].

Different breast cancer subtypes display various molecular miRNA signatures [54]. There are oncogenic, tumor-suppressive, and metastatic-influencing miRNAs that have been reported to have different roles in the pathogenesis of cancer [57]. The mechanism of specific miRNAs involvement in carcinoma invasion and metastasis is described in multiple publications [58]. This mechanism might include as a necessary step transition from epithelial to mesenchymal cells (EMT) that is a prerequisite for carcinoma invasion and metastasis [59], which would allow these specific miRNAs to be used as biomarkers for cancer spread. Tumor metastasis may also be promoted in breast cancer by enhanced expression of prometastatic miRNAs and/or downregulation of antimetastatic miRNAs. These molecules are not only important for regulating cancer biology but could serve in tumor samples as biomarkers to predict the prognosis and treatment sensitivity in various tumor types [29].

miRNAs Signatures Identified

miRNA profiling in breast cancer started with quantitative analysis of breast tissue using primary tumors and its surrounding tissue [43], or metastatic sites [60]. Expression levels in these cases had to be first normalized for quantitative microRNA analyses in formalin-fixed paraffin-embedded (FFPE) tissue. The analysis results when using tissue samples is, however, complicated because of tissue samples heterogeneity.

Circulating miRNAs are increasingly recognized as promising biomarkers, due to the ease with which miRNAs can be isolated and because of their structural stability under different conditions of sample processing and isolation. miRNAs are present in the circulation of cancer patients and can potentially be used for disease monitoring. However, accurate quantification of circulating miRNAs in body fluids still currently have challenges because of their low abundance and the small size of the molecules [52].

In cancer, miRNAs play a role in ontogenesis, metastasis, and resistance to various therapies and can be classified as oncogenes (oncomirs) or tumor-suppressors [59-61]. The majority of the differentially expressed miRNAs have attenuated expression levels in tumor samples. This global repression of miRNAs in cancerous tissue relative to normal tissue has been reported previously and suggests that most miRNAs have a tumor suppressive function [53], some of which exhibit anti-metastatic properties as well.

The loss of several tumor suppressor miRNAs resulted from their global downregulation (miR-206 (ER signaling), miR-17-5p (proliferation), miR-125a, miR-125b, miR-200 (signaling), let-7 (proliferation), miR-34 (proliferation) and miR-31 (metastasis)) and the overexpression of certain oncogenic miRNAs (miR-21, miR-155, miR-10b, miR-373 and miR-520c) have been observed in many breast cancers [47]. miRNAs are deregulated in all stages of breast cancer and thus can be used as diagnostic as well as prognostic and predictive biomarkers [54-56].

Oncogenic miRNAs. Several oncogenic miRNAs associated with different breast cancer subtypes (miR-21, miR10b, miR155, and Let-7a) are found to be dysregulated in sera of patients compared to healthy individuals, see.[7, 54,56,57]. In particular, oncogenic miRNAs, miR-21 and miR-10b, are listed in several publications [7,47,54,57] and references within. miRNAs miR-21 promotes cell apoptosis, migration, invasion and metastasis [62], and miR-10b promotes cell migration, invasion and metastasis [63].

Tumor-suppressive and metastasis-suppressive microRNAs. Tumor-suppressor miRNAs exhibit a lower expression in cancer cells ("global downregulation of miRNAs in cancer"). These molecules suppress oncogene expression, thereby controlling cellular differentiation [62]. The regulation mechanism describing the miR 200 family involvement in transitions from epithelial to messenchymal cells (EMT) as a step to metastatic cancer is explained in [59] and the references [63-65].

The list of breast tumor-suppressive and metastasis-suppressive microRNAs from [56] is shown below in Table 1.

Table 1: List of major tumor suppressive microRNAs in breast cancer from [56].

miR-125b-Inhibits cell proliferation and differentiation, migration and invasion)
miR-205-Suppresses proliferation and invasion
miR-17-92 -Promotes cell antitumoral activity and reduces metastasis
miR-206 -Reduces migration, invasion and metastasis
miR-200-Reduces tumor growth, metastasis ref 38-40 [62-6 5]
miR-126-Reduces metastasis and angiogenesis
miR-335-Suppresses metastasis and migration
miR-31-Inhibits several steps of the invasion-metastasis cascade in breast cancer

miRNAs as Potential Biomarkers Specific to Different Breast Cancer Subtypes

Several oncogenic miRNAs are found to be specific to different breast cancer subtypes. (Oncogenic miRNAs Let-7f, Let-7c, and miR-10a are associated with luminal A, and miR155, miR-93, miR-18a, and miR-135b are associated with basal subtype [56]. On the other hand, miR-150 and miR-142-3p are associated with the HER2-positive subtype, while miR-153, miR-10b, miR-26a, and miR146a, are shown to be potential biomarkers of the triple negative subtype [56]. miR-4734 has also recently been identified in breast cancer by extensive next-generation sequencing analysis and encodes within the ERBB2/Her2 gene, which is upregulated in HER2-positive breast cancer patients [7]. At this point, however, there is no real agreement between authors regarding specific miRNAs biomarkers for each subtype.

A detailed early study to identify subtype-specific miRNAs in tumor samples was performed in [56 and references within] using pair-wise comparisons according to the single sample predictor (SSP) that defined molecular subtype classification. The results are shown in Table 2.

Table 2: Identification of subtype-specific miRNAs [53, 66] at P value cut-off level<0.01; and maximal false discovery rate, 15%.

Basal-like: hsa-miR-135b#, hsa-miR-135b, hsa-miR-934, hsa-miR-577, hsa-miR-501-5p, hsa-miR18a#, hsa-miR-92a, hsa-miR-106a, hsa-miR-17, hsa-miR-18b, hsa-miR-18a, hsa-miR-20a, hsa-miR-17#, hsa-miR-15b#, hsa-miR-19a, hsa-miR-500
Luminal A: hsa-miR-148a, hsa-miR-219-5p
Luminal B: hsa-miR-30d#, hsa-miR-30d, hsa-miR-342-3p
Normal-like: hsa-miR-136#, hsa-miR-497, hsa-miR-139-5p, hsa-miR-99a#, hsa-miR-145#, hsa-miR-195, hsa-miR-143, hsa-miR-145, hsa-miR-335, hsa-miR-125b-2#, hsa-miR-139-3p, hsa-miR-7-2#, hsa-miR-216b, hsa-miR-487b, hsa-miR-100, hsa-miR-410, hsa-miR-204, hsa-miR-376a, hsa-miR-99a, hsa-miR-337-3p, hsa-miR-27a#, hsa-miR-411, hsa-miR-656, hsa-miR- 495, hsa-miR-551b#, hsa-miR-770-5p, hsa-let-7b#, hsa-miR-378, hsa-miR-215, hsa-miR-127-3p, hsa-let-7c#, hsa-miR-379, hsa-miR-422a, hsa-miR432, hsa-miR-299-5p, hsa-miR-494, hsa-miR-378, hsa-miR-511, hsa-miR-23a#, hsa-miR-452

Large numbers of miRNAs for Basal-like subtype and especially for Normal-like subtype might indicate that these groups are not uniform and represent several, more specific subtypes. Some results shown in Table 2 are correlated with earlier data [67]. For example, miR-135b and miR-106a are upregulated in Basal-like breast cancers in both studies. Also, miR-100 and miR-145 show comparable expression patterns in both studies, with elevated expression in the Normal-like samples.

Molecular miRNA signatures, which distinguish between different breast cancer subtypes, were described for the first time by Blenkiron and colleagues (2007). The authors profiled 309 miRNAs in 93 breast tumors with different molecular subtypes and identified 31-46 miRNA signatures, which allowed them to be able to distinguish the different breast cancer subtypes.

Table 3 presents subtype-specific microRNAs found from three independent studies derived from analysis by Dvinge et al. [68], Blenkiron et al. [67], and de Rinaldis et al. [69]. An identified 31-miRNA signature was able to distinguish the different breast cancer subtypes. The differential miRNA expression resulted in a correct classification of basal versus luminal subtypes.

Table 3: Common subtype-specific microRNAs found by meta-analysis of three independent studies [56].

Luminal A	Basal	HER2	Normal-like
let-7c	miR-18a	miR-142-3p	miR-145
miR-10a	miR-135b	miR-150	miR-99a
let-7f	miR-93		miR-100
	miR-155		miR-130a
Common subtype-specific microRNAs (miRNAs) derived from analysis by Dvinge et al. 68, Blenkiron et al. 67, and de Rinaldis et al. 69. No common miRNAs for the luminal B subtype could be found			

miRNAs in HER2-Positive Breast Cancer (current study patient) and Cancer Resistance to Treatment

One of the earliest and broadly characterized miRNAs in HER2-positive breast cancer is miR-21. It is one of the oncogenic miRNAs that targets tumor suppressor genes and activates oncogenic transcription factors (see reviews and references in [6], 2019; [7], 2019, [47], 2010, and [56], 2015, where the detailed analysis and listing of references on miR-21 up-regulation can be found).

miR-21 is consistently overexpressed in many tumors, including in breast cancer when compared to the matched normal breast tissues, among 157 human miRNAs analyzed by real-time RT-PCR arrays. This suggests that miR-21 may function as an oncogene playing an important role in tumor genesis. This miRNA is also associated with invasive and metastatic breast cancer. miR-21 suppresses multiple anti-metastatic genes including tumor suppressor protein tropomyosin 1 (TPM1), which is known to be down regulated in breast cancer epithelial cell lines, as well as other tumor suppressive genes, including phosphatase and tensin homolog (PTEN) [47] that reduces invasiveness in metastatic breast cancer lines. These

findings further establish miR-21 as an oncogenic miRNA and suggest that miR-21 has a role not only in tumor growth but also in invasion and tumor metastasis by targeting multiple anti metastatic genes. Significantly higher expressions of miR-21 is found in invasive ductal carcinoma (IDC) compared with normal breast tissue, which positively associated with tumor size, stage and grade. A higher miR-21 expression also correlated with ER negativity and HER2 positivity.

miR-21 is overexpressed not only in breast tumor tissues but in sera of patients as well when compared with healthy individuals. The expression of circulating miR-21 in serum demonstrated it clinical significance. It is one of oncogenic miRNAs that is targeting tumor suppressor genes and activating oncogenic transcription factors. and it is associated with invasive and metastatic breast cancer [56] and [70]. It promotes invasion, metastasis, migration and epithelial- mesenchymal transition (EMT) [71] and can be used as potential biomarkers for the monitoring of breast cancer patients [57,72]. The current body of evidence indicates an important role for miR-21 as a predictive biomarker for resistance to treatment by trastuzumab [6].

miRNAs may Reverse Resistance to Drugs, and Represent Potential Therapeutic Targets for Cancer

Since miRNAs play regulatory roles in breast cancer progression they have the potential to reverse resistance to drugs [6]. The expression profiles of miRNAs in tamoxifen and trastuzumab-sensitive breast cancer cell lines by qRT-PCR-array analysis was performed in [6] to explain the common molecular mechanism and differences of these two drugs. Tamoxifen has been used for over 30 years to reduce the risk of breast cancer recurrence. However, HER2+ positive tumors show resistance to tamoxifen [73]. Trastuzumab (Herceptin) is a FDA-approved antibody currently used as a therapy for HER-2 overexpressing breast cancer patients.

Several studies have investigated the relationship between drugs and miRNAs. It was found that miR-210 levels in plasma might be associated with trastuzumab resistance in patients, and that trastuzumab affected the expression of miRNAs [6,74].

Since miRNAs may play effective roles in disease progression, they represent potential therapeutic targets for cancer as well. Modulating miRNA expression levels could provide effective diseases therapies, [6,75].

The authors in [6] have shown that HER2 signaling was one of the targets of miR-770-5p, and that overexpression of miR-770-5p potentiated the effect of trastuzumab. miR-770-5p expression level in breast tumor tissue is significantly downregulated in tumor samples compared to normal samples. Thus, upregulation of the expression of miR-770-5p in tumor cells is a strategy to explore its molecular function in cancer cells. HER2 protein levels decreased significantly when the cells were treated with a combination of trastuzumab and miR-770-5p.

MicroRNA profiling has helped in enhancing breast cancer classification and in stratifying patients according to their response to therapy [76]. The authors in [77] utilized miRNA microarrays to identify nine differentially expressed miRNAs between recurrent and nonrecurrent breast cancer patients and developed a 2-miRNA model using miR-4734 and miR-150-5p to generate prognostic signature that might be a reliable prognostic biomarker for patients with HER2-positive breast cancer. This signature successfully classified patients into two groups based on the risk of tumor recurrence, independent of clinical characteristics, and predicted the five-year disease-free survival, comparatively better than other clinicopathological factors, thus adding prognostic value to the TNM staging system (T describes the tumor, N describes nodes, and M-metastasis). While elevated miR-150 expression is associated with poorer clinical outcome in a triple negative breast cancer [78], miR-4734 has been recently identified in breast cancer by extensive next-generation sequencing analysis and encodes within the ERBB2/Her2 gene, which is upregulated in HER2-positive breast cancer patients [79].

In [29] the authors identified 13 differently expressed miRNAs in serum of HER2+ metastatic breast cancer patients with different response to trastuzumab and selected four miRNAs to predict survival using Lasso model, including miR-940 (from the tumor cells), and miR-451a, miR-16-5p and miR-17-3p from immune cells. The model predicts the therapeutic benefits of trastuzumab for HER2+ MBC patients. Overexpression of miR-940 or silencing miR-451a, miR-16-5p, or miR-17-35 (collectively housekeepers) reduced the sensitivity of tumor cells to trastuzumab. These four miRNAs are predictive biomarkers and also functional regulators of the sensitivity of breast cancer to trastuzumab. Thus, the data suggest that the serum-based 4-miRNA signature can effectively distinguish HER2+ metastatic breast cancer patients who are sensitive to trastuzumab from the resistant ones, which is an important practical result since the techniques of examining serum miRNAs are now widely used in clinics.

The references [80-83] describe miRNAs that reverse tamoxifen resistance and can be useful biomarkers for breast cancer survival.

Latest Progress

In the last 2-3 years, starting from 2018, medical research has exploded, with a great number of new publications focused on miRNA's role in cancers start and development, response to treatments, drug resistance, and all aspects of metastatic cancers. Several new reviews are currently available, some with more than 450 references; see [83,84,86].

Currently, miRBase database (release 22.1) reports information about 2656 mature miRNAs (http://www.mirbase.org/) with~ 2300 that have been validated in a recent study [87].

The involvement of miRNAs in breast cancer development and progression has been well demonstrated, thus miRNAs may be considered a potential diagnostic and prognostic markers, as well as therapeutic targets. To date, in November 2020, "miRNAs and breast cancer" keywords in PubMed database yielded 6227 papers, of which about 96% were published during the past decade (https://pubmed.ncbi.nlm.nih.gov/).

Application of Sub-Terahertz Vibrational Spectroscopy for analysis of HER2+ Breast Cancer progression (Vibratess)

Using the support from DOD SB Grants and Contracts, Vibratess has developed the frequency-domain spectroscopic instrument prototype (Vibr-2) operating at room temperature in the sub-

THz spectral region between 315 and 490 GHz, where multiple specific spectroscopic signatures from biological molecules, including microRNAs are present. This instrument with imaging capability includes an electronically tunable radiation source and a microdetector, both based on Schottky frequency multipliers (Virginia Diode, Inc), and a sub-micron precision motorized stage for three-dimensional scanning of the detector relative to a multi-channel chip sample holder (Figure 1). This instrument takes advantage of using a very strong local enhancement of the electro-magnetic field due to the discontinuity edge effect and the extraordinary transmission of a sub-wavelength-slit conductive structure with multiple channels in the sample holder chip [88-92]. This allows for increased coupling of the THz radiation with the sample biomaterials and improving the sensitivity [91]. The attached micro-syringe allows for accurate positioning of a micro liter of sample material onto a sample holder chip.

A sample holder is installed into the THz spectrometer (Figure 1) and a background frequency scan over the frequency range of the instrument is performed at one location on the channel array. Time for one scan is ~25 min at a standard spectral resolution. A micro-syringe needle is then positioned over this point to deposit a portion of sample material (normally ~ 0.03 µl, usually as a solution in isopropyl alcohol). Once the sample has dried for ~ 2 min, another scan is taken in the same location, where the background has been recorded. Based on the channel width in the array (10-15 µm), the channel depth ~ 3-5 µm, and the detector opening width (~200 µm), a sample volume of roughly 10 pL is interrogated in each spectroscopic measurement. Optical transmission (T) of a sample material is calculated as a ratio of a sample to a background scan signals. Absorbance (A), used to compare different sample materials, is calculated as -log (T).

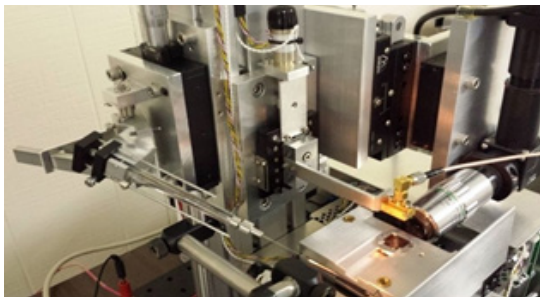

Figure 1: Vibratess spectrometer-Vibr2 with micro-syringe attached for accurate positioning of sample material. Micro-syringe is on the front left (not in dispensing position). Microfluidic chip with sample material is on the movable table (at right bottom of the picture) with the microdetector in a metal case (yellow) above it and the microscope objective behind it.

The instrument has been used for over 7 years to characterize sub-THz signatures of many biological materials [91-99], including comparisons of normal and cancer cells and tissue [100-105].

Experimental sample characterization was performed in parallel with simulation to predict the spectroscopic signatures for several miRNAs known from scientific publications to be important for breast cancer. The prediction results help us in confirmation and analysis of experimental signatures. The procedure to simulate absorption spectra of molecules is described in [90,97,102,105]. For the present study, saliva samples from the patient were taken before and after treatment procedure described above (section II). These samples were stored in a freezer at -16°C until use for characterization and mixed with isopropyl alcohol, if needed. The list of samples characterized can be found in the supplemental information.

Sigma-Plot program was used for data presentation. The most important results are presented below.

Figure 2 demonstrates example of signature from patient saliva sample using Vibratess Spectrometer (file E220) measured in 2016, 4 years before diagnosis of HER2+ and any treatment started. It also shows results scalability with material amount in the sample.

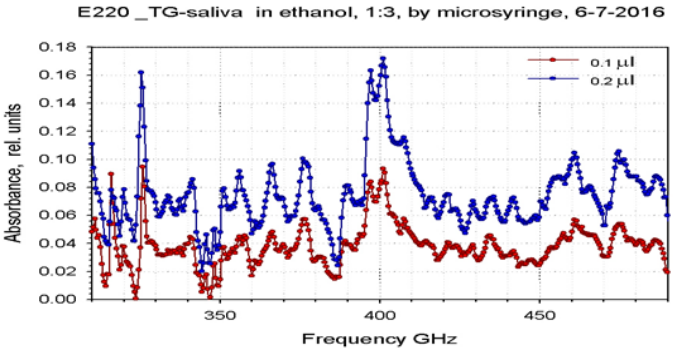

Figure 2: Scalability of absorbance measurement results: breast cancer samples in saliva.

Figure 3 compares spectroscopic signature from the same saliva sample shown in Figure 2 with absorbance spectrum of a commercially available breast cancer cell line sample (MDA-231), characterized at the same time as sample in the file E220. MDA-231 represents HER2- (negative) breast cancer subtype. The results demonstrate some similarities of the two shown spectra, especially the presence of most intense central features near 400 GHz, which might belong to molecular signature of breast cancer. If this is correct, at least two conclusions can be made from comparison of these two spectra in Figure 3. The presence of the specific structure near 400 GHz in both spectra might indicate that the patient already had breast cancer four years before diagnosis, although early mammograms had not detected the tumor. There are additional similarities below 380 GHz, as well as absorption peak at ~475 GHz However, there are enough differences to discriminate these two molecular signatures, including a very specific absorption structure between 380 and 390 GHz in MDA-231 that is absent in sample E220 signature. These differences might be explained since the two spectra in Figure 3 belongs to different subtypes: MDA-231 is HER2- negative subtype, and our patient was diagnosed 4 years later with a HER2 Positive Breast Cancer. The results presented in the following text will confirm this analysis.

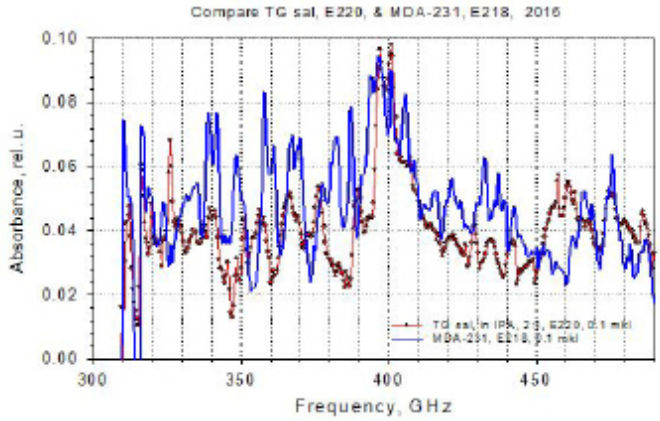

Figure 3: Absorption signature of TG saliva from Figure 2 (in red) compared with the spectrum of purchased material MDA-231 (in blue).

In our previously published work focused on epithelial ovarian cancer [102] we demonstrated the involvement of the microRNA-200 family (miR-200a, 200b, 200c, and miR-141) in serum, in accordance with many studies of other cancers too [103]. Expression levels of serum microRNAs are reproducible and consistent among individuals. In addition, microRNA signatures are different between ovarian carcinoma histotypes. We conducted molecular dynamics simulation in [102] to predict the sub-THz absorption signatures from specific microRNAs of the microRNA-200 family. Imperfect structures of the microRNA molecules without loop but with mismatches were used for simulation. Figure 4 gives simulation results for two molecules, miR-200a and miR-200c. Both microRNA molecules have strong absorption lines near 400 GHz and many other common absorption features for comparison with experimental spectra, for example at frequencies 355-360 GHz and 455-470 GHz in the sample E220 on Fig.ure 3. It might be that the pattern of combined spectra from several miRNA of this family simulated in [102] can be used as prognostic biomarkers for early breast cancer too.

The next set of spectra in Figure 5 demonstrates how the absorption signature from the patient saliva samples changed between 2016 (in red) and 2020 (green), files E220 and E243. In January 2020, the presence of a breast tumor was identified for the first time in the patient's mammogram which had been taken regularly once a year. This observed mass resulted in a breast cancer diagnosis. It is obvious that the molecular signature changed significantly between 2016 and 2020, likely due to disease progression. These changes occurred even before any treatment started after the initial diagnosis of breast cancer. The spectral changes include not only absorption peak intensity being reduced (green curve), but also changes in the absorption feature's structure around 400 GHz and beyond. Note that a much larger amount of material from the sample was required in 2020 to obtain the spectrum. These changes represent a significantly reduced contribution from the microRNA-200 family. This could be expected since the

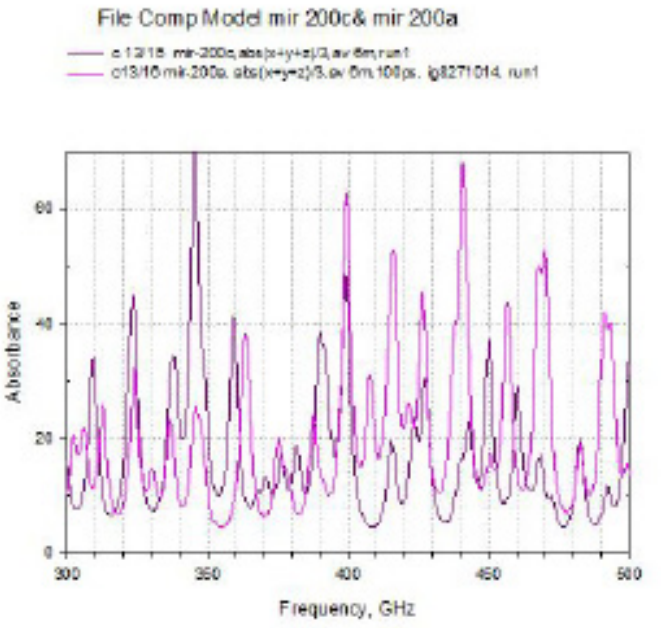

Figure 4: Simulated spectra of miRNAs 200a and 200c

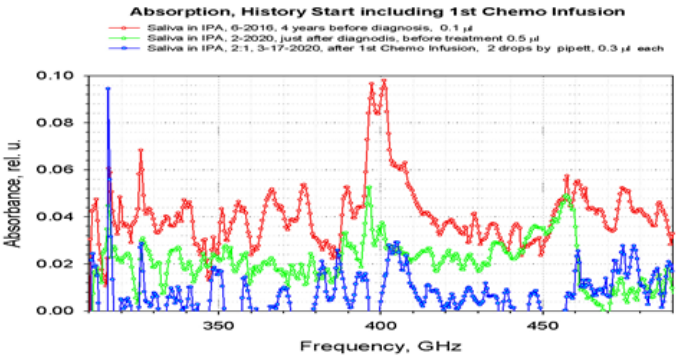

Figure 5: Absorption history (in scale): E220 (2016) in red, E243 (2020)-green before treatment started,and E248 (2020) - blue, after the first chemo-infusion.

miR-200 family have a tumor suppressive function (so-called housekeeping molecules), and the loss of these molecules agrees with the global downregulating of these molecules associated with cancer progress (see table I above and references 38-40 in [55,61,63]).

After the first infusion of chemotherapy a sample was collected the same day and the measurement results are also shown in Figure 5 (blue). The central peak at 400 GHz has completely disappeared, and the entire signature completely changed with significantly reduced intensity of absorption peaks over the entire spectral range (in scale). These results are not surprising and could be expected. From this point after lost of all protective microRNAs, only the chemotherapy can help a patient.

Our sub-THz spectroscopic analysis has confirmed that Chemotherapy is a very powerful technology and has suggested that not only is the chemotherapy affecting the tumor/cancer, but other signatures in the spectra potentially due to RNA viruses - such as the coronavirus, might also be affected by the chemotherapy treatment.

Conclusion

The results from the sub-THz spectroscopy performed in this work demonstrate dramatical modifications of the spectroscopic signatures from patient samples following disease development and the very initial steps in the course of treatment. These modifications are due to deep global regulation (reductions) of the initially participating microRNAs in general agreement with the types of microRNA changes discussed in Section III: MicroRNA and Breast Cancer. Literature Review.

In this collaborative work we demonstrate that clinical implementation of Sub-THz resonance spectroscopy for fast visualization and quantification of molecules in body fluids provides a complimentary approach to traditional analysis. The details provided by the spectroscopic analysis could revolutionize the discovery of effective new molecular biomarkers for clinical diagnosis of early-stage breast cancer.Sub-THz spectral signatures could also be useful in helping with disease classification, allowing doctors to follow disease progression, potentially predict the resistance to treatment, and indicate metastasis of the cancer to new sites. The experimental results confirm our earlier results for ovarian cancers [102,105-107]. which identified spectroscopic signatures in this spectral range from molecules circulating in bodily fluids as exclusively representing extra-cellular small RNA molecules released from patient cells.

Spectral signature results correlating with a known disease state warrant further investigation and suggest that continued analysis of experimental results from samples collected throughout the course of treatment may provide additional insights. Such insights may reveal the utility of clinical sub-THz spectroscopy to follow the course of treatment especially for metastatic breast cancer and detail how well a particular course of treatment is working for each patient.

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Supplemental Information

The list of samples used in this research for supplemental information.

NN	Sample material, when prepared	When used	Preparation details	Files of measurement
1	Breast cancer cells purchased, MDA-231	5-20-2016	300 c/uL in IPA MicroSyr@0.05uL dr	E217
2	Breast cancer cells purchased, MDA-231	5-23-2016 5-24-2016	MicroSyr@0.05uL dr	E218
3	Breast cancer cells purchased, MDA-231	6-1-2016	--"---	E219
4	TG-salava in ethanol 1:3	6-6-2016	---"--	E220
5	BP-saliva	6-7-2016	--'--	E221
6	TG-urine	6-17-2016	---"---	E222
7	---"---	6-20-2016	---"---	E223
8	Breast cancer cells purchased, MDA-231	8-24-2016	300 c/uL, 0.1 uL	E228 round chip
9	---"--	8-25-2016	300 c/uL, MicroSyr @ 0.1uL	E229 big circle chip
10	---"--	8-27-2016 8-29-2016	MicroSyr @ 0.1 uL 0.1 uL	E230 chip circle 8-25-16
11	---"--	8-30-2016		E231 --"--
12	---"--	8-31-2016	MicroSyr@0.1 uL	E232 --"--
13	---"--	9-1-2016	MicroSyr@0.1uL	E233 -"--
14	TG-U2	05-03, 2018	0.5 uL by micropipette	E238, chip copper foil 5-6-2016
15	Wax-TG in Isopropil alcohol+ water 1:1	06-03-2018	Drop wooden stick	E239, chip copper foil 5-6-2016
16	Wax BG bad eah, in IPA & a little water	06-13-2018 06-16-2018	Drop wooden stick	E240, chip-negative resist E240, water add

2020				
17	TG saliva in Isopropil	02-22-2020	0.5 uL by micropipette	E243 chip
18	TG saliva in IPA taken	03-07-2020		E244-06 chip, Positive resist,
19	TG Saliva in IPA taken before start treatment in MJH at 03-13-2020	03-07-2020		E244-06, chip Pos. resist, 5-18-18
20	TG Saliva in IPA taken before start treatment 03-13-2020			E245, same chip and sample as E244
21	No sample material Signal vs sample hight position	03-14-2020		E246-E247
22	TG-saliva in IPA, 2:1 Taken on 3-13-20, just after the 1st infusion (3-13-20) & before the 2nd (3-20-20)	Used 03-17-2020 03-19-2020 03-21-2020	0.3 ul by micropipette New portion of the same 2 more drops 0.3 ul each	E248, col 07-14 E248, col 15-25 E248, col 26-30 E248, col 31-36
23	TG saliva in IPA, 2:1. Prep 3-19-2020 just before the 2nd infus.(3-20). Same type chip, same position	03-19-2020	0.3 ul by micropipette Sal TG 3-19-20, 2:1 in IPA	E249, col 09-11
24	TG-saliva in IPA, 2:1. Prep 3-19-2020, just before the 2nd infusion. 3-20-2020	03-28-2020	Microsyringe 0.03 ul	E250, col 04-07