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REVIEW

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A review of the applications of Raman spectroscopy for breast cancer tissue diagnostic and their histopathological classification of epithelial to mesenchymal transition

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Abstract

Breast cancer is one of the leading cancers in women worldwide. Notwithstanding the clear advances being made in treatment, early diagnosis of the disease can certainly be expected to reduce morbidity and mortality. With increasing evidence of the role of epithelial to mesenchymal transition (EMT) in tumour progression, early detection of this phenomenon is suggested to be important given that the majority of breast cancer deaths are due to tumour invasion and metastasis. Although histopathology and biomedical imaging techniques continue to be used as standard procedures in breast cancer diagnosis, these techniques have a number of disadvantages, including being time-consuming, the imaging in particular having attendant limited resolution, sensitivity, and specificity, leading to results that are prone to errors in human interpretation. Due to its rapidity and high specificity, Raman spectroscopy has emerged as a diagnostic tool for breast cancer, useful in identifying malignancy of breast cells, correlated with the EMT phenotype, expressed at the molecular level. Detailed biochemical information from tissue biopsies can also be provided from use of this technique. The use of Raman spectroscopy in breast cancer investigations over the past 10 years and more, including in the study of EMT, is reviewed in the present work also listing the corresponding Raman peaks reported in the literature in seeking to better facilitate identification of peaks of interest.

KEYWORDS

breast cancer, diagnostic, epithelial to mesenchymal transition (EMT), molecular characterisation, Raman spectroscopy

1 | INTRODUCTION

In women within the age range of 33–55 years, breast cancer is the second most prevalent cause of cancer death, preceded only by lung cancer.[1,2] Many factors, including genetics, are involved in the initiation of breast cancer, but mortality is largely due to metastatic disease. The heterogeneity of breast cancers adds to a less than comprehensive description of progression of the biological features of the disease at first diagnosis, although the different biological behaviours and metastatic patterns observed among the distinct breast cancer phenotypes may suggest different mechanisms of invasion and metastasis. Thus said, specific genomic alterations have

been utilised by other researchers[3–6] to define subtypes of breast cancer. However, the large number of genetic alterations present in tumour cells complicates the discrimination between genes that are critical for preserving the disease state and those that are merely coincidental. However, if breast cancer is detected and treated in its initial phase, statistics indicate some 90% of the patients can be cured.[7]

The gold standard for diagnosis of breast cancer usually involves fine needle aspiration cytology, core needle biopsy, and surgical excision followed by histopathological optical microscopic analysis of the excised tissue. Although the methods are well established, they require accurate sample preparation and expert breast pathologist interpretation. As such, pathological diagnoses are to some degree subjective, variability among pathologists having been observed in clinical practice. The pathologist looks for particular morphological features or patterns in order to determine malignancy, each of which is associated with biochemical changes in the tissue. Concomitant with the latter, nanoscale changes in the structure associated with disease can also be used to discriminate between different tissue types, highlighting the importance of the tumour-associated stroma in breast cancer initiation and progression. The main component of such stroma is abnormal collagen, the orientation and amount of which can directly influence epithelial growth and transition, including response to neoadjuvant therapy.[8–10] The mechanism is similar to the epithelial to mesenchymal transition (EMT) process that has been characterised in embryonic development.[11] By means of EMT, the epithelial cancer cells acquire molecular alterations that facilitate the loss of epithelial features and gain of mesenchymal phenotype. Such transformation promotes cancer cell escape from the primary tumour and dissemination into the circulation. Despite recent advances and technologies, the EMT phenomenon in breast cancer progression remains poorly understood as multistep and complex processes are involved, with EMT representing only part of the tumour invasion and metastasis process.[12]

At present, studies on developing techniques and their optimisation for diagnosing breast cancer have been undertaken by several groups. Various techniques have been investigated, for instance, X-ray mammography, ultrasonography, positron emission tomography, and magnetic resonance imaging (MRI), all of help in detecting tumour presence and supporting analysis of changes in tissue morphology and composition. However, none of these techniques can both localise suspect areas and characterise the morphological and compositional changes in a single measurement without the use of contrast agents. As an instance, there is little or no contrast between X-ray attenuation properties of normal and abnormal collagen. In addition, these conventional techniques display several limitations, providing poor spatial resolution and relatively low sensitivity and specificity, the outcome of which is that they are unable to interrogate the classification of malignancy of a tumour.

Development of a technique in identifying abnormal collagen could have profound impact on breast cancer diagnosis and treatment; it being suggested that Raman spectroscopy (RS) could provide quantitative biochemical and morphological information about tissue composition in situ, comparable to the information used by a pathologist. As the metabolic features in breast tumour cells plays a crucial role in cancer progression, there exists a need to establish alternative methods that are user-friendly, cost-effective, rapid, and unambiguous for medical diagnosis of metastatic breast cancer cells. RS can provide for this based entirely on biochemical analysis of vibrational properties of tissue, the instrumentation being now available in a sufficiently compact and manageable form to make it an entirely realistic prospect as an effective tool within the pathology lab.

RS has unique potentials in the study of breast cancer as it can provide sensitive, quantitative, and specific chemical information of biological components in cellular and tissue samples.[13] Tumour cells commonly show specific changes in protein, lipid, nucleic acid, and carbohydrate quantities and/or conformation.[14,15] RS can potentially identify the functional groups and chemical bonds that are present in the tissues and/or cells. Hence, in particular, it is possible to evaluate the structure of the protein, lipids, nucleic acids, and carbohydrates in the tissues and/or cells and also the changes in their chemical structure at the molecular level due to the process of cancer metastasis. The application of RS in breast cancer research has been widespread,[16–20]; however, to our knowledge, limited studies have been carried out on EMT in breast cancer tissue. Thus herein, in assessing the RS technique, it can be summarised that molecular characterisation of breast cancer tissue can be analysed accurately, distinguishing between normal and cancerous types, as well as to understand the mechanism that drive transformation of a EMT phenomenon, and as a possible diagnostic tool.

The present work will review the role of EMT in cancer and previous attempts by a number of researchers over the past decade and more in seeking demonstration of the existence of EMT in breast cancer. The principle of RS and its application in the study of breast cancer and EMT in breast tissue are outlined, including listing of Raman peaks reported by others in such previous investigations.

2 | THE PRINCIPLE OF EMT

EMT is defined as a biological process in which epithelial cells lose their cell polarity and cell–cell adhesion and become mesenchymal cells. EMT has been classified into three types. Type 1 EMT occurs during embryogenesis and organ development.[21–23] Type 2 EMT is involved in wound healing, organ fibrosis, and tissue regeneration.[21,22,24] Type 3 EMT is involved in cancer progression occurring in epithelial cancer cells. The presence of the EMT process in cancer suggests a critical role in metastatic progression.[21,22,24] Over the past few decades, intensive studies have been made to understand how cancer metastasises, with emerging work suggesting that EMT has an important role in progression of cancer. Notably, the majority of human malignant tumours develop from epithelial tissue. During epithelial cancer progression, cells lose epithelial features and gain mesenchymal characteristics, enabling the cancer cells to spread and express invasive properties. The tumour cells leave the initial site and invade the basement membrane. Then, the tumour cells intravasate into blood or lymph vessels and move through the circulation to distant organs. The cells then extravasate from the circulation and move into the secondary site. At the secondary site, the cells can extravasate and either form micrometastasis and stay dormant, or they can grow and form a new tumour colony (macrometastasis) through mesenchymal to epithelial transition (MET).[25–27] Initiation of metastasis requires the carcinoma cells to invade and spread to another distant organ, enabled by EMT–MET. The EMT and MET involvement in metastatic processes is illustrated in Figure 1.

As most of the evidence linking breast cancer progression with EMT is evaluated from cell lines and/or animal models, the relevance in human cancer tissues is not well established and still under debate.[28] One of the possible reasons that has made EMT difficult to be precisely identified in human tissue samples is the reversibility of the EMT–MET process. MET is a reverse process of EMT, converting migratory mesenchymal cells to polarised epithelial cells. EMT is necessary in migration and invasion, whereas MET is necessary to colonise the metastatic site.[29,30] Evaluating EMT in human cancer tissue is a difficult task as tissue samples obtained may have either not yet undergone EMT or have undergone the reverse process MET, respectively.[24,28,31] The role of EMT in cancer is further complicated as EMT is not a uniform process defined by a single pathway. Several signalling pathways of EMT have been discovered in tumour progression. The process of EMT involves a loss of epithelial markers such as E-cadherin, claudin, occludin, plakophilin, cytokeratin, and desmoplakins, gaining mesenchymal markers such as Vim1 (Vimentin), SNA1 (sometimes referred to as Snail), N-cadherin, Zeb 1, and Zeb 2. [22,32] A summary of EMT markers and roles is provided in Table S1 (supporting information).

2.1 | EMT in breast cancer

The majority of deaths (~90%) among breast cancer patients result from invasion and metastasis, two features that are related to EMT.[29,33] The underlying and multiple causes of remaining 10% deaths in breast cancer patients include external causes (e.g., accidents, suicide,

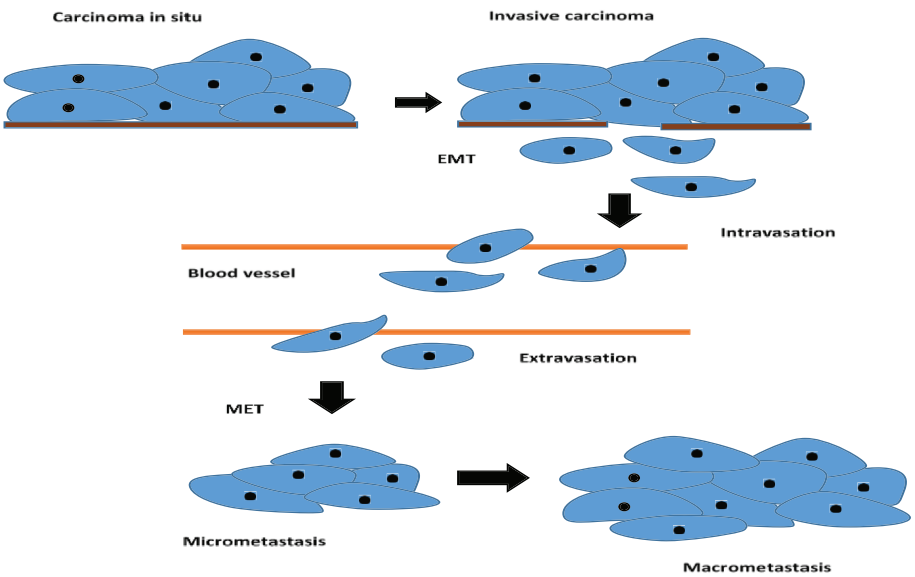

FIGURE 1 The involvement of EMT and MET in the metastatic process in cancer. EMT, epithelial to mesenchymal transition; MET, mesenchymal to epithelial transition

and iatrogenic causes), heart failure, diseases of pulmonary circulation, and gastrointestinal disease.[34,35] To date, the EMT process seems to have been best known in regard to the metastases of epithelial cancers including breast cancer, with evidence building substantially. In epithelial cancers, the EMT process is currently characterised based on the loss of some epithelial markers (downregulation of E-cadherin, occludins and claudins, desmoplakin, and epithelial cytokeratins, including 8, 18, and 19) and the acquisition of new mesenchymal markers (up-regulation of vimentin, N-cadherin, and fibronectin) both by western blot analysis and immunohistochemistry.[24,36–38] A number of experiments suggests that EMT is an intrinsic component of neoplastic progression to invasion and metastases,[39–41] citing an indicative number of published studies, as in Table S2 (supporting information). Overall, previous investigations of EMT in breast tissue [see Table S1 (supporting information)], suggest that EMT markers are associated with the invasion and metastasis of breast cancer. Based on the prototypical spindle-shaped morphology of fibroblasts and histopathological analysis, these can apparently differentiate between fibroblasts and epithelial cancer cells. However, epithelial cells that have undergone EMT and have transformed to the mesenchymal type of cells are difficult to distinguish from fibroblasts.[25] Hence, in current clinical studies, it has remained a challenge to assess EMT in tumour progression. Verifying biomarkers related to EMT and use of recent technologies and strategies are expected to be highly important for targeting EMT-associated cancer progression and in developing better therapies. As shown in Table 2, evidence linking EMT to breast cancer progression has been obtained via in vitro studies in cell lines and blood samples and via in vivo studies using mouse models of breast cancers. Hence, pathologic significance of EMT in human cancer remains unclear and is still being debated.[28] Regardless of current technologies, the EMT process in cancer progression and metastasis remains obscure, with cancer involving multistep processes with EMT being just one part of this. Analysing gene expression and validating biomarkers that induce EMT process in human cancer are also expected to shed further light on breast cancer progression and metastasis and identification of more effective treatment protocols to inhibit metastasis. Hence, it is highly crucial to develop a technique that can be used to monitor and analyse the EMT process at the molecular level. RS would seem well-suited to this purpose, further acknowledging its ability for rapid analysis, retaining high sensitivity and specificity in the early detection and diagnosis of metastatic breast cancer cells.[42]

3 | THE UNDERLYING PHYSICS OF RAMAN SPECTROSCOPY

Optical spectroscopy, extensively studied as a potential in vivo diagnostic tool, can provide information about both the chemical and morphological structures of tissue in near real time. Although most in vivo studies have concentrated on elastic scattering and fluorescence spectroscopies obtained rapidly with good signal-to-noise ratios, RS, an inelastic scattering process, has also been demonstrated to be a highly effective diagnostic tool. The spectrally narrow features of RS offer relationships with the specific molecular structure of the sample and for present interests allows exploration of the lipid phenotype associated with breast cancer malignancy. Of associated importance is that over the past decade, RS instrumentation has become available in compact form, sufficiently so that it now provides for the entirely realistic prospect of such instrumentation being established within the typical pathology lab setup.

If one may briefly reflect on how optical spectroscopy has come into existence, the first published report dates back to the 17th century, Isaac Newton using a glass prism to obtain spectral dispersion, the term spectrum being introduced in the classic volume Opticks.[14] In due course, discovery has given rise to a range of analytic techniques that for present purposes allows investigation of interactions of electromagnetic radiation with atoms and molecules The vibrational technique of RS based on inelastic light scattering (atoms and molecules being set into vibration as a result of use of an incident interrogating beam) allows identification of chemical structural and physical features, altered from that of the characteristic spectral pattern of a pristine substance, a fingerprinting analysis.

RS light scattering involves a two-step process, that of the incident and scattered photons. Although most scattered photons have same energy as that of incident photons, referred to as elastic or Rayleigh scattering, occasionally the scattered photons gain or lose energy resulting in wavenumber shift. This shift results from inelastic scattering (resonant and nonresonant Raman scattering, the details of which do not require present description). The relatively weak phenomenon of Raman scattering, involving approximately one in every 10^6–10^8 scattered photons,[43,44] was first predicted by Adolf Smekal in 1923 [45] and was first observed experimentally in 1928 by Raman and Krishnan.[46] When illuminated with an optical beam, the technique can be used to probe the vibrational energy levels of molecules within a sample. When this occurs, the photon donates energy to, or receives energy from the molecule, producing a change in the molecular vibrational state, the exiting photon

having an altered energy level and thus an altered wavelength. This change in photon energy, known as the "Raman shift," is measured in wavenumbers. Photons interacting with different biochemical bonds undergo specific Raman shifts that, when considered together, form the Raman spectrum of scattered intensity as a function of energy difference between incident and scattered photons (the Raman shift), a direct function of the molecular composition of the material studied. The loss (or gain) in photon energies corresponds to the difference in the final and initial vibrational energy levels of molecules participating in the interaction. Typically, Raman peaks are spectrally narrow (of the order of just a few wavenumbers) and in many cases can be associated with the vibration of a particular chemical bond (in normal mode dominated by the vibration of a single functional group) in a molecule.

Notably, RS is a rapid, reagent-free and nondestructive alternative for the analysis of cell biology systems Because of these advantages, RS has been used extensively in biology and biochemistry to study the structure and dynamic properties of important molecules in solution, single living cells in cell cultures, and more recently in tissues. When applied to biological tissue, the technique can distinguish between pathologies based on the differences in their biochemical makeup. Recent advances in RS have given rise to a wide range of biomedical applications including in regard to cancer diagnostics. Its ability to detect variance related to DNA/RNA, proteins, and lipids have also made it an excellent tool for quantifying changes on the cellular level, as well as differentiating between various cell fingerprints over the entire Raman spectral range. The collection of spectra can be performed in vitro, ex vivo, or in vivo without disrupting the cellular environment. This is a major advantage, as most biological assays utilise chemical biomarkers and often require conditions non-native to the biological environment. The positions and relative intensities of various spectral bands can be used to probe primary, secondary, tertiary, and quaternary structures of large biological molecules. The fingerprint spectral region, from approximately 700 to 1,900 cm^{-1}, contains a series of sharp bands that can be used to characterise a particular molecule and in some cases to identify the composition of complex, multicomponent samples.

Figure 2 shows a schematic and functional diagram of the main components of a typical Raman spectrometer, the assembled system consisting of an excitation source (monochromatic and most typically that offered by a laser), the optical path system, the Rayleigh (elastic scattering) rejection filter, a monochromator/dispersing element (spectrograph), and a detector. Through use of the excitation source, the sample is irradiated with monochromatic light that impacts on and thereby interacts with sample molecules, most essentially with the electron cloud and bonds (setting them into vibration), resulting in scattered light. The spectrometer processes this scattered light, eliminating the unwanted portion (as in for instance the elastically scattered component) and dispersing the remainder into its constituent wavelengths. Given that the intensity of the Raman (inelastic) scattering component is very weak compared to that of the elastic component, a notch filter or high performance long pass filter is used to block the Rayleigh-scattered light because otherwise this would easily overpower the weak signal of the Raman-scattered light. The Raman-scattered light is then detected, most typically through use of a deep depletion charge-coupled device detector. Finally, through use of a computer, a Raman spectrum in digital form is displayed, generally as a function of energy (frequency) difference between incident and scattered photons. The wavenumber

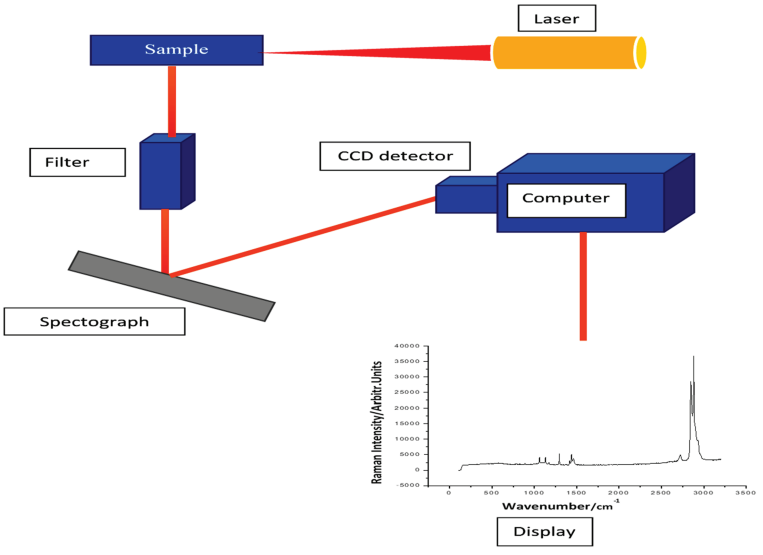

FIGURE 2 Schematic diagram of Raman spectrometer and a resulting Raman spectrum

difference between incident light and Raman-scattered light is known as the Raman shift, as previously mentioned. The Raman shift, expressed in wavenumber (per centimetre), is unique for individual molecules of a particular type. The molecules in the material have specific vibrational modes allowing the Raman spectrum of each material to be used for material identification and analysis.

4 | THE APPLICATION ON RAMAN SPECTROSCOPY ON BREAST CANCER AND DETECTION OF EMT IN BREAST CANCER

That RS is capable of differentiating noncancerous, precancerous, and cancerous tissues at high sensitivity and specificity also points to the possibility of harnessing the technology in investigations of cases that are symptomatic of cancer, as an adjunct in the early detection of malignancy. However, the lesions identified using this technique will have been from biopsy samples, the samples also being evaluated histopathologically to determine the presence of malignancy. Contrasting with this is a number of current screening techniques for breast cancer detection, including mammography, ultrasound, and MRI. In evaluation of the performance of such modalities, each of the techniques has associated drawbacks and inaccuracies.[14,47–49] For example, although mammography is the most common technique for breast cancer diagnosis, 10–14% of clinical diagnosis of cancers are not detectable using this approach.[14,49] Additionally, mammography can result in high false positive or negative outcomes, the prevailing sensitivity and specificity producing uncertainty.[50,51] Ultrasound imaging is not as sensitive or specific as mammography[14] and shares with MRI the problem of a low imaging resolution (3 mm in the case of MRI, 40 μm for digital mammogram techniques,[52] 70 μm for the amorphous silicon mammography,[53] and 1.0–1.5 mm for breast ultrasound imaging) and[54] MRI also being expensive.[50] The limitations of each of the modalities together with the potential albeit low radiation risk from mammography encourages development of a non-invasive, rapid, cost-effective, highly sensitive technique, notes being made of RS in breast cancer studies and also its potential as a diagnostic tool. Table S3 (supporting information) shows a survey of RS breast cancer research work carried out over the past decade, demonstrating the potential of RS to distinguish normal tissues in comparison with pathological tissues that achieved high sensitivity and specificity. The works also revealed that the normal breast tissue has higher content of lipids compared to benign and malignant tissues. In addition, the protein level was found higher in both benign and malignant tissues compared to normal tissues. The Raman spectra can be interrogated using principal component, linear-discriminant analysis[55] and also in an environments such as MATLAB® that compresses the information held by the spectra without losing any relevant data;[56] linear-discriminant analysis accentuates the differences in spectra measured from the different pathological groups, minimising these spectral differences within each group.[57]

Despite that the EMT process associated with tumour progression is now becoming well accepted, the molecular and cellular events that initiate the EMT have not been clearly elucidated. [58] The metabolism changes of EMT have been studied through analysis of mRNA and protein levels of key metabolic enzymes. Even though previous work on gene expression provides important understanding on metabolic changes during EMT in cancer, direct visualization of the relevant metabolites is lacking especially at the single-cell level. [59] In studies of EMT metabolic changes, important in developing cancer therapy strategies and drug resistance,[42,60,61] it is crucial to develop alternative analytical techniques, RS being well-positioned in this respect. Table S4 (supporting information) reviews key published literature focusing on RS of EMT in cancer, each demonstrating utility of the method. Generally, the studies listed in Table 4 showed the capability of RS in monitoring the biomolecular changes in EMT, and it was shown that the lipid content in the cells was correlated with the EMT process. The RS studies of EMT in breast cancer, although limited in number, all point to the considerable advantages of RS applied in breast cancer diagnostics and EMT studies [see Table S5 (supporting information)]. It is nevertheless apparent that further studies on the relationships between EMT markers and breast cancer are needed, helping to identify early stage breast cancer metastasis biomarkers.

5 | RAMAN CHARACTERISTIC PEAKS IN BREAST CANCER STUDIES

A detailed database of Raman peaks is fundamental to understanding the chemical fingerprint of samples, researchers largely depending on prior studies that have focused on defining the band assignments of the different peaks. Table S6 (supporting information) provides such information, detailing the major Raman peaks within the spectral range of interest to studies of breast cancer.

6 | CONCLUSION

Despite current technologies, breast cancer remains one of the most common causes of death among women throughout the world. Histopathology and biomedical imaging are the current techniques that have been used as common practice for breast cancer diagnosis. However, these techniques reflect some limitation and drawbacks such as being time-consuming, being expensive, having a poor resolution, and leading to vague results prone to human interpretations. The demand for highly sensitive, accurate, and rapid techniques urges the researchers to develop Raman spectroscopic techniques in breast cancer diagnosis. RS has emerged as a diagnostic tool in breast cancer studies due to its non-invasive, rapid, and high specificity and sensitivity. Over the past few decades, RS have been intensively used to distinguish between normal, benign, and malignant breast tissue based on the chemical changes (e.g., in lipids, proteins, and nucleic acids) in the molecular level.

Many studies have been conducted to provide the evidence of EMT in breast cancer progression and metastasis in vitro (cell lines) and in vivo (mouse models). However, the relevance of EMT in human cancer is much unknown due to its difficulty to identify the reversibility of EMT–MET in human samples. Based on previous studies, RS proved to be a useful technique to monitor the EMT process in cancer progression and metastasis. More studies need to be done as the study on EMT by means of RS seems to be limited specifically in breast cancer.

In this article, we have reviewed the applications of RS in breast cancer studies including the process of EMT in cancer progression and metastasis for the past 10 years. The previous conducted studies show that the RS is feasible to distinguish normal and pathological breast tissues. The RS has shown a high ability to stratify malignancy of breast cell that is correlated with the EMT phenotype, and this technique is capable to monitor the biochemical composition of breast cancer cells during EMT process. We also provided a list of Raman peaks reported in previous studies of breast cancer by means of Raman spectroscopic methods. We believed this list will help other researchers to define Raman peaks obtained in future studies on breast cancer by means of RS.

However, more work is still required before this method can be used in clinical practice, requiring comprehensive research in translating Raman data as the current technique of spectroscopic needs to employ several multivariate statistical tools. Therefore, it is crucial to develop a convenient methodology to standardise protocol for data acquisition and data analysis into the clinical setting being easily interpretable and producing rapid and accurate results. Another interesting work in RS research should be done comprehensively is the real time early stage cancer diagnosis by using the optical fibres coupled with a biopsy needle and incorporated into Raman spectrometer. A number of studies have already demonstrated the effectiveness of so called "optical needle biopsies."[62–64] In vivo Raman spectroscopic diagnosis is capable of providing rapid diagnosis, reducing the number of biopsies and high-resolution information of the biomarkers responsible for the cancer progression. Hence, this in vivo method needs to be developed and fabricated for the clinical practice.

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