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Abstract. Tryptophan is investigated as the key native marker in cells to determine the level of metastasis competence in breast cell lines using native fluorescence spectroscopy. The ratio of fluorescence intensity at 340 nm to intensity at 460 nm is associated with aggressiveness of the cancer cells. We found that the fluorescence of aggressive breast cancer cell has a much higher contribution from tryptophan compared with that from the normal cells and nonaggressive breast cancer cell. © 2014 Society of Photo-Optical Instrumentation Engineers (SPIE) [DOI: 10.1117/1.JBO.19.3.037005]

1 Introduction

The ability for cancer tumors to metastasis is a fateful characteristic of certain malignant tumors, which accounts for the majority of cancer-related deaths. Metastasis is the primary cause of mortalities among cancer patients due to spreading of cancers to various organs. One third of the people will receive a diagnostic of cancer during one's life, and one third of them will die of this cancer due to metastasis.[1] In 2007, ~12.7 million cancer cases and 7.6 million cancer deaths are estimated to have occurred, which is the second most common cause of death only after heart disease. Moreover, breast cancer is the leading cause of cancer death among females.[2] There is an active search by scientists to find methods to determine aggressive cancer *in situ* worldwide. One promising method to diagnose cancers without removing tissue is based on optical spectroscopy.[3–6] The field using optical spectroscopy for biomedical samples has been coined "optical biopsy," which is becoming commonplace to determine the state of tissue *in vivo* and *ex vivo*. To characterize the properties of normal, benign, and malignant cancers from tissue and cells, the major focus in optical biopsy is by measuring native fluorescence (NFL) spectra, also termed autofluorescence, and by measuring Raman spectroscopy.[3–11] The main fluorophores building blocks in tissues are tryptophan, reduced nicotinamide adenine dinucleotide (NADH), flavin adenine dinucleotide (FAD), collagen, and elastin. The latter two molecules are present in tissue, not in cells. Tryptophans are the essential amino acid for all the cells. NADH and FAD are two of the principal electron donors and acceptors in cellular metabolism, respectively. These molecules may appear in different amounts in tumor evolution and structure and these changes can be revealed by NFL spectra. These key intrinsic molecules in cells and tissues have unique spectral profiles for absorption and emission from the ultraviolet to visible range.[3–5,12–14] The emission from tryptophan is clearly the main fluorescence over the other molecules upon exciting the tissues and cells using the light at $\sim \leq 300$ nm.[4]

The objective of this study is to demonstrate that tryptophan level in cells is an important biomarker for determining aggressiveness by studying two different breast cancer cell lines and one normal cell line. The NFL spectroscopy is used as an effective approach to distinguish two types of breast cancer cell lines with different metastatic ability as well as normal cell line based on their tryptophan level. Upon excitation with 300 nm, the emission spectrum was measured and the normalized tryptophan level was obtained by calculating the ratio of tryptophan intensity at 340 nm to intensity at 460 nm for various breast cell lines. The experiment and analysis results indicate that the relative content of tryptophan reflected by NFL can be used to determine the aggressive nature of cancer metastasis. Our study provides a possible diagnosis method that the relative content of tryptophan in cell indicates the metastasis competence and the risk level of cancer in the patients.

2 Material and Method

2.1 Sample Preparation and Cell Lines

Three cell lines were used in this study: normal human fibroblast cells (Coriell CellTech, New Jersey),[15] nonaggressive breast cancer cells MCF-7 (ATCC, Virginia),[16] and aggressive breast cancer cells MB-MDA-231 (ATCC, Manassas, Virginia).[17] The MB-MDA-231 and fibroblastic cells were cultured in Dulbecco's modified Eagle's medium (Sigma, Missouri),

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supplemented with 10% fetal bovine serum (FBS) (Thermo Scientific, Massachusetts), 3% penicillin streptomycin, and 1% L-glutamine (Sigma, Missouri). The MCF-7 cells were cultured in minimum essential medium Eagle (Sigma, Missouri), with 10% FBS, 2 mM L-glutamine, 1.5 g/L NaHCO$_3$, 4.5 g/L glucose, 0.1 mM nonessential amino acid (NEAA), and 1.0 mM Na-pyruvate. All cell lines were incubated at 37°C under a 5% CO$_2$ atmosphere. At >95% cell confluence (3 to 5 days), the cells were harvested by the treatment with 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA) (Sigma, Missouri) within 1 min and were washed away from the bottom of the flask with 5 ml cell culture media. Centrifuge the solutions at 125 g for 5 min and aspirate the supernatant with remaining trypsin; at that time, the remaining centrifuged cells is only ∼100 ul. Resuspend the cells again with 5 ml phosphate buffered saline (PBS, Sigma, Missouri), centrifuge the solution, and aspirate the supernatant (almost all the solution), and this time the remaining centrifuged cells is only ∼100 ul. Resuspend the cell to ∼3.6 × 10^6 cells/ml with PBS. Based on the above steps, the concentration of the trypsin-EDTA was diluted from 0.25 to ∼0.0001%; few trace of trypsin remains. The cells were transferred to a $1 \times 1 \times 4$ cm^3 (the inside dimensions) quartz cuvette (NSG Precision Cells, New York) for the fluorescence experiments. The cell suspensions were vortexed evenly before each measurement was taken. Trypan blue solution was used to estimate the living cells rate before and after the experiment.

2.2 Measurement Instrument

The measurements of NFL spectra of the cells were performed by using the Fluorolog®-3 spectrofluorometer system. The excitation light with 5 nm spectral width was focused on samples with a spatial size of ∼3 × ∼1 mm^2 and 0.5 μW power deposition. Each scan was <1 min at a scan speed of 300 nm per minute. The fluorescence was collected with a spectral resolution of ∼1 nm. To check background fluorescence due to cell sample preparation, the emission spectra of the quartz cuvette and supernatant from cell samples were also measured. The intensity was only 0.1 to 1.5% compared with the intensity at 340 nm and was also subtracted from the cell suspension spectrum for final results. The 300-nm excitation was selected because, among all the fluorophores, tryptophan has a higher quantum yield (QY) of 0.2 to 0.35 when excited at ∼300 nm than the other fluorophores, such as QY for NADH of 0.019 to 0.05.[18] The relative tryptophan levels were calculated by using the ratio of intensities at 340 over 460 nm as reference signal for each sample type.

2.3 Data Analyzing Methods: Support Vector Machine

To classify the metastasis competence between these three types of cell lines, support vector machine (SVM) is used to categorize the three groups of data. SVM is one of the most useful techniques for data classification.[19,20] In general, the SVM classifier is determined by a number of components for most effectively discriminating the support vectors, which are located at the boundary of the group of data. The goal of the SVM is to find an optimal $n - 1$ hyperplane to separate the data into two categories for an n-dimensional vector.[19] In this study, $n = 2$; the hyperplane is, thus, a scalar value. By using the ratio obtained from NFL spectra, a classifier, a

hyperplane $w^T s = b$, in the subspace is obtained by SVM with a linear kernel. Our previous study has already presented[21] in detail the SVM analysis by using MATLAB®, and the codes are available at http://www-ee.engr.ccny.cuny.edu/www/web/ysun/NanoscopeLab/Codes/BSS-JBO2012/BSS.htm. The sensitivity and specificity would be calculated. Subsequently, the receiver operating characteristic (ROC) curve was generated to further evaluate the performance of SVM.

3 Results and Discussion

3.1 Native Fluorescence Spectra of Fibroblast Cells, MCF-7 Breast Cancer Cells and MDA-MB-231 Breast Cancer Cells

The average fluorescence spectral profiles in the range of 320 to 580 nm from fibroblast cells ($n = 16$), MCF-7 cells ($n = 17$), and MDA-MB-231 cells ($n = 18$) under 300-nm excitation wavelength are displayed in Fig. 1 as solid, dash, and dash-dot lines, respectively. Fifty-one samples were run. It shows that the spectra have consistency for the same type of cells, but significant differences exist among these three types of cells. The living cells rate before and after the measurement was estimated by trypan blue and it was 99 and 98%, respectively. We believe that all the measurement was performed on living cells.

The main emission peaks of all three kinds of cell lines were found around 340 nm, which is known as the emission peak of tryptophan.[12] A small peak at 460 nm was also observed, which is known as the characteristic emission peak of NADH,[11,12] whose emission spectral range is from 420 to 480 nm. The major difference of the profiles between MDA-MB-231 and MCF-7 or fibroblastic cell is that a much higher fluorescence intensity of MDA-MB-231 was observed compared with MCF-7 or fibroblastic cells at the peak of 340 nm. Intensity of MCF-7 is lower than intensity of MDA-MB-231 at 340 nm, but higher than intensity from fibroblastic cells at 340 nm. Also, there is no significant difference between intensities from all the samples at 460 nm in the profiles. The relative tryptophan levels were calculated by using the ratio of intensities at 340 over 460 nm as reference signal for each sample and the differences compared in Fig. 2. The average ratio R of all MDA-MB-231 breast cancer cells
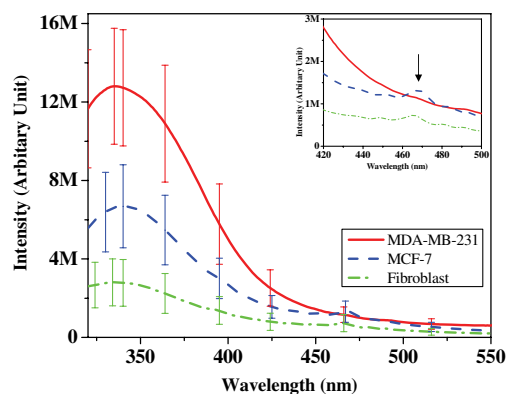
Fig. 1 Native fluorescence spectra of all three types of cells with standard deviation error bars: MDA-MB-231 (solid line), MCF-7 (dash line), and fibroblast (dotted line) excited at 300 nm wavelength. The black arrow in the small window indicates the weaker peak between 460 and 470 nm.
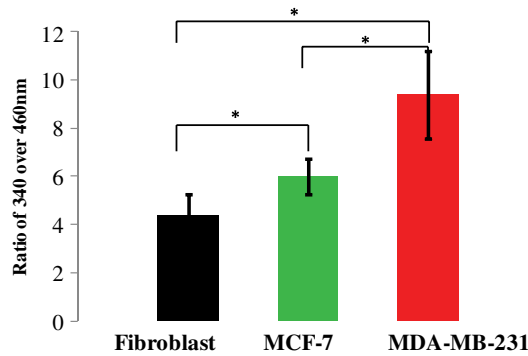
Fig. 2 There is an increment of ratio R of 340 over 460 nm: normal cells (fibroblast), nonaggressive cancerous cells (MCF-7), and aggressive cancerous cells (MDA-MB-231). Significant difference (*t*-test, $p < 0.001$) exists between fibroblast and MCF7, fibroblast and MDA-MB-231, MCF-7 and MDA-MB231. There were no significant differences among these three groups (ANOVA, $F \gg 1$, $p < 0.001$).

were 11.19 ± 3.8, R of MCF-7 breast cancer cells were 5.99 ± 0.7, and R of fibroblast cells were 4.40 ± 0.9. The average ratios are $R_{231} > R_{MCF-7} > R_{fribroblast}$. Other reference signals were also used to check if the 460 nm can cause unreliable results. When intensity at 530 nm was selected as a reference signal, the average ratio of intensity at 340 over 500 nm for MDA-MB-231, MCF-7, and fibroblast cells were 19.8, 15.4, and 11.8. The emission about 530 nm is associated to FAD. The ratios associated with different reference signals at 440 and 500 nm were also calculated and listed in Table 1. We found that with different reference signals at 440, 460, 500, and 530 nm, the ratio R of MDA-MB-231 was much higher than R of MCF-7 or fibroblast cells, and R of MCF-7 was a little higher than fibroblast cells. The relative tryptophan level is more prominent in aggressive cancer cells compared with nonaggressive cancer cells as well as normal cells. It is in good agreement with our past observation on breast tissue, which used the ratio of 340 to 440 nm in cancer tissues, that the aggressive breast cancer tissue has a much higher relative tryptophan level than normal or benign tissue has.[4] Figure 2 shows the relative tryptophan level exhibits an increase of ratio for aggressive cancer cells. This reflects higher tryptophan level and contained higher grade of aggressive cancer cells in comparison with the lower-grade nonaggressive cancer cells as well as normal cells. In cancer diagnosis and cancer research, identification of the metastatic competence of cancer is crucial to determine the stage and therapeutic method.[12] T-test and analysis of variance (ANOVA) were applied to analyze the differences among the ratio of three types of cells by using the JMP software. T-test

was performed between three groups—fibroblast cells and MCF7, fibroblast cells and MDA-MB231, MCF-7 and MDA-MB231—all with $p < 0.001$, which means there is a significant difference between each group. In the ANOVA test, the result is $F = 70.7 \gg 1$, and $p < 0.0001 \ll 0.05$. This means there is a significant difference between three types of cells and no significant difference within the same type of cells. This statistic result shows that our result is consistent and reliable. This study provides a highly relevant methodology to predict the metastatic potential of cancers by measuring the tryptophan spectra.

3.2 Native Fluorescence Spectra Data Analysis of Aggressive, Nonaggressive, and Normal Cells by SVM Method

The SVM classifier was employed to analyze the ratios of tryptophan from native florescence spectrum of different cell lines to determine if the tryptophan ratios can be used to classify the cell with different metastasis competence. SVs were chosen from the components of ratio R between $R_{min}^{agg} - \kappa$ and $R_{max}^{non-agg} - \kappa$ from both groups, where $R_{min}^{non-agg}$ is the minimal ratio of the aggressive cancer cells, $R_{max}^{non-agg}$ is the maximum ratio among all the nonaggressive cancer cells and normal cells, and κ is a self-defined threshold value for optimum, for identifying aggressive cancer cells and others. Same method was applied for chosen SV for identifying cancer cells of both aggressive and nonaggressive from normal cells. Since there are limited samples, all these data are applied to be trained by the SVM machine-learning algorithm to establish categorizing standard. This standard will be used to classify an unknown data set in the future study when large number of sample is available.
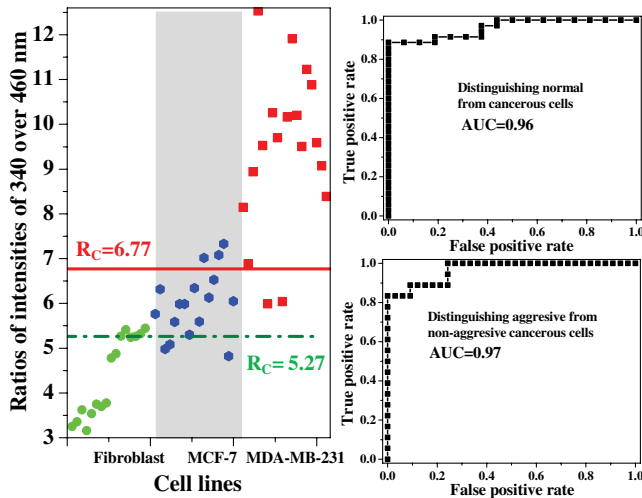
During a diagnosis of cancer metastasis competence, one may meet with the problem whether the results are positive (cancer) or negative (healthy). It is necessary to eliminate statistical errors, such as false positive or false negative. False positive is defined as a test result that is erroneously classified in a positive category and false negative is defined as a test result that is erroneously classified in a negative category. The sensitivity and specificity can be calculated by

$$\text{Sensitivity} = \frac{\text{No. of true positive}}{\text{No. of true positive} + \text{No. of false negative}},$$

$$\text{Specificity} = \frac{\text{No. of true negative}}{\text{No. of true negative} + \text{No. of false positive}}.$$

To set the diagnosis standard for distinguishing the positive or negative results and evaluating the metastasis level of the cancer, ratio R_C was used as the criteria by applying the SVM algorithm on all 51 (three types) cell samples. It was obtained that $R_C = 5.26$ and $R_C = 6.77$ for distinguishing normal versus cancer, and nonaggressive versus aggressive, respectively, which is shown as solid lines in Fig. 3(a). According to these two categorizing values, the sensitivity and specificity for these two cases can be calculated.

The ROC curves were used to evaluate the performance of criterion of the calculated R_C of the relative tryptophan level combined with SVM for distinguishing the metastasis competence of cancerous cells as well as normal cells. Accuracy can be measured by the AUC (area under the ROC curve). ROC curves shown in Figs. 3(b) and 3(c) were generated from the cases of aggressive versus nonaggressive cells, and cancer versus normal cells, respectively. The AUC values of the ROC curves were

Table 1 Ratio of 340 nm over different reference signals at 440, 460, 500, and 530 nm.

Ratio	MDA-MB-231	MCF-7	Fibroblast
I_{340}/I_{440}	7.3	5.1	4
I_{340}/I_{460}	11.2	5.9	4.4
I_{340}/I_{500}	16	10	7.7
I_{340}/I_{530}	19.8	15.4	11.8

Fig. 3 (a) The criteria R_C, for 340 over 460 nm of total three types of cells. The separating lines indicating the value of R_C were calculated using support vector machine. Accuracy evaluated by receiver operating characteristic curve using the R_C for distinguishing significant criterion to classify the cell samples into two groups for (b) cancer versus normal cells and (c) aggressive versus nonaggressive cancerous cells.

then calculated to evaluate the accuracy. The sensitivity, specificity, and the AUC values for using the calculated ratios combined with SVM for detecting cancerous cells metastasis competence are summarized in Table 2. The excellent sensitivity, specificity, and AUC values demonstrate the excellent efficacy using the ratios combined with SVM for separating different metastasis competence cancerous cells as a promising diagnostic tool for early cancer detection.

The results indicate that there is a higher tryptophan content in aggressive cancerous cells, which relates to the physiology phenomena in cancer. Tryptophan is an essential amino acid necessary for protein synthesis. It cannot be synthesized by mammalian cells and, thus, the mammalian cells depend on transport machineries for uptake.[22–24] Tryptophan is transported into cancer cells via large amino acid transporter system (LAT1/CD98),[25] degraded to kynurenine in the cells by enzyme indoleamine-2,3-dioxygenase. Numerous studies pointed out that the tryptophan consumption by cancer was involved in suppressing the immune response to cancer cells.[26–29] It is known that aggressive cancer cells have more large amino acid transporters on the cell membrane, which can more efficiently uptake tryptophan from surrounding environment.[30,31] An increasing number of studies shows that the fast progress of tumor is due to a failure of immune system control over the growth of tumor

cells.[27,29,32,33] The immune system T cells are particularly susceptible to low tryptophan concentrations, resulting in energy and apoptosis, so that cancer cells can escape the immune detection and survive.[29] Moreover, there is a pair of receptor and ligand proteins, PD-1 receptor on T cells and PD-L1/2on cancer cell, which is one of the causes that the cancer cells can escape from the immune systems because PD-1 receptor is activated in low tryptophan environment. When the PD-1 binds to PD-L1/2, this interaction suppresses the T cell activity, causing T cell apoptosis.[24,27] These proteins are shown to provide a protective shield surrounding the cancer to protect against the T cells from the immune system.[30] In the above T cells "death by binding" paradigm, the cancer cells escape the immune detection from T cells and develop toward increasingly aggressive forms.[23] Therefore, direct monitoring of the tryptophan level in cells/tissue can be used as a key to investigate the immune escaping ability of the cancer cells and the metastasis ability of mild and aggressive breast and other cancer cells in prostate.

4 Conclusion

NFL spectra were investigated for potential diagnosis of the aggressiveness of breast cancer cell lines. According to our knowledge, this is the first time to catalogue the aggressive breast cancer cells, nonaggressive breast cancer cells, and normal cells by measuring the relative contents of tryptophan using NFL. Our results demonstrate that aggressive breast cancer cells have relatively higher contribution from tryptophan than other cells. Tryptophan level can be evaluated using the emission ratio of 340 to 460 nm excited by 300 nm. This research indicates that measuring the NFL of tryptophan within the breast tissue may be used as a fingerprint for monitoring different metastasis cancers. The outcome of this research can result in a novel screening optical NFL tool where cells can be extracted from organs, such as breast, prostate, brain, and colon, and measured ratio fluorescence intensity at ∼340 over 460 nm to evaluate the risk level of tumor.

Table 2 Evaluation of performance for criterion using ratios of 340 over 460 nm combined with support vector machine for categorizing different types of cells.

Evaluated components	Sensitivity (%)	Specificity (%)	AUC
Normal versus cancerous cells	91.4	81.3	0.96
Nonaggressive versus aggressive cancerous cells	88.9	90.9	0.97

Note: AUC, area under the receiver operating characteristic curve.

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