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Nanoscale structural alterations in cancer cells to assess anti-cancerous drug effectiveness in ovarian cancer treatment using TEM imaging

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Abstract

Understanding the nanoscale structural changes can provide the physical state of cells/tissues. It has been now shown that increases in nanoscale structural alterations are associated with the progress of carcinogenesis in most of the cancer cases, including early carcinogenesis. Anti-cancerous therapies are intended for the growth inhibition of cancer cells; however, it is challenging to detect the efficacy of such drugs in the early stages of treatment. A unique method to assess the impact of anti-cancerous drugs on cancerous cells/tissues is to probe the nanoscale structural alterations. In this paper, we study the effect of different anti-cancerous drugs on ovarian tumorigenic cells, using their nanoscale structural alterations as a biomarker. Transmission electron microscopy (TEM) imaging on thin cell sections is performed to obtain their nanoscale structures. The degree of nanoscale structural alterations of tumorigenic cells and anti-cancerous drug treated tumorigenic cells are quantified by using the recently developed inverse participation ratio (IPR) technique. Results show an increase in the degree of nanoscale fluctuations in tumorigenic cells relative to non-tumorigenic cells; then a nearly reverse of the degree of fluctuation of tumorigenic cells to that of non-tumorigenic cells, after the anti-cancerous drugs treatment. These results support that the effect of anti-cancerous drugs in cancer treatment can be quantified by using the degree of nanoscale fluctuations of the cells via TEM imaging. Potential applications of the technique for cancer treatment are also discussed.

Keywords

mesoscopic physics; inverse participation ratio; ovarian cancer; anti-cancerous drugs; tight binding model; structural disorder

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I. Introduction

TEM imaging and probing nanoscale changes in cancer

TEM imaging is a method where we can probe ~1 nm resolution within the sample, and this has been used for imaging of cells at the nanoscale to see the inner structures of the cells, in general, qualitatively. It has been established that the cancer progression is associated with the nanoscale structural alteration in a cell due to rearrangements of the building blocks of the cell such as DNA, RNA, and lipids. Therefore, TEM imaging with spatial nano-resolution is a good modality to look at the cell structures at nanoscale and measure cancerous changes in cells at the nano to submicron scales.

The structural alterations in cells have been characterized by visible-light microscopy techniques within the diffraction limit. For example, the newly developed spectroscopic microscopy technique has shown good success in probing these alterations with some degree that are prominent in early carcinogenesis (1–6). However, looking at the actual nanoscale structures of the cell and then characterizing the structural disorder properties of the cells are not well studied or addressed in distinguishing the progress carcinogenesis or effect of anti-cancerous drugs in carcinogenesis.

The recently developed light wave localization technique, ‘inverse participation ratio’ (IPR) using TEM imaging, has shown success in quantifying the degree of structural changes at a few nano to submicron length scales in one parameter, known as the degree of structural disorder (7,8). In particular, using the IPR method, a TEM image is used to construct a disordered 2D mass matrix, and from this, we generate a 2D refractive index matrix. Optical waves are then solved for their eigenvalues and eigenfunctions using the refractive index matrix with closed boundary conditions. The light localization properties are measured by the ensemble averaging of inverse participation ratio, $\langle\langle IPR(L) \rangle\rangle$, and standard deviation, $\sigma(\langle IPR(L) \rangle_{cells})$, of the eigenfunctions of the light waves in these samples (see Method). It is shown that the degree of structural disorder is proportional to the $\langle\langle IPR \rangle\rangle$ or $\sigma(\langle IPR(L) \rangle_{cells})$ (9,10). Therefore, the IPR-TEM technique can be used to measure the degree of nanoscale structural disorder and to monitor structural change in cells under diseases condition. The IPR method is a very versatile approach. The IPR method, using TEM imaging, has been recently generalized to study the structural changes in brain and colon cells in chronic alcoholism (11,12). Furthermore, the IPR method also extended to study the molecular specific (DNA, histone, etc.) structural changes in cells by using molecular specific fluorophores and confocal microscopy imaging (13,14).

Ovarian cancer

Ovarian cancer (OC) ranks the 5th in cancer related deaths among women and accounts for more deaths than any other cancer of the female reproductive system. The American Cancer Society (ACS), estimated new cases of OC in the USA in 2019 would be 22,530, whereas estimated deaths would be 14,000. Most OC cases are diagnosed at a very late stage, of which 51% are diagnosed as stage III and 29% are diagnosed as stage IV (15). The exact cause that triggers OC is not clearly understood but there are several risk factors such as fertility therapy, late pregnancy, family history, hormone therapy after menopause, etc. are

associated with the development of OC. Metabolic alterations, suppression of tumor suppressor genes, and oncogenic activations are also considered as triggering factors for OC initiation and progression of the disease (16,17). Although initially sensitive to chemotherapy treatment, however, the majority of OC patients develop chemo resistant. 10 years survival rate for most patients of all stages of OC is ~30%. Development of chemoresistance, widespread disease during the time of diagnosis and tumor recurrence are the major challenges in the therapeutics of ovarian cancer (18).

In this study, we are focusing on analyzing the impact of novel anti-cancerous drug treatment in the tumor forming OC cell line *in vitro*. HSulf-1 knockdown OV202 cells are selected for this study for their aggressive tumor forming ability and high proliferation rate (19,20). This method of analysis is aimed to understand the effect of anti-cancerous drugs on cells in the early phases of treatment. Here we propose a novel approach to assess the impact of anti-cancerous drugs in cancerous cells by quantifying the degree of nanoscale structural disorder.

II. Method

An analytical formulation for the inverse participation ratio (IPR) technique from TEM images

TEM experiment has a resolution of ~1nm and can identify the nanoscale architectural alterations inside the cells which take place in normal cells when affected by cancer. These nano-alterations happen in the cells due to the rearrangement of the basic building blocks of the cells, such as DNA, RNA, lipids, macromolecules, etc. This results in mass density fluctuations in the cells. Using a thin slice of a cell (~100nm), the mass density variations can be probed by TEM imaging. The IPR calculation is an efficient technique to measure and quantify the cancerous level of aggressiveness in a cell through its mass density fluctuations. A higher $\langle IPR \rangle$ or $\sigma(\langle IPR(L) \rangle)_{cells}$ value indicates an increasing amount of the nanoscale mass density fluctuations in cells. The IPR technique is described in detail in earlier publications (7,8,10,11). However, for a self-sufficient and completeness of this paper, we will describe the IPR technique in brief.

The refractive index of a thin cell slice at a point $n(x,y)$ with a constant thickness dz has a voxel of volume $dV=dx dy dz$ (dx and dy are the lengths and width of a voxel) which can be written as $n(x,y) = n_o + dn(x,y)$, where n_o is the average refractive index and $dn(x,y)$ is the fluctuation of refractive index at (x,y) indicated voxel.

In case of thin sample, a voxel of area $dx dy$ at position (x,y) and thickness dz , the transmission TEM intensity: $I(x,y) = I_0 \exp(-\alpha dz/z_0)$; or $I_{TEM}(x,y) \approx I_0(1 - \alpha dz/z_0) = I_0 - I_0 \alpha dz$, where α is a transmission related constant depends on the mass of the voxel. TEM image intensity at any voxel point (x,y) for a thin cell sample of voxel area $dx dy$ is represented as $I_{TEM}(x,y)$ and can be expressed as $I_{TEM}(x,y) = I_{0tem} + dI_{TEM}(x,y)$, where I_{0tem} is the average pixel intensity and $dI_{TEM}(x,y)$ is the fluctuation part of the pixel intensity. Here, the intensity fluctuation $I_{TEM}(x,y)$ is less than the average intensity I_{0tem} , we define: $I_{0TEM} = \langle I(x,y) \rangle_{(x,y)}$ and $dI_{TEM}(x,y) = I_{TEM}(x,y) - \langle I(x,y) \rangle_{(x,y)}$. Similarly, the fluctuation of refractive index $dn(x,y)$ is less than the average refractive index n_o .

Optical parameter refractive index $n(x,y)$ of the scattering substances is linearly proportional to the mass density of a biological cell for the thin samples (7,8). Therefore, the intensity of a TEM image is linearly proportional to the mass, M , and refractive index of the voxel:

$$I_{TEM}(x, y) \propto M(x, y) \propto n(x, y) \quad (1a)$$

$$I_{0TEM} + dI_{TEM}(x, y) \propto M_0 + dM(x, y) \propto n_0 + dn(x, y) \quad (1b)$$

From this, we can calculate the optical potential of the voxel point as $\varepsilon_i(x,y)$ to generate an optical lattice:

$$\varepsilon_i \propto dn(x, y)/n_0 = dI_{TEM}(x, y)/I_0 \quad (2)$$

Knowing the optical potential at every point, the Anderson disordered tight binding model TBM Hamiltonian (21–23) can be generated as follows:

$$H = \sum_i \varepsilon_i |i\rangle\langle i| + t \sum_{\langle ij \rangle} (|i\rangle\langle j| + |j\rangle\langle i|) \quad (3)$$

Where $|i\rangle$ and $|j\rangle$ are the eigenvectors of i -th and j -th lattice sites, $\varepsilon_i(x,y)$ or simply ε_i is the i -th lattice site optical potential energy and t is the overlap integral between sites i and j . The eigenfunctions (E_i 's) can now be generated from the above Hamiltonian by its diagonalization. For a sample size of length L in the TEM image, we have $L \times L$ sample area in the TEM image. We calculate the average IPR at length L where L is defined as $L = N_L \times dx$ ($dx=dy$). As there are $N=N_L \times N_j$ numbers of lattice points, thus, there will be N eigenvalues, as well as the same number of eigenvectors. Finally, the average IPR value of the sample of length L or size $L \times L$ is defined as (7–10,24,25):

$$\langle IPR(L) \rangle_{L \times L} = \frac{1}{N} \sum_{I=1}^N \int_0^L \int_0^L E_i^4(x, y) dx dy, \quad (4)$$

where E_i denotes the i -th eigenfunction of the Hamiltonian, N is the total number of potential points on the refractive index matrix (i.e., $N=(L/dx)^2$). Eqn (4) presents the average value of one IPR pixel at length (L) that is constructed from the $L \times L$ TEM area or N pixels of the TEM image. From this formulation, if there is $L \times L$ sample length or N number of TEM pixels, this will provide one pixel of the IPR image of length L , defined in Eq. (4) as $\langle IPR(L) \rangle = \langle IPR(L) \rangle_{L \times L}$. It has been shown that the ensemble average or STD (i.e., averaging or STD over several similar type of cells) values: $\langle \langle IPR(L) \rangle \rangle$ or $\sigma(\langle IPR(L) \rangle)$, is proportional to the degree of structural disorder $L_d = d_n \times l_c$, where d_n is the STD of all $n(x,y)$ point of cell samples/ensemble and l_c is the average spatial correlation decay length of the $n(x,y)$ over the sample (7,8). Then,

$$\langle \langle IPR(L) \rangle \rangle \equiv \langle \langle IPR(L) \rangle_{L \times L} \rangle_{cells} \sim L_d = d_n \times l_c \quad (5a)$$

$$\sigma(\langle IPR(L) \rangle) \equiv STD(\langle IPR(L) \rangle)_{cells} \sim L_d = d_n \times l_c \quad (5b)$$

Our statistical analysis involves calculating the average and standard deviation of the disorder strength of $\langle IPR(L) \rangle_{L \times L}$ values, i.e. L_d values over the cell samples for the length of a given sample. Using this structural disorder strength $\langle\langle IPR(L) \rangle\rangle$ or $\sigma(\langle IPR(L) \rangle)$ or L_d as a biomarker, we study the structural properties of ovarian cancer cells with anti-cancerous drug treatments. In particular, we expect an increase in the structural disorder with the growth of cancer and the reversibility of the structural disorder when the cancerous cells are treated with the anti-cancerous drug if the nanoscale structural disorder is a good cancer stage biomarker.

III. Sample Preparation and TEM imaging

Ovarian normal and cancer cell lines

OV202 cell line is a low-passage primary ovarian cancer cell line established at the Mayo Clinic (19). OV202 NTC (expressing HSulf-1) and Sh1 cells (HSulf-1 deficient) are developed by Dr. Shridhar's group at Mayo Clinic and is described earlier elsewhere (20). Subcutaneous injection of OV202 Sh1 cells resulted in tumor formation in nude mice, whereas HSulf-1 expressing OV202 NTC cells did not form tumors (19). Both cells were grown in minimum essential medium alpha 1X (Cellgro) supplemented with 20% fetal bovine serum (Biowest) and 1% penicillin streptomycin (Cellgro). All cells were grown in the presence of 1 µg/ml puromycin as a selection marker for the HSulf-1 shRNA cells were treated with 10 µl of AACOCF3 or MAFP (cPLA2 inhibitors; Cayman chemicals) for 24 hours. Following this treatment, cells were washed twice with PBS and then fixed in Trump's fixative containing 4% formaldehyde and 1% glutaraldehyde in phosphate buffer pH ~7.3, post-fixed in 1.0% OsO₄, dehydrated with ethanol gradation, and transitioned into propylene oxide for infiltration and embedding into super epoxy resin.

TEM imaging

Cell samples were fixed in Trump's fixative (pH 7.2) at 4°C overnight, spun down and the supernatant removed. They were re-suspended in agarose which was cooled and solidified. The cells in agarose were then post-fixed in 1% OsO₄, dehydrated through a graded series of ethanol and embedded in Spurr resin. 100nm (or 0.1µm) ultra-thin sections were mounted on 200-mesh copper grids, post-stained with lead citrate, and observed under a JEOL JEM-1400 transmission electron microscope at 80kV.

IV. Results and discussions

The TEM images of the ovarian cancer cells are obtained as described in the above section. IPR analyses were performed for the samples on different length scales of TEM images. The IPR averaging of a sample length L , $\langle\langle IPR(L) \rangle\rangle$ over a single cell, then over the different cells, were performed for obtaining the ensemble averaging: $\langle\langle IPR(L) \rangle\rangle$. As discussed above, the $\langle\langle IPR(L) \rangle\rangle$ value for each TEM image was calculated and provides the degree of the structural disorder strength at a defined sample length L .

Figure 1(a)–(d) are the representative grayscale TEM images of a thin section (~100nm) of a cell from the following ovarian control/cancer cell lines: (i) non-tumorous NTC, (ii) tumorous Sh1, (iii) Sh1 treated with drug AACOCF3 (Sh1-AACOCF3), and (iv) Sh1 treated

with drug MAPF (Sh1-MAFP). For each case (i.e. for a particular cell line) study, 8 different cells were taken from the cell line for averaging. Figure 1(a')–(d') are the corresponding $\langle IPR(L) \rangle$ images of Figure 1(a)–(d), at a length scale of 165nm and sample size of $165 \times 165\text{nm}^2$. It is reported that structural alteration in carcinogenesis happens around sample length $L \sim 100\text{nm}$ or sample size $L \times L$ (11), which is around the basic building blocks of the cell. Therefore, we have taken a length scale (165nm) that is higher than the 100nm . For a better understanding of the IPR images at different length scales, we have also added IPR images at two different length scales (one $82\text{nm} < 100\text{nm}$ and another $206\text{nm} > 100$ and 165nm), in the Supplementary Information (SI). As can be seen from Figure 1, $\langle IPR(L) \rangle$ images represent different intensities of disorder patterns in the cell line which are distinct from conventional grayscale TEM images. In the IPR images, intensities patterns of higher fluctuations in the cells are represented by the red spots and lower intensities with blue. In the figure, it can be seen that the increasing fluctuation or $\langle IPR(L) \rangle$ value increases from the less proliferating NTC cells to highly proliferating Sh1 cells, and decreasing of the fluctuations or $\langle IPR(L) \rangle$ values decrease with the treatment of two different anti-cancerous drugs, AACOCF3 and MAFP. The drug effect can be distinctly visualized in the IPR images.

Figure 2 shows the length (L) dependent fluctuations with the sample size ($L \times L$). We plotted, variations of the standard deviation $\sigma(\langle IPR(L) \rangle)$ vs L with the increase of sample lengths: $L = 41, 82, 123, 165, 206, 247, 288\text{ nm}$. As the deviation started appearing in the mean and STD of $\langle IPR(L) \rangle_{L \times L}$ at $L=100\text{nm}$, therefore we have plotted the $\sigma(\langle IPR(L) \rangle)$ vs L , systematically well below and above 100nm . These lengths are for the cells from the following cell lines: non-tumorigenic (OV202 NTC); tumorigenic (OV202 Sh1); AACOCF3 treated tumorigenic Sh1 (Sh1-AACOCF3); and MAFP treated tumorous Sh1 (Sh1-MAFP). As can be seen from the figure that the deviation in the degree of nano-fluctuations between non-tumorigenic cells NCT and tumorigenic cells Sh1 started becoming prominent around the length scale $\sim 100\text{nm}$. Interestingly, the degree of nano-fluctuations of anti-cancerous drugs treated Sh1 tumorigenic cell reverse to that of the non-tumorigenic NTC cells. This confirms the efficacy of these two anti-cancerous drugs.

Figure 3 presents the bar graphs of the standard deviation of calculated $\sigma(\langle IPR(L) \rangle)$ or L_d value, of the ovarian cells at the fixed length scale 165nm . The variations are similar at lower sample length scales $>165\text{nm}$, however, we have chosen 165nm to show a prominent difference. Statistically, the standard deviation is the more reliable marker than the average, as it only depends on the width of the distribution, irrespective of the mean position. The result shows the standard deviation of the degree of structural disorder $\sigma(\langle IPR(L) \rangle)$ value increased by 70% from NTC to Sh1 cells. Furthermore, when Sh1 cells were treated with 2 different anti-cancerous drugs, AACOCF3 and MAFP, the $\sigma(\langle IPR(L) \rangle)$ values decreased by around 58% for AACOCF3 and 54% for MAFP, relative to the $\sigma(\langle IPR(L) \rangle)$ value of the Sh1 tumorous cells. In particular, with the treatment of the anti-cancerous drug, the structural biomarker parameter $\sigma(\langle IPR(L) \rangle)$ or L_d value decreased nearly back to the normal value. The normalcy detection of these anti-cancerous drug treated cancerous cells may require further investigations using different modalities. It has been earlier shown that AACOCF3 is a better anti-cancerous agent producing more anti-cancerous treatment in OC cells compared to MAFP (26). It can be seen in Fig. 3 that similar trend of bar graphs which show a reduction in the degree of structural disorder $\sigma(\langle IPR(L) \rangle)$ value for AACOCF3

(58%) > MAFP (54%), consistent with the known hierarchy of the effectiveness of the drugs, in this length scale. Hence, the quantitative analysis technique, called IPR, quantifies the nanoscale structural disorder $\sigma(\langle IPR(L) \rangle)$ or L_d as an important biomarker to study the degree of structural alterations at the nanoscale level and has potential to detect the effect of anti-cancerous drugs in ovarian cancer.

IV. Conclusions

The nanoscale mass-density fluctuations are quantified with the progression of ovarian carcinogenesis, as well as the effects of two anti-cancerous drugs on non-tumor forming OV202NTC and tumor forming OV202Sh1 cells are studied using the TEM imaging and IPR technique. The nanoscale fluctuations are quantified by the STD value of the $\langle IPR(L) \rangle_{\text{cell}}$, $\sigma(\langle IPR(L) \rangle)_{\text{cell}}$ performed over similar types of cells or ensemble of the samples. Results show an increase in the nanoscale fluctuations or $\sigma(\langle IPR(L) \rangle)$ value from non-tumorous NTC to tumorous Sh1 cells. The $\sigma(\langle IPR(L) \rangle)$ values for two different drugs treated tumorous cells, Sh1-AACOCF3 and Sh1-MAFP, have reduced value from tumorous cells Sh1 and the reduced values are nearly same to the NTC non-tumorous cells. Earlier IPR analysis of a different cell line has verified the increase of nanoscale structural disorder with the progression of cancer (7,8). Based on the results presented, we investigate the potential applications of the IPR technique in measuring and quantifying the effectiveness of different anti-cancerous drugs on ovarian cancer treatment. This quantification of the effectiveness of anti-cancerous drugs in ovarian cancer treatment could enhance better drug treatment modalities at its earliest and helps to control the deadly ovarian cancer. Although this study is based on ovarian cancer cells, however, the technique can be applied to the varieties of cancers to assess the effectiveness of different anti-cancerous drugs in treatment.

Our technique is based on the linear transmission of the TEM intensity through thin cell samples as described in the Method. The TEM intensity transmits well till ~500nm of thin cell samples, the technique works until that thickness of the samples. Because of the thin sample, this method can be applied as well as to heterogeneous cell samples.

A cell is around 5 microns thick and the TEM sample thickness is around 100nm, therefore, 50 TEM slices per cell can be generated. Thus, it is important to consider ensemble averaging correctly by choosing the similar types of TEM micrographs. For each cell, TEM micrographs are chosen around the middle of the cell by observing the maximum size of the nucleus. The maximum changes in a cancerous cell can be captured by considering the largest portion of the cell or nucleus. Not considering proper ensemble averaging, results may vary.

At present, the super resolution optical microscopy techniques have achieved an order of 5–10nm resolutions targeting particular types of molecules in a cell (27–29). However, in the case of TEM imaging, we can target whole cells or nuclei with ~1nm resolution. This brings advantages of the TEM imaging for the quantification of structural disorder and its alterations at submicron to nanoscales in the nucleus/cell, in progressive carcinogenesis or any other cell abnormalities. It would be interesting to compare the results of TEM-IPR

analysis with the super resolution microscopy in progressive carcinogenesis or abnormalities of a cell, in the near future.

As discussed in the introduction, there are other techniques which enable us to probe the structural disorder in biological samples such as spectroscopic microscopy, confocal microscopy techniques. However, the advantage of the high resolution of TEM imaging facilitates the real view of the cell structures at nano scales, that cannot be performed by standard optical microscopies. The complicated sample preparation steps and heavy intrumentation setup of TEM are the disadvantages. However, this technique will provide an understanding of actual nanoscale structures in cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig 1:
(a)-(d) are the TEM images and (a')-(d') are their respective IPR images at the sample length ($L \times L = 165 \times 165 \text{ nm}^2$) from ovarian cells of the following: non-tumorous (OV202 NTC); tumorous (OV202 Sh1); AACOCF3 treated tumorous Sh1, Sh1-AACOCF3; and MAFP treated tumorous Sh1 Sh1-MAFP. IPR images are distinct from the TEM images.

Fig. 2:

Variations of the standard deviation $\sigma(<IPR(L)>)$ with the increase of sample length L , for cell lines non-tumorigenic (OV202 NTC); tumorigenic (OV202 Sh1); AACOCF3 treated tumorigenic Sh1 (Sh1-AACOCF3); and MAFP treated tumorous Sh1 (Sh1-MAFP). It can be seen that the deviation between NTC and Sh1 started to become prominent around the sample length/length scale $\sim 100nm$. Interestingly, the drug treated Sh1 tumorigenic cells fluctuation degrees reverse to the non-tumorigenic cells.

Fig. 3:

Bar graph representation of the standard deviation of the degree of structural disorder strength $L_d \sim \sigma(<IPR(L)>)$ calculated from the TEM images for: non-tumorous (NTC), tumorous (Sh1), AACOCF3 treated tumorous (Sh1) cells Sh1-AACOCF3, and MAFP treated tumorous cells (Sh1) Sh1-MAFP. The normal cells, IPR analysis was performed at the sample size $165 \times 165 nm^2$. The result shows L_d value increases from non-tumorous to tumorous cells, then it decreases when these tumorous cells are further treated with anti-cancerous drugs AACOCF3 and MAFP, interestingly the L_d value returns almost back to the same value of the non-tumorous cells (p-value $< .05$). This may imply that anti-cancerous drugs are working well in ovarian cancer treatment.