Intratumoral cycling hypoxia inhibits expression level of estrogen receptor to promote the formation of heterogeneous sub-clones in luminal A breast cancer

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Abstract

Background

The expression level of estrogen receptor (ER) is positively correlated with chemoresistance in patients with Luminal A tumor (LAT). ER expression level alters frequently after neoadjuvant chemotherapy which may be related with hypoxia. The spatial and temporal heterogeneity of tumor sub-clones is one of the major contributors to treatment failure. It is essential to investigate how the intratumoral heterogeneous sub-clones survive hypoxic microenvironment in LAT.

Material and Methods

LAT with largest cross section was divided into micro-sections, the expressions of hypoxia inducible factor-1 (HIF-1) and ER were then detected by immunohistochemistry. The intratumoral distribution of micro-vessels was assessed by 3D reconstruction and stained CD34; the cycling hypoxia model of MCF-7 was established in a step fashion to investigate the changes in HIF-1 and ER expressions. All statistical analyses were performed using SPSS software (version 17.0 for Windows).

Results

There was a negative correlation between the expressions of ER-alpha and HIF-1alpha ($c=-2.40$; $p=0.044$) as assessed by mean optical density values in the maximal cross section of tumor. As shown by 3D ultrasound image, the center of tumor had less functional micro-vessels compared with the periphery ($P<0.05$). From the periphery to the center of tumors in the large sections from 8 patients with LAT, the expressions of ER, progesterone receptor and CD34 were gradually decreased with HIF-1alpha expression showing an opposite direction. There was a negative correlation between expressions of ER-alpha and HIF-1alpha, and between expressions of CD34 and HIF-1alpha. No significant difference was noted in the outcomes of cytometry between the hypoxia and control groups. ER-alpha expression gradually decreased with the time course of cycling hypoxia with HIF-1alpha showing an opposite direction.

Conclusion

LAT is composed of heterogeneous sub-clone cells which can be distinguished by ER-alpha and HIF-1alpha. The distribution of heterogeneous sub-clone cells was closely related with heterogeneous microenvironment of hypoxia / reoxygenation.

Keywords: Luminal A breast cancer, cycling hypoxia, estrogen receptor, heterogeneous sub-clones.

1. Background

The rate of pathological complete response (pCR) in patients with luminal A tumor (LAT) is far lower than that in those with triple negative breast cancer or human epidermal growth factor receptor 2 (HER2)-enriched tumors, as generally acknowledged by physicians involved in neoadjuvant chemotherapy (NAC).^[1] The expression level of estrogen receptor (ER) is positively correlated with the resistance to NAC.^[2] Meanwhile, the level of ER expression alters frequently post neoadjuvant chemotherapy.^[3] It has been found that MCF-7 breast cancer cells were resistant to doxorubicin, which was associated with activation of hypoxia inducible factor-1 (HIF-1).^[4] As demonstrated in a recent study, hypoxia inhibited ER expression in ER-positive breast cancer cells, the molecular mechanism, however, remained unclear.^[5]

In a clinical context, although difference exists, the agreement rates between core needle biopsy (CNB) and surgical specimens in immunohistochemically detecting the expressions of ER, progesterone receptor (PR) and HER2 were as

high as 81.3%, 92.9% and 89.3%, respectively. ^[6] A review of 45 studies on expressions in lymph node metastasis, distant metastasis and local recurrence revealed that the average phenotypic drift in ER, PR, and Her-2 expression was from 6.6–32.0%. ^[7] The resulting spatial and temporal heterogeneity becomes the major contributor to treatment failure, and poses a huge challenge to comprehensively understand the characteristics of the tumor. ^[8]

If the intratumoral heterogeneity occurs from the tumorigenesis, the extratumoral heterogeneity could be easily understood. Thus, it is essential to investigate how the LAT intratumoral heterogeneous sub-clones survive under hypoxia. Besides, the intratumoral heterogeneous hypoxic zones are believed to evolve dynamically. The model of hypoxia / reoxygenation, termed as “cycling hypoxia”, is a well-recognized phenomenon in animal and human solid tumors. ^[9] Therefore, the aim of the present study was to investigate intratumoral heterogeneity by assessing expressions of HIF-1 and ER sub-clones under intratumoral cycling hypoxia in LAT. HIF-1 and ER expressions were detected using immunohistochemical technique from micro-blocks and large sections; the intratumoral distribution of micro-vessels was observed and assessed using 3D reconstruction and stained CD34; and the ladder-type cycling hypoxia model of MCF-7 was established.

2. Methods

2.1. The expression maps of ER-alpha and HIF-1alpha in tumor tissue of micro-blocks

On 12 August 2015, a piece of tumor tissue with a maximal cross-sectional area was obtained paralleled to the pectoralis major in an open surgery from a patient who was diagnosed with LAT via preoperative biopsy. The patient was labeled as “Patient One” (Table 1). The thickness of the “pancake” like tissue was approximately 3 mm. Fat tissue was manually removed before the sample was divided into micro-blocks of less than 3 mm x 3 mm x 3 mm and numbered according to the cross-sectional shape of tumor (Fig. [1](#), A-1 to A-3). Each micro-block was detected by immunohistochemistry (IHC) for ER-alpha (1:200 dilution; ZETA, Bosterbio, USA) and HIF-1alpha (ab82832#, ABCAM, UK), respectively, in the Department of Histology and Pathology of the hospital. The details of surgical protocols were published elsewhere. ^[10] Integrated Optical Density (IOD) was calculated as the sum of grey values for each pixel in the detected area, divided by the total area covered by breast cancer cells. ^[11] The expressions of ER-alpha and HIF-1alpha were represented as the Mean Optical Density (MOD) of each micro-block which obtained from more than five independent views.

Table 1
Patient characteristics

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Age(yrs)	43	52	39	48	42	57	56	40	46
Menopause									
Postmenopausal		●				●	●		
Premenopausal	●		●	●	●			●	●
Family History									
No	●	●		●	●	●	●	●	●
Yes			●						
Quadrant									
Areolar					●				
Inner upper									
Inner lower									
Outer lower		●					●		
Outer upper	●		●	●		●		●	●
Diameter (cm)	2.5	2.8	2.3	3.0	2.5	3.4	2.5	2.7	3.8
Histological Grade									
I									
II	●				●				●
III		●	●	●		●	●	●	
Cancer Thrombosis									
Negative		●			●		●	●	●
Positive	●		●	●		●			
Nodal Metastasis									
Negative		●	●		●	●	●	●	
Positive	●			●					●
ER(%)	80	60	80	90	90	80	70	90	60
PR(%)	70	50	80	90	80	70	70	90	50
Ki67(%)	15	10	10	15	10	7	10	3	15
Operation									

Characteristics	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6	Patient 7	Patient 8	Patient 9
Mastectomy	●	●			●	●	●		●
Tumorectomy			●	●				●	
Study	Expression maps	Intratumoral heterogeneity							

2.2. Intratumoral heterogeneity and oxygen supply (vascularization) study of luminal A breast cancer

Between 1 February and 30 June 2016, 20 patients treated in the Department of Breast Surgery, Shengjing Hospital of China Medical University, Shenyang, China were investigated. The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University. The study consisted of two sections. Inclusion criteria of the first section were: 1) no previous history of breast cancer or other malignancies; 2) not pregnant at the time of diagnosis; 3) no history of NAC; 4) BI-RADs 4C or 5 as determined by the preoperative comprehensive imaging assessment (mammography and ultrasound);^[12] and 5) the longest diameter of the tumor > 2 cm.

Conventional ultrasound and 3D vascular images were obtained using the Philips IU22 system with a linear 3D transducer. QLAB vascular analysis (Philips) was used to calculate the volume of tumor and quantitative vascularization index (VI). Small spheres of varying sizes were drawn in the center of the tumor (Fig. 2, A-1 to A-3). All tumors underwent intraoperative instant frozen histopathology to confirm malignancy.

The inclusion criteria for the second section were: 1) invasive ductal carcinoma; 2) ER ≥ 50%; 3) Ki67 < 20%; and 4) HER2 negative. Tumors from eight patients met the above criteria thus were completely embedded and made into large sections.^[13] The characteristics of these patients are listed as Patient 2 to 9 in Table 1. The expressions of ER, PR (1:200 dilution; ZETA, Bosterbio, USA), CD34 (1:500 dilution; ZM-0046, Zhongshanjinqiao, China) and HIF-1alpha (ab82832#, ABCAM, UK) were assessed via immunohistochemically staining. Through the view fields of the microscope, the tumors were arranged from the edge to the center based on the boundary of the tumor, and were rotationally divided into non-overlapping zones (Fig. 2, C-1 to C-13). The expression levels of these markers in each zone were analyzed to assess the intratumoral heterogeneity.

2.3. Ladder-type cycling hypoxia and cytometry

MCF-7 cells (provided by Professor Bing, originally obtained from the ATCC (HTB-22, Rockville, MD, USA), the passage number < 6 and the mycoplasma screening was safe) were routinely maintained in Dulbecco's modified Eagle's medium/Ham's F12 (Sigma-Aldrich, Irvine, UK) supplemented with 10% fetal bovine serum (FBS) and 1 × penicillin and streptomycin. Cells were randomly allocated into two groups: cycling hypoxia group and control group (normoxic environment). Four cycles of hypoxic culture (1–2% O₂, 5% CO₂, and 93–94% N₂) were performed for 24, 36, 48, and 72 h respectively in a hypoxic workstation (YQX-15 II, Shanghai Qiaofeng Corp, China), followed by normoxic culture (21% O₂, 5% CO₂, and 74% N₂) for 24 h to induce the MCF-7CH. Cell passaging was initiated after hypoxic cycle in MCF-7CH group. Cytometry for cell passaging in the control group underwent at the same time point (i.e. 84, 156 and 276 h, respectively after the test started) with the hypoxia group (Fig. 3).

2.4. Heterogeneous sub-clones in vitro were induced by cycling hypoxia

The expressions of HIF-1alpha and ER-alpha were examined by Western Blot (n = 3). The resulting blots were probed with rabbit monoclonal (#EPR4097, Estrogen Receptor alpha, Abcam, UK), rabbit polyclonal (#ab82832, Anti-HIF-1 alpha, Abcam, UK), and secondary antibodies (#ab6940, Goat polyclonal Secondary Antibody to Rabbit IgG; H&L, Abcam, UK), respectively (Fig. 4, A-1 & A-2). The expressions of ER-alpha (1:200 dilution; ZETA, Bosterbio, USA) for MCF-7CH and MCF-7 were also examined by immunofluorescence assay for three times. Fluorescent gathered in the nucleus of ER positive cells. Photos were taken for more than 100 cells before analyzed by ImageJ (Fig. 4, B-1 & B-2).

2.5 Statistical analysis

All the cell experiments were performed three times. P value < 0.05 was considered to be statistically significant. All statistical analyses were performed using SPSS software (version 17.0 for Windows). Pearson correlation test was used to investigate the relationship of expressions between the markers.

3. Result

3.1. The Expression map of ER-alpha and HIF-1alpha in LAT

ER and HIF-1alpha expressions of each micro tissue block were assessed by ImageJ for an MOD value, and were endowed with a blue and red color, respectively. The greater the MOD value, the darker the blue or red color was endowed. The expression map of ER-alpha (MOD) on the maximal cross section of the tumor was presented in Fig. [B-1](#). ER staining for tissue block #40 and #9 was shown in Fig. 1, B-2 and B-3. The expression map of HIF-1alpha (MOD) of the tumor with the maximal cross section was presented in Fig. [C-1](#). HIF-1alpha staining for tissue block #21 and #51 were shown in Fig. 1, C-2 and C-3. There was a significantly negative correlation ($c = -2.40$; $p = 0.044$) between the expressions of ER-alpha and HIF-1alpha of the tumor with the maximal cross section, as shown in Fig. [D-1 & D-2](#).

3.2. Intratumoral heterogeneity and oxygen supply study for Luminal A breast cancer

The intratumoral microvascular distribution in one patient with LAT was investigated using the 3D ultrasonic imaging. The tumor volume was 5.1 ml with a microvascular density (MD) of 1.5% (Fig. 2, A-1). If a small virtual sphere was built at the tumor center, the tumor volume and MD were 0.53 ml and 0.3%, respectively (Fig. [A-2](#)). If a large homocentric virtual sphere was built, the volume and MD were 0.9 ml and 1.0%, respectively (Fig. 2A-3). The mean MDs of the tumors from eight patients who met the criteria were presented in Fig. 2B. The volumes of tumors ranged from 4.0 to 8.0 ml, whereas the volumes of the large and small virtual spheres ranged from 0.8 to 2.0 ml and 0.2 to 0.7 ml, respectively. The difference was statistically significant ($p < 0.001$).

The pathological report for Patient #4 showed that the expressions of ER, PR, and Ki67 were 90%, 90% and 15%, respectively, and the Her2 expression was negative by the routine IHC (Fig. 2, C-1). Part of the tumor tissue was used for big pathological section to investigate intratumoral heterogeneity of ER, PR, CD34 and HIF-1alpha expressions. Zone #3, #21 and #24 represent the edge, medium and center of tumor, respectively (Fig. [C-2 to C-13](#)). There was a negative correlation between the expressions of ER and HIF-1alpha ($c = -0.433$, $p = 0.027$) (Fig. 2, C-14) and a negative correlation between the expressions of CD34 and HIF-1alpha ($c = -0.621$, $p = 0.001$) (Fig. 2, C-15). From the edge to the center of the tumor, the expressions of ER, PR and CD34 were gradually reduced in all patients (Fig. 2, D-1 to D-3), whereas the expression of HIF-1alpha was increased (Fig. 2D-4).

3.3. Heterogeneous sub-clones in vitro in relation to cycling hypoxia

No significant difference was noted in the outcomes of cytometry between the hypoxia and control groups, as assessed at the some checking points for the two groups (Fig. 3). With the increasing cycles of cycling hypoxia, the expression of HIF-1alpha by Western Blot was increased, whereas the expression of ER-alpha was decreased (Fig. 4, A-1 & A-2). The fluorescence intensity of ER-alpha by immunofluorescence assay was decreased after 4 cycles of cycling hypoxia treatment ($p < 0.001$) (Fig. 4, B-1 & B-2).

4. Discussion

By studying micro-block tumor tissues, we have found that the expressions of ER-alpha and HIF-1alpha were heterogeneous (Fig. 1). ER is the most important marker to predict prognosis, guide treatment for patients with breast cancer, and the HIFs master the transcriptional response to local tissue hypoxia, a hallmark of solid tumors.^[14, 15] Previous studies revealed that hypoxia could inhibit ER expression in ER-positive breast cancer cells.^[16] We also demonstrated a negative correlation between the expressions of ER-alpha and HIF-1alpha (Fig. 1 and Fig. 2). It was well established that the energy consumption differs within the tumor tissue with predominance of glycolysis in the zone of central necrosis and phosphorylation in surrounding microenvironment.^[17] This phenomenon is closely related to the heterogeneous distribution of micro-vessels.^[18] As shown by the 3D ultrasound images, the center of tumor was free of functional micro-vessels (Fig. 2). Generally, most human tumors are heterogeneous, consisting of cellular clones of different natures, which present different characteristics at varying frequencies.^[19] In recent years, growing number of studies have used large sections to investigate intratumoral heterogeneity.^[20, 21] From the edge to the center of the tumor, the gradually decreased expressions in ER, PR and CD34 were shown on the large sections with the expression of HIF-1alpha showing an opposite trend (Fig. 2). The expression of CD34 and HIF-1alpha was negatively correlated (Fig. 2). As an important marker of vascular endothelial cell, CD34 can, to some extent, represent microvessel density.^[22] In addition, the distribution of intratumoral microvessels is a potential prognostic indicator for patients with breast cancer.^[23] Our findings suggested that LAT tissues were composed of cell population with different sub-clones which can be distinguished by the expression levels of ER-alpha and HIF-1alpha. In 1955, Thomlinson and Gray first explained tumor hypoxia. They proposed the concept of gradually decreasing oxygen gradient from the periphery to the center of the tumor sphere. They found that cancerous cells grew in the periphery of the vascular stroma, while the central region of larger tumor was necrotic, and was surrounded by intact cells with ring appearance.^[24]

In 1992, Semenza and Wang discovered HIF in the extracts of hypoxic hepatocellular carcinoma cell line Hep3B.^[25] HIFs family is responsible for the adaptation of cellular metabolism to hypoxia.^[26] Recent evidence revealed that chronic repeated exposure to hypoxia and reoxygenation seems to be beneficial to tumor growth.^[27] After the initial trial and error, we established a cycling hypoxia model in a step fashion to induce the MCF-7 phenotypic drift, and the hypoxia/reoxygenation ratio was adjusted from 1:1 to 1:3. The step-type cycling hypoxia potentially protected the cell survival from long term hypoxia (Fig. 3). Furthermore, the HIF-1alpha expression level was gradually increased with the time course of cycling hypoxia, and the ER expression trended in an opposite direction (Fig. 4). In fact, different phenotypic cells with varying expression levels of ER-alpha and HIF-1alpha could be obtained artificially by adjusting oxygen supply. These in vitro cultured cells were similar to the large section cells obtained from patients. Therefore, it seems reasonable to hypothesize that the intratumoral heterogeneous oxygen supply induces the production of heterogeneous sub-clone cells which are adapted to the hypoxia / reoxygenation microenvironment. If the hypothesis is true, that is, tumors are composed of dynamic sub-clones from the scratch under the pressure of hypoxia, which definitely will help to comprehend its spatial and temporal heterogeneity.

Oxygen is vital for the most living organisms. William G. Kaelin and Gregg L. Semenza of the United States and Sir Peter J. Ratcliffe of the United Kingdom, the winners of the 2019 Nobel Prize in Physiology or Medicine, discovered

pathways through which cells perceive and adapt to oxygen.^[28] Tumor as a “society” composed of heterogeneous sub-clones induced by heterogeneous oxygen supply, is well prepared for future changes in the hypoxia / reoxygenation microenvironment. For example, cancer composed of predominant sub-clones at the periphery and non-predominant sub-clones at the center of tumor; at least one sub-clone becomes a new dominant clone by adapting to changes in the tumor microenvironment caused by the therapeutic stress, such as chemotherapy or radiotherapy. In previous preclinical and clinical studies, positive ER expression in breast cancer cells was associated with decreased sensitivity to chemotherapy.^[29, 30] We speculate that LAT could adapt to chemotherapy by reducing the expression level of ER. Surely, huge complicated work is yet to be done, e.g. how to convert the chemotherapy or radiotherapy stress to hypoxia; how the HIFs family inhibits the ER; and why the cells loss the ER phenotype and gain an aggressive phenotype.

5. Conclusions

In summary, we used micro-block, large section and Doppler imaging technique and preliminarily conformed that LAT is composed of multiple heterogeneous sub-clones which can be distinguished by HIF-1 and ER. This study simulated the formation process of heterogeneous sub-clones in vitro under cycling hypoxia, suggesting different heterogeneous sub-clones are adapted to their hypoxia / reoxygenation microenvironment.

Abbreviations

NAC
neoadjuvant chemotherapy
pCR
pathological complete response
LAT
luminal A tumor
HER2
human epidermal growth factor receptor 2
ER
estrogen receptor
HIF-1
hypoxia inducible factor-1
CNB
core needle biopsy
PR
progesterone receptor
IHC
immunohistochemistry
IOD
Integrated Optical Density
MOD
Mean Optical Density
VI
vascularization index
FCS
fetal calf serum

Declarations

Ethics approval and consent to participate

The study was approved by the Ethics Committee of Shengjing Hospital of China Medical University.

Consent for publication

All presentations have consent for publication.

Availability of data and material

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

All authors declare no conflict of interests.

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Authors' contributions

LJY and SB designed the proposal and drafted the manuscript. ZGF, KLN and WZR participated in data collection. KY and SH participated in immunohistochemical stain and analysis. MY carried out the ultrasound analysis. SB, LJY, ZY and JS participated in total data analysis. All authors read and approved the final manuscript.

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Figures

Figure 1

The Expression maps of ER-alpha and HIF-1alpha in luminal A tumor. Tumor sample obtained from a patient with Luminal A tumor was divided into micro-blocks of less than 3mm x 3mm x 3mm, as shown in by photo A-1, 2 & 3. Each micro-block was detected by immunohistochemistry for ER-alpha and HIF-alpha, as shown in B-1,2,3 and C-1,2,3. Mean Optical Density (MOD) values of both markers from each micro-block were analyzed according to the number order, the correlation was shown in D-1&2.

Figure 2

Intratumoral heterogeneity and oxygen supply. The 3D reconstruction was established by the doppler ultrasound for a breast tumor which was probably malignant assessed by the ultrasound. A-1 shows that the tumor's volume was 5.1ml and the microvascular density (MD) was 1.5%; A-2 shows that small sphere's volume was 0.53ml and the MD was 0.3%, which was in the core of the tumor; A-3 shows that the big sphere's volume was 0.9ml and the MD was 1.0%, which was homocentric sphere with the small sphere. The average MD of eight tumors that met the criteria is presented in B. The tumor was diagnosed with Luminal A breast cancer and the detail pathological information postoperative is shown at the top of the C-1. The tumor was made into large section as shown at the bottom of photo C-1. In the large

section the expressions of Estrogen Receptor (ER), Progesterone Receptor (PR), CD34 and Hypoxia Inducer Factor-1alpha (HIF-1a) were detected immunohistochemically. Because of the large section, the tumor boundary was determined visually and established a roadmap corkscrew to the tumor center and the width of the road was more than a view under the microscopic with 100X. Each view was observed and recorded from the edge to the center and numbered from 1 to 26. The expressions of the markers at tumor edge, medium and center for sample number 3, 21 and 24 are presented in C-2 to C-13. C-14 and C-15 show correlation between expressions of ER and HIF-1alpha, and between HIF-1alpha and CD34, respectively. D-1 to D-4 show expressions (measured by Mean Optical Density) of ER, PR, CD34 and HIF -1a from eight tumors that met the criteria.

Figure 3

Ladder-type cycling hypoxia and cytometry. After 4 cycles of cycling hypoxia, the MCF-7 cells were named as MCF-7CH. Passing cells would be carried out after hypoxia cycle in MCF-7CH group. The control group would be passing cells at the same time point (after the beginning for 84 hours, 156 hours and 276 hours) for cytometry. The cell counting results of the two groups were shown. The red squares represent hypoxia and the blue squares represent reoxygenation.

Figure 4

Heterogeneous sub-clones in vitro were induced by cycling hypoxia. After 4 cycles of cycling hypoxia, the MCF-7 cells were named as MCF-7CH. The expressions of HIF-1alpha and ER-alpha detected by Western Blot of each cycle of MCF-7 are presented in A-1 & A-2. And the blots were cropped to improve the clarity and conciseness of the presentation. The expressions of ER assessed by immunofluorescence for both groups are shown in B-1 & B-2.

Supplementary Files

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