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Estrogen signaling and prediction of endocrine therapy

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Abstract Estrogen plays an important role in the growth and progression of human breast cancer. Understanding the whole picture of estrogen signaling is a very important goal towards clarifying the biology of this disease. On the other hand, hormonal therapy for breast cancer has been progressing rapidly with the advent of drugs such as selective estrogen receptor (ER) modulators and aromatase inhibitors. Prediction of individual response to these hormonal therapies is becoming important for the management of breast cancer patients. To help address these basic and clinical issues, we are developing several new tools such as the focused microarray and the green fluorescent protein-reporter cell system. We first carried out expression profiling of approximately 10,000 genes in ER-positive breast cancer cells. Based on the results, estrogen-responsive genes (ERG) were selected and a custom-made cDNA microarray consisting of 200 genes from a narrowed-down subset was produced. Using this microarray, we investigated various aspects of estrogen signaling such as the effect of estrogen-antagonists on ERG expression profile and functional analysis of ERβ and novel estrogen responsive gene EGR3. Furthermore, expression levels of several candidate genes selected from the custom-array contents were analyzed by real-time RT-PCR and immunohistochemistry using breast cancer tissues to determine novel predictive factors for responsiveness to hormone therapy in primary breast cancer patients. Expression of several genes, such as HDAC6, significantly correlated with disease-free and overall survival of patients treated with adjuvant tamoxifen therapy. We are currently developing a new tool for analyzing the effects of novel aromatase inhibitors in individual breast cancer patients using estrogen-responsive element-green fluorescent protein-indicator cells. We hope that these approaches may provide not only new clues for elucidation of estrogen-dependent growth mechanisms of cancer, but also clinical benefits to patients by assessment of individual responses to endocrine therapy.

Keywords Estrogen · Breast cancer · Microarray · Endocrine therapy · Aromatase

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Introduction

In recent years, endocrine therapy for breast cancer has been extensively developing in the settings of advanced disease, adjuvant therapy after surgery and primary systemic therapy. Since effectiveness of endocrine therapy for hormonal cancers is closely associated with the biological disposition of malignant cells, e.g., hormone-dependent cell growth, this therapy is extremely valid in hormone-responsive but not in unresponsive cases. Thus it is important to be able to predict the efficacy of this therapy in each patient before commencing treatment. In breast cancer, the presence of the estrogen receptor (ER)α has been widely used as a predictive marker for endocrine therapy together with clinicopathological factors. However, it is known that approximately 30–40% of ERα-positive patients do not respond to endocrine therapy, while some ERα-negative patients are responsive to this therapy (Fig. 1). The former patients might be well advised to choose some treatment other than antihormone therapy, and the latter group may benefit from antiestrogen drugs. Hence, a readily reliable diagnostic technique for sensitivity evaluation of antihormone among individual patients is warranted.

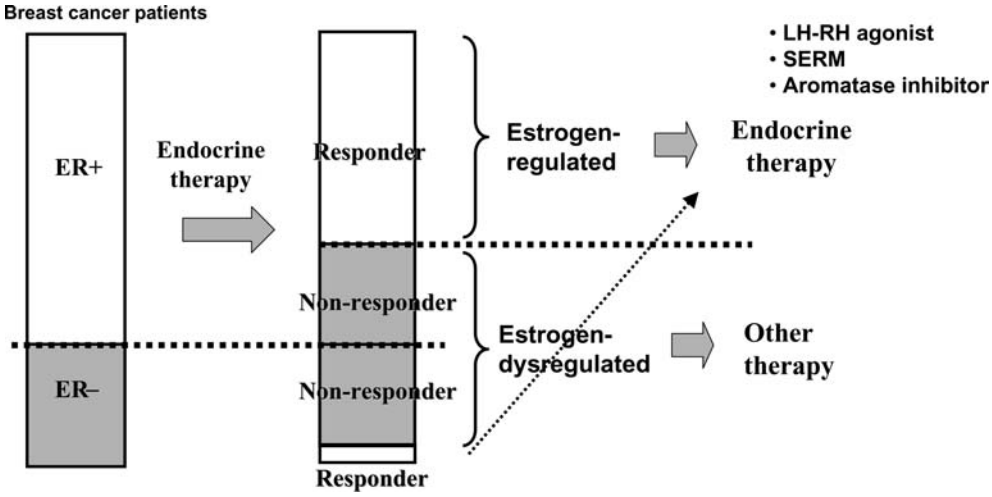

Fig. 1 Discrepancy between expression status of estrogen receptor (*ER*) and efficacy of endocrine therapy. Approximately 30–40% of ER-positive patients do not respond to endocrine therapy. Reprinted with permission of Elsevier from Hayashi [6]. (*SERM* selective estrogen receptor modulator, *LH-RH* Luetinising hormone releasing hormone, *ER* estrogen receptor)

As a comprehensive analytical technique of gene expression, DNA microarray has been rapidly developed and widely applied to various basic and clinical studies. This technique might be useful not only for finding genes that significantly impact clinical outcomes, but also as a new diagnostic tool for cancers. Several laboratories have carried out cDNA microarray analyses of breast cancers [2, 13, 16, 17], and a novel gene whose expression status is highly correlated with prognosis of patients was identified. Nonetheless, there is little information on how many markers are sufficient and which markers are suitable for accurate prognosis and diagnosis of breast cancer, especially regarding sensitivity to antihormone therapy. The microarray system used in previous reports consisted of several thousand clones and therefore is obviously powerful for basic cancer research, but is too complicated and too expensive for clinical application; it would be prohibitive to use such a microarray glass slide for the diagnosis of each patient. On the other hand, a focused microarray of limited numbers of genes has several advantages for further specific studies of estrogen signaling in terms of both basic and clinical research. We first analyzed the expression profiles of genes in human breast cancer cells in response to estrogen. Based on the results, we are currently developing custom-made microarray systems consisting of the selected genes, and attempting to apply them to basic and clinical research on breast cancer.

DNA microarray analysis of estrogen-responsive genes (ERG)

Some years ago, we analyzed estrogen-responsive genes (ERG) expression profiles among ER-positive cancer cell lines using DNA microarray consisting of 9182 human cDNA clones [6, 8]. The vast majority of the transcripts did not reveal substantial changes on treatment with 17β-estradiol: 96% of genes revealed only small (<2 fold) differences between the cells with and without 17β-estradiol. Nevertheless, 240 and 126 genes were found clearly to be induced or repressed by 17β-estradiol treatment, with relative expression levels of >2 and <-2, respectively.

According to the information obtained from the large-scale DNA microarray analysis, approximately 200 genes were selected for custom microarray to study estrogen responsiveness [8]. The time-course of expression of ERG in MCF-7 cells clearly distinguished them into early and late response types, whereas genes showing reduced expression by estrogen treatment did not show apparent subgroups. Expression of many functionally interesting genes was strongly repressed by estrogen stimulation. Using this microarray consisting of a narrowed-down subset, we studied several aspects of estrogen signaling such as the effect of estrogen antagonists and endocrine disruptors on ERG expression profile [8, 15] and functional analysis of ERβ [10] and novel estrogen responsive gene EGR3. We found that transcription factor EGR3 is the bona fide target gene for ERα and is involved in the estrogen-signaling pathway in breast cancer cells [9]. We are currently applying this microarray to various studies such as basic research on estrogen signaling and clinical application for diagnosis of estrogen-dependent cancer (summarized in Fig. 2). We are also focusing particular attention on the development of a predictive microarray for endocrine therapy. We are analyzing paired biopsy samples from primary breast cancer patients before and after administration of aromatase inhibitor, which is used as neoadjuvant primary systemic therapy. The laser capture microdissection technique has been applied for carefully collecting cancer cells, and the TALPAT method [1] is used for the quantitative amplification of mRNA. These approaches have enabled us to investigate correlations between the expression profiles of ERG in breast cancer tissues and efficacy of endocrine therapeutic agents such as aromatase inhibitors.

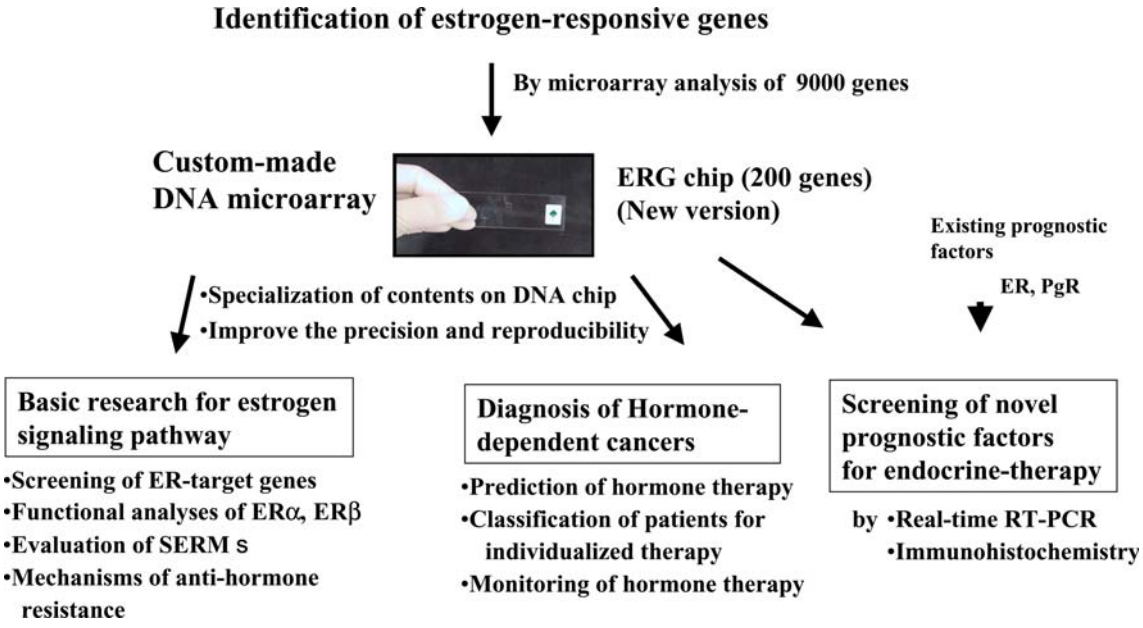

Fig. 2 Development of custom-made estrogen-responsive cDNA microarray and its application to basic and clinical studies including prediction of response to endocrine therapy. Reprinted with permission of Elsevier from Hayashi [6]. (*ERG* estrogen-responsive genes, *SERM* selective estrogen receptor modulator, *PgR* progesterone receptor)

Identification of predictive factors for endocrine therapy

According to the results obtained from the microarray analysis, we selected 11 genes as candidate factors for predicting efficacy of endocrine therapy. Their expression levels in breast cancer tissues were assessed by real-time RT-PCR, and the results analyzed by cluster analysis. ER-positive patients were clearly divided into two subgroups that were expected to represent different estrogen responsiveness [18]. The same subgroups were almost reproducible with a few representative genes in this 11-gene subset. Therefore, we examined the expression of these genes by immunohistochemical analysis in patients treated with adjuvant tamoxifen therapy so as to assess the impact on prediction of the effect of this endocrine therapy. Expression of HDAC6 significantly correlated with disease-free survival rate in tamoxifen-treated patients [12, 18, 20]. Further analysis revealed that the estrogen-inducible HDAC6 in ER-positive breast cancer cells caused deacetylation of α-tubulin and induced cell motility [12].

Green fluorescent protein (GFP) reporter cell system for assessment of cancer estrogen-microenvironment

In postmenopausal breast cancers, aromatase, a key enzyme of estrogen synthesis, is highly expressed in the adipose stromal cells adjacent to tumors. These stromal cells provide intratumoral estrogen signals and affect the efficacy of hormone therapy. To analyze the estrogen signals and detect the ER-activating ability of adipose stromal cells for individual human breast cancers, we developed a new reporter cell system. To visualize ER activation, we first established a stable transformant, named E10, of human breast cancer MCF-7 cells by transfection with the estrogen-responsive element (ERE)-GFP gene [5]. E10 cells specifically express GFP when ER is activated by estrogen or by coculture with adipose stromal cells isolated from breast tumor tissues in the presence of testosterone, an aromatase substrate. Treatment of adipose stromal cells with dexamethasone, a stimulator of aromatase gene expression, resulted in increased expression of GFP in E10 cells in the coculture. Using this system, we characterized the adipose stromal cells of 67 human breast cancers and found that GFP expression levels vary among cases (Fig. 3), suggesting that the ability of adipose stromal cells to activate ER depends on individual breast cancers. Aromatase inhibitors, which have been approved as a first-line treatment for hormone-dependent advanced breast cancer, inhibited induction of GFP expression in the coculture, but sensitivities to these drugs also varied among individual cases. Aromatase gene expression levels in adipose stromal cells did not always correlate with their ability to induce GFP. These results suggest that this system to detect total ER activation based on the interaction with adipose stromal cells is a useful tool for analyzing local estrogen signals and tumor–stromal interactions, and for predicting the efficacy of aromatase inhibitors.

Conclusion

So far, we have studied the molecular mechanisms of estrogen-dependent breast carcinogenesis [7] specifically from the viewpoints of ERα gene expression [3, 14, 19] and functional modulation of ERα in breast

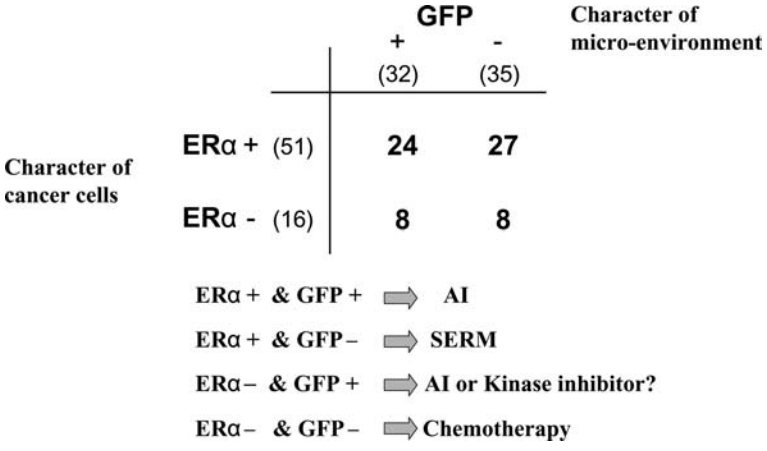

Fig. 3 Possible diagnosis by combination of estrogen receptor and green fluorescent protein (*GFP*) status. In cases where GFP expression is inducible, aromatase inhibitors may be effective regardless of ER status (*ER* estrogen receptor, *GFP* green fluorescent protein, *AI* aromatase inhibitors, *SERM* selective estrogen receptor modulator)

cancer [4, 11]. We have recently studied the function of ERβ in ERα-positive breast cancer cells using our custom-made cDNA microarray, and found that this microarray is a valuable tool for analyzing estrogen signaling in ERα-expressing breast cancer cells [10].

This downsized microarray may also be especially useful for predicting the efficacy of endocrine therapy in individual breast cancer patients. Several candidate genes selected from the contents of our custom-made microarray exhibited clinical impact to survival of breast cancer patients, indicating factors that could distinguish between patients who may and may not be responsive to tamoxifen therapy among ER-positive populations. Although, further study is needed, the microarray technique and other related methods could provide not only new clues for the elucidation of the mechanisms of estrogen-dependent carcinogenesis and development of breast cancer, but also clinical benefits to patients by assessment of their individual responses to endocrine therapy.

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