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ScienceDirect
European Journal of Obstetrics & Gynecology and Reproductive Biology 139 (2008) 11–15
ejog European Journal of Obstetrics & Gynecology and Reproductive Biology
www.elsevier.com/locate/ejogrb

Expert opinion

''Stem cell like'' breast cancers—A model for the identification of new prognostic/predictive markers in endocrine responsive breast cancer exemplified by Plexin B1

Abstract

The identification of new biological markers for breast cancer has adopted a new dimension by the use of novel techniques such as global gene expression profiling. While important results have been achieved by these methods not all hopes for a more precise assessment of patients' prognosis have yet been accomplished and validation of prognostic or predictive gene signatures is still often difficult. Several recent approaches suggest that comparisons of differential gene expression could be more instructive if prior classifications of tumors based on molecular or biological characteristics were applied. We previously reported a subtype of breast cancer by using a cluster of coordinately expressed genes many of which has been associated with the mammary epithelial stem cells. While a stringent inverse link of ER status and proliferation of the tumor was observed among those ''stem cell like'' (SCL) tumors, this link was ''uncoupled'' in about half of the Non-''stem cell like'' (Non-SCL) tumors. This subgroup of SCL tumors can be used as a reference system to analyze changes in the ER pathway by comparing the expression of genes dependent on the ER status.

By using this strategy we identified Plexin B1, a cell-surface receptor for the semaphorin Sema4D, whose expression is reduced in the group of ''uncoupled'' tumors. Loss of Plexin B1 is associated with a poor prognosis in both univariate (all patients: $p = 0.0062$; ER positive: $p = 0.0107$) and multivariate analyses (all patients: $p = 0.032$; ER positive: $p = 0.022$). In conclusion those strategies of gene expression analysis in a context of biological meaningful classifications could be helpful to reveal new prognostic/predictive markers.

Keywords: Plexin B1; Stem cell like breast cancer; Prognosis; Gene expression

1. Global gene expression profiling of breast cancer

Gene expression profiling of malignant breast tumors is a powerful new technique. The global analysis of gene expression offers the opportunity to assess regulation of the entire human genome. This characteristic promised to identify ''master genes'' to give more insight into either key players of malignant transformation, prediction of prognosis and therapeutic efficacy, or new therapeutic targets. Gene expression profiling revealed that breast cancer is a very heterogeneous disease. Sorlie et al. identified an intrinsic gene signature by hierarchical clustering and found at least five tumor subgroups (luminal A and B, basal-like, normal-like and erbB2-like) which showed significant differences in clinical course of disease [1]. Other authors identified prognostic and predictive gene signatures which could discriminate patients with a good and poor prognosis or treatment response, respectively [2–5]. However, comparisons of all these different signatures often failed to identify overlapping genes. Moreover, statistical re-analysis of the signatures revealed that many genes showed comparable results in stratifying tumors with good or poor prognosis [6]. Further drawbacks of the clinical implementation of this new technique were caused by observations that several signatures could not be validated in other patient cohorts or when using different chip platforms. Thus, while important results have already been achieved by the use of gene expression profiling and there are many ongoing clinical trials investigating prognostic and predictive gene signatures, many hopes for a better understanding of tumor development and a more precise assessment of patients prognosis helping in treatment decisions have not yet been accomplished. In light of these circumstances different approaches of the analysis of expression data might be evaluated. We propose a strategy that is more based on biological hypotheses for the classification of tumors. One such specific phenotype that we describe here is the expression of stem cell markers.

0301-2115/$ – see front matter
doi:10.1016/j.ejogrb.2008.02.015

2. "Stem cell like" breast cancers and the estrogen receptor

Endocrine responsiveness is one of the most important characteristics of breast cancer. The negative association between ER expression and proliferation detected in normal breast is frequently lost in breast cancers resulting in receptor-independent growth and poor patients prognosis. The ability to study ER function largely depends on an appropriate reference system to recognize alterations in steroid mediated cell responses.

There is evidence that some breast cancers arise from mammary stem cells [7,8], but these cells have yet not been fully characterized [9]. The existence of distinct ER-positive and ER-negative stem or progenitor cell populations has been postulated [10,11]. Those proposed mammary stem/progenitor cells with distinct ER status could represent such a unique reference to analyze the functional characteristics of the ER pathways.

Microarray analysis of 171 breast cancer samples allowed us to identify two subtypes of stem cell like (SCL) tumors [12]. Both subtypes express the known stem cell markers (e.g. cytokeratins CK-5, -6, -14, -17, ITGA6, S100A1, CD24 as well as markers of the NOTCH and WNT pathways) which was confirmed by real time PCR and immunohistochemistry. However, the two subtypes of SCL tumors are distinguished by their positive and negative ER status, respectively. The ER-negative type of SCL tumors are more related to the proposed primitive stem cell and recently isolated cells which can reconstitute a complete mammary gland in vivo ("mammary repopulating units", MRU) [9]. These ER-negative SCL tumors are furthermore characterized by high proliferation (Fig. 1). The other subtype of SCL tumors which are characterized by ER positivity consistently displays low proliferation. Thus, the inverse link of ER expression and proliferation is perfectly conserved within the SCL group. On the other hand, among the group of Non-"stem cell like" (Non-SCL) tumors about one half of the ER-positive samples are characterized by high proliferation despite their ER positivity. In order to analyze these "uncoupled" tumors the SCL tumors provide a unique reference system to dissect estrogen receptor signaling pathways.

3. Identification of new prognostic markers associated with endocrine responsiveness

Given the crucial role for ER in growth control and response to endocrine therapy, the finding of a highly related group of tumors (SCL tumors) with perfect inverse correlation between proliferation and ER status strongly suggests a widely undistorted estrogen-dependent signaling. Thus, we performed gene cluster analysis of 828 ER-regulated genes and compared the ER dependency of their expression in the SCL and Non-SCL groups. We noted two larger gene clusters, which were uniformly regulated among ER^+/SCL, but show differential expression in the remaining

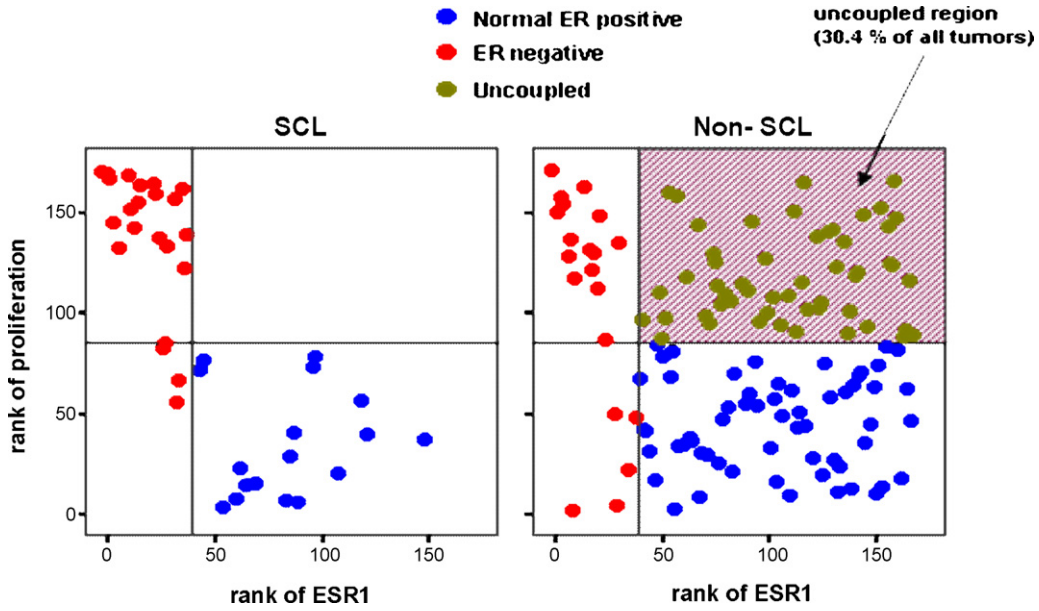

Fig. 1. Correlation of estrogen receptor expression and proliferation in "stem cell like" and Non-"stem cell like" breast cancers. All 171 tumor samples were ranked according to the expression of the estrogen receptor (ESR1) as well as their proliferation state using a series of proliferation markers. Two separate scatter plots of these ranks are given for samples from the "stem cell like" (SCL) group (left) and the Non-SCL group (right). A stringent inverse correlation of proliferation and ESR1 expression was observed in the SCL group, while this link is uncoupled among the Non-SCL tumors. Two vertical lines in the scatter plots represent the boundaries between ER-negative (red) and ER-positive (blue) samples. In addition two horizontal lines tiling the whole collective in two equal sized groups allow the definition of a region containing "uncoupled" tumors with high proliferation despite a positive ER status. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

estrogen receptor positive tumors (ER^+/Non-SCL). This characteristic allowed the identification of novel markers for disturbed estrogen response including HER2-linked cell adhesion molecules and genes involved in maturation of hormone receptors. Among those genes were AHSA2 [13], which is essential for maturation of protein kinases as well as hormone receptors including PR (progesterone receptor), and RNPC2 [14], an important transcriptional coactivator for ERα and ERβ as well as Plexin B1 [15,16], a cell adhesion molecule recently shown to be stably associated with HER2 [17].

4. Plexin B1—pathophysiology, expression and estrogen-dependent regulation

Plexins are cell surface receptors for semaphorin molecules. They have been shown to be widely expressed in epithelial cells and their interaction governs cell adhesion and migration in a variety of tissues (for recent reviews see Kruger et al. [18] and Bussolino et al. [19]). Plexins belong to the c-Met family of scatter factor receptors but lack an intrinsic tyrosine kinase domain. Their ligands, the semaphorins, are cell surface and secreted proteins and were first identified as repulsive axonal guidance molecules governing neuronal growth. Later on it was recognized that these ligand receptor pairs regulate cell motility in many cell types. Semaphorin 4D (Sema 4D) and its receptor Plexin B1 trigger invasive growth, a complex programme that includes cell–cell dissociation, anchorage-independent growth and branching morphogenesis. The observation that Plexin B1 couples with the receptor tyrosine kinases MET [16] and ERBB2 [17] might suggested that Plexin B1 may trigger invasive growth of epithelial cells. However, there is only limited knowledge about the expression of plexins and semaphorins in breast cancer, their regulation and role for disease prognosis and prediction.

A survey of Plexin B1 gene expression among Affymetrix microarray data from 79 human tissues revealed Plexin B1 expression in a variety of human tissues. Highest levels were obtained in several regions of the brain, placenta, prostate, heart, colorectal adenocarcinoma, liver, lung, kidney, and thyroid. Moreover, in a previous microarray analysis of breast cancer samples Plexin B1 was identified among 828 genes which are most dependent on the estrogen receptor status of the tumor [20].

When the subgroup of SCL tumors were used as a reference for a functional ER pathway and these tumors were stratified by their ER status, it is evident that Plexin B1 expression is tightly linked to ER positivity of the tumor. However, when these results were compared to the group of Non-SCL breast cancers, reduced Plexin B1 expression values were observed in the ‘‘uncoupled’’ ER-positive cancers. Moreover, the tight link of ER positivity and Plexin B1 expression allows the definition of biological plausible cutoff value for Plexin B1-positive tumors (Fig. 2).

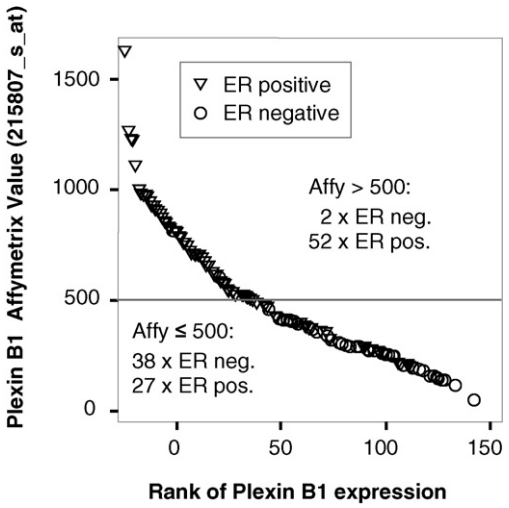

Fig. 2. Correlation of ER status and Plexin B1 expression. A scatter plot of Affymetrix expression values of Plexin B1 (probe set ID 215807_s_at) versus their ranking among 119 non-selected breast cancer samples is given. ER-positive samples are represented by triangles, ER-negative samples by dots. The horizontal line represents the cutoff value of 500, which was adopted as a biological threshold based on Plexin B1 expression in ER-negative samples. The absolute numbers of ER-positive and ER-negative samples above and below this threshold are given.

However, since both microarray and real time PCR measurements of gene expression are based on bulk biopsies containing different cell types, an important question is whether Plexin B1 is expressed by the cancer cells or the surrounding non-tumor tissue. Thus immunohistochemical analysis was performed to demonstrate that in those tumors positive for Plexin B1 the protein is expressed in the epithelial cancer cells, not in the stromal compartment [21].

5. Loss of Plexin B1 predicts poor outcome in estrogen receptor positive breast cancer

On the basis of these findings we were able to investigate the prognostic role of Plexin B1 expression in $n = 119$ breast cancers and performed a verification in an independent gene expression dataset ($n = 295$, van de Vijver et al. [2]) even across two different platforms [21].

When comparing Plexin B1 in univariate analysis to standard parameters, Plexin B1 mRNA expression (Affymetrix value > 500) displayed the highest prognostic value for disease-free survival. As presented in Fig. 3, this result was obtained for both the whole sample group ($p = 0.0062$; Fig. 3A) as well as the subset of ER-positive patients ($p = 0.0107$; Fig. 3B).

Moreover, a significant prognostic value Plexin B1 expression both among all patients and the ER-positive subgroup was also observed in the validation data set (Fig. 3C and D).

In a stepwise multivariable Cox regression model starting with all standard parameters only Plexin B1 ($p = 0.032$ and 0.022), tumor size ($p = 0.017$ and 0.026) and HER2

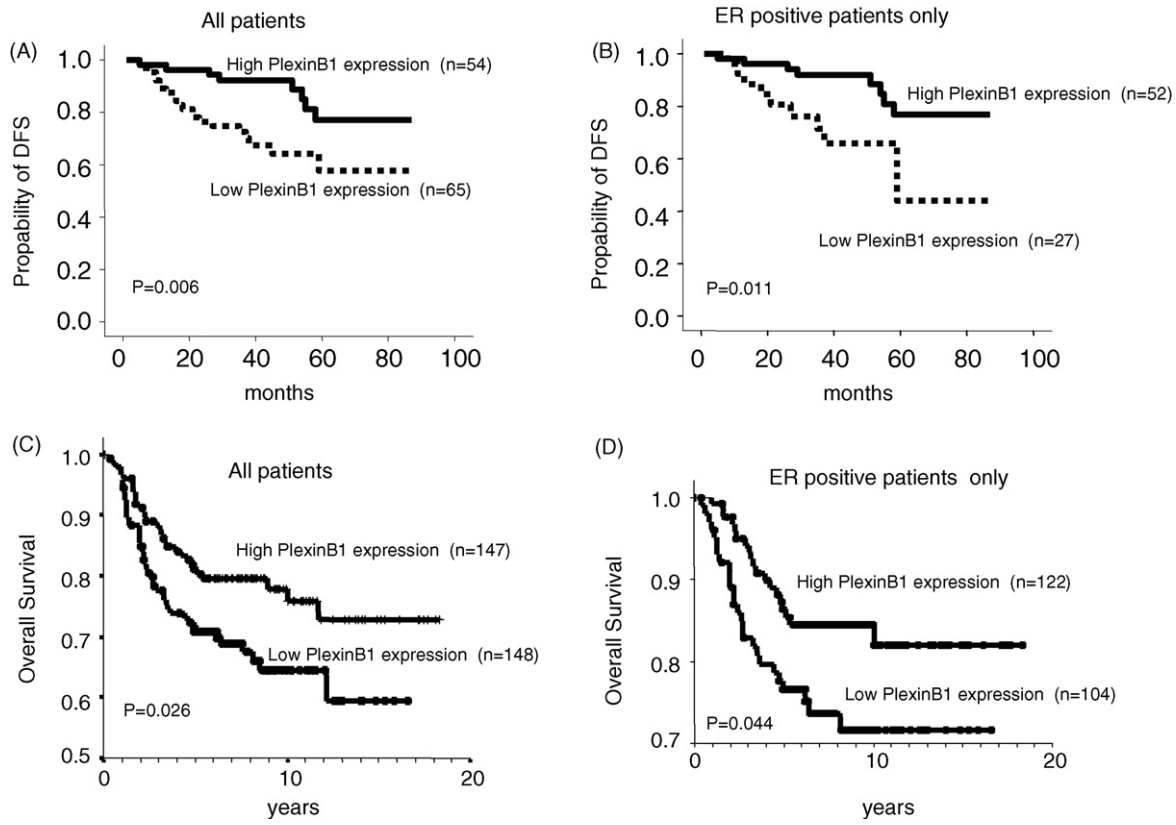

Fig. 3. Prognostic significance of loss of Plexin B1 expression for disease-free survival. (A and B) Kaplan–Meier estimates of the disease-free survival of patients with non-selected tumors stratified by Plexin B1 expression (Affymetrix threshold of 500) are given. Individual curves are presented for all patients (A) and the ER-positive subgroup (B) [21]. (C and D) Validation of the prognostic value of Plexin B1expression in published microarray data sets. The sample group of 295 breast tumors from the study of van de Vijver et al. [2] was median split according to the expression value of the Plexin B1 reporter (AB007867) on their microarray. Kaplan–Meier estimates of survival of patients according to this stratification are given both for all patients (C) and the ER-positive subgroup (D).

($p = 0.048$ and 0.004) remained significant among all patients as well as ER-positive patients only.

These data demonstrate that loss of Plexin B1 is associated with a poor outcome and therefore might serve as a new prognostic marker. In addition the predictive value of this marker in relation to specific treatment options as e.g. endocrine or cytotoxic therapy in adjuvant or neoadjuvant settings should be investigated.

6. Definition and comparison of tumor subgroups—a clue for a successful gene expression analysis

Gene expression analysis uncovered that breast cancer is a heterogeneous disease. This might also be the reason for the difficulties in identifying molecular key players of malignant transformation or signatures which have prognostic/predictive value for all subsets of breast cancers. The strategy presented here aims for a primary discrimination of clearly defined breast cancer subtypes on a molecular and biological basis. This can allow a neat identification of differentially expressed genes, which should be of advantage for detecting new markers and understanding the mechanisms of growth and treatment response. The classification of tumors according to gene pathways is another approach in this direction. In summary, the analysis of differentially expressed marker genes in pre-defined subtypes of breast cancers might be helpful in identifying new prognostic and predictive markers, as well as new therapeutic targets.

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22 August 2007