Review Article
PAR Genes: Molecular Probes to Pathological Assessment in Breast Cancer Progression

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Taking the issue of tumor categorization a step forward and establish molecular imprints to accompany histopathological assessment is a challenging task. This is important since often patients with similar clinical and pathological tumors may respond differently to a given treatment. Protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR), is the first member of the mammalian PAR family consisting of four genes. PAR1 and PAR2 play a central role in breast cancer. The release of N-terminal peptides during activation and the exposure of a cryptic internal ligand in PARs, endow these receptors with the opportunity to serve as a “mirror-image” index reflecting the level of cell surface PAR1&2-in body fluids. It is possible to use the levels of PAR-released peptide in patients and accordingly determine the choice of treatment. We have both identified PAR1 C-tail as a scaffold site for the immobilization of signaling partners, and the critical minimal binding site. This binding region may be used for future therapeutic modalities in breast cancer, since abrogation of the binding inhibits PAR1 induced breast cancer. Altogether, both PAR1 and PAR2 may serve as molecular probes for breast cancer diagnosis and valuable targets for therapy.

1. Introduction

The classification of a tumor differentiation level is routinely based on histopathological criteria whereby poorly differentiated tumors generally exhibit the worst prognoses. However, the underlying molecular pathways that regulate the level of breast tumor development are as yet poorly described. Until now the pathological tissue criteria that entail tissue traits have not been defined by an appropriate set of genes. A challenging task is to take the issue of breast tumor categorization a step forward and establish molecular imprints to accompany histopathological assessment. This is important since often patients with similar clinical and pathological tumors may have a markedly different outcome in response to a given treatment. These differences are encoded by and stem from the tumor genetic profile [1]. Individual gene signature may complement or replace the traditional pathological assessment in evaluating tumor behavior and risk. This is the basis for optimizing our approach to personalized care whereby genomic finger prints may refine the prediction of the course of disease and the response to treatment [2]. Oncotype Dx is a clinically validated and widely used multigene assay (there are also other commercially available gene panels such as Mammaprint; Agenda Amsterdam, Netherland, and THEROS H/I; Biotheranostics, San Diego, CA), that quantifies the likelihood of breast cancer recurrence. This gene profile has been developed specifically for women with hormone receptor-positive (estrogen and progesterone receptor; ER, PR) and lymph node-negative disease. The gene profile consists of 21 genes that are associated with disease recurrence. Sixteen are cancer-related genes and 5 serve as reference genes. This gene panel is used to calculate the recurrence score (RS), a number that correlates with the specific likelihood of breast cancer recurrence within 10 years from the original diagnosis. Therefore, an ongoing goal is to identify important genes that play a central part in breast cancer biology and determine their relative function during the course of breast cancer progression [3]. Identification of these genes will significantly contribute to the prospect of treatment making choices.

Protease-activated receptor-1 (PAR1), a G protein-coupled receptor (GPCR), is the first and prototype member of
the mammalian PAR family consisting of four genes. The activation of PAR1 involves the release of an N-terminal peptide and the exposure of an otherwise hindered ligand, resulting in an exclusive mode of activation. This mode of activation serves as a general paradigm for the entire PAR family [4–6]. While a well-known classical observation points to a close link between hyperactivation of the coagulation system and cancer malignancies, the molecular mechanism that governs procoagulant tumor progression remains poorly defined [7–10]. Thrombin is a main effector of the coagulation cascade. In addition to cleaving fibrinogen, it also activates cells through at least three PARs: PAR1, PAR3, and PAR4. In contrast, PAR2 is activated by multiple trypsin-like serine proteases including the upstream coagulant proteases VIIa—tissue factor (TF) and Xa, but not by thrombin. It is now becoming well established that human Par1, hPar1, plays a central role in epithelial malignancies [13, 14, 16]. PAR2, the second member of the family, is also emerging with central assignments in breast cancer [11, 12]. High levels of hPar1 expression are directly correlated with epithelia tumor progression in both clinically obtained biopsy specimens and a wide spectrum of differentially metastatic cell lines [13, 14]. PAR1 also plays a role in the physiological invasion process of placental cytotrophoblasts during implantation into the uterus decidua [15]. Trophoblast invasion shares many features with the tumor cell invasion process. It differs, however, by the time-limited hPar1 expression, which is confined to the trophoblast-invasive period and is shut off immediately thereafter, when there is no need to invade [13]. This strongly supports the notion that the hPar1 gene is part of an invasive gene program. Surprisingly, the zinc-dependent matrix-metalloprotease 1 (MMP-1), a collagenase that efficiently cleaves extra cellular matrix (ECM) and basement membrane components, has been shown to specifically activate PAR1 [16]. PAR1-MMP1 axis may thus provide a direct mechanistic link between PAR1 and tumor metastasis. The mechanism that leads to hPar1 gene overexpression in tumor is yet unclear and under current extensive investigation. Although the impaired internalization of PAR1 that results with persistent signaling and invasion was previously suggested for several breast cancer lines [17], an imbalanced expression between hPar1 repressors and activators was proposed, suggesting transcriptional regulation [18]. We found that the mechanism of hPar1 overexpression involves enhanced transcriptional activity, whereby enhanced RNA chain elongation takes place in the aggressive cancer cells as compared with the nonaggressive, low metastatic potential cells [19]. Indeed, we have identified the Egr-1 transcription factor as a critical DNA-binding protein eliciting hPar1 expression in prostate cancer cells and the wt p53 tumor suppressor as an hPar1 transcription repressor [19, 20]. The wt form of p53 thus acts as a fine-tuning regulator of hPar1 in cancer progression.

2. Prognostic Parameters of PARs

The PARs act as delicate sensors of extra cellular protease gradient to allow the cells to respond to a proteolytically modified environment. The fact that PAR1 gene and protein overexpression are associated with the aggressiveness of a tumor, in vivo, reflect its potential role in cancer dissemination. Furthermore, it assigns PAR1 as an attractive target for anticancer therapy. On the other hand, the release of an N-terminal peptide during activation and the exposure of an otherwise cryptic internal ligand in PARs endow these receptors with the opportunity to serve as a “mirror-image” index reflecting in body fluids the level of PARs on the surface of cancer cells. Hence, PAR1 and PAR2 peptides in the blood directly imitate PAR expression serving as a faithful indicator for the extent of cancer progression. While the overexpression of both PAR1 and PAR2 takes place on the surface of cancer cells that are being constantly turned over in the body, yet there is no current information as to the half-life of the released peptides. It is envisioned that measuring the level of released peptides may underline the severity of cancer. Another aspect is that the followup levels of PAR1-released peptides may be instrumental in demonstrating the effectiveness of a given treatment. For example, determining the level of the released PAR1 and PAR2, through repeated measurements in the blood stream, may serve as a baseline for a patient, and a sensitive indicator for response to a treatment. If the released PAR peptides are becoming gradually low and finally disappear, it may reassure that the tumor is indeed regressing until finally the cancer disappears. In contrast, if the level remains unchanged, it may indicate that the tumor is progressing despite of a given treatment. A critical aspect, however, that needs to be addressed is the prospect of high released PAR1/S2 peptides present during inflammation [21, 22]. Therefore, the repeated followup of PAR released peptides is necessary for the purpose of demonstrating that during inflammation the high PAR-released peptide level is transient and disappears when the inflammatory response is over. In contrast, in the case of a tumor, the level of PAR-released peptides remains constantly high. The relative contribution of PAR1 versus PAR2 during the process of tumor progression is as yet unknown and is under current investigation. One approach to decisively address this issue is by immunohistological staining (of anti-PAR1 and anti-PAR2 antibodies, separately) utilizing tissue microarray biopsy specimens on a large pool of primary breast cancer biopsy specimens representing invasive carcinoma. Such analysis will determine the relative percentage of PAR-positive individuals in a given cancer patient pool. Whether PARs join the triple negative population (ER-, PR-, and Her-2/Neu, an indicator of disease aggressiveness)—or perhaps stands independently as a prognostic marker—needs to be evaluated.

3. PARs as Target for Therapy

Importantly, PAR1 cellular trafficking and signal termination appear to occur in a different mode than other GPCRs. Instead of recycling back to the cell surface after ligand stimulation, activated PAR1 is sorted to the lysosomes where it is degraded [23, 24]. While cellular trafficking of PAR4 impinges on the extent and mode of signaling, the identification of individual PAR1 signaling partners and their contribution to breast cancer progression remain to be elucidated.
Breast cancer progression
Steps in epithelia tumor progression

![Diagram showing steps in breast cancer progression and PAR signaling](image)

**Figure 1:** Steps in breast cancer progression. Subtypes definition of breast cancer according to ER, PR, and Her2/neu status. Additional categorization is suggested including PARs status.

We have adopted the approach of utilizing a truncated form of hPar1 gene devoid of the entire cytoplasmic tail to demonstrate the significant role of PAR1 signaling in breast tumor progression. This was demonstrated in a xenograft mice model of mammary gland tumor development, *in vivo* [25]. Along this line of evidence, we have identified PAR1 C-tail as a scaffold site for the immobilization of signaling partners. In addition to identifying key partners, we have determined the hierarchy of binding and established a region in PAR1 C-tail critical for breast cancer signaling. This minimal binding domain may provide a potent platform for future therapeutic vehicles in treating breast cancer. The above-described outcome is a brief summary of the detailed experimental approach illustrated below.

The functional outcome of MCF7 cells overexpressing various hPar1 constructs *in vivo* was assessed by orthotopic mammary fat pad tumor development. MCF7 cells overexpressing either persistent hPar1 Y397Z or wt hPar1 constructs (e.g., MCF7/Y397Z hPar1; MCF7/wt hPar1) markedly enhanced tumor growth *in vivo* following implantation into the mammary glands, whereas MCF7 cells overexpressing truncated hPar1, devoid of the entire cytoplasmic tail, behaved similarly to control MCF7 cells in vector-injected mice, which developed only very small tumors. The tumors obtained with MCF7/wt hPar1 and MCF7/Y397Z hPar1 were 5 and 5.8 times larger, respectively, than tumors produced by the MCF7/empty vector-transfected cells. Histological examination (H&E staining) showed that while both MCF7/wt hPar1 and MCF7/Y397Z hPar1 tumors infiltrated into the fat pad tissues of the breast, the MCF7/Y397Z hPar1 tumors further infiltrated the abdominal muscle. In contrast, tumors produced by empty vector or truncated hPar1-transfected cells were capsulated, with no obvious cell invasion. Tumor growth can also be attributed to blood vessel formation [26, 27]. The hPar1-induced breast tumor vascularization was assessed by immunostaining with anti-lecin and anti-CD31 antibodies, showing that both MCF7/wt hPar1 and MCF7/Y397Z hPar1 tumors were intensely stained. In contrast, only few blood vessels were found in the small tumors of empty vector or truncated hPar1. Thus, both MCF7/wt...
Figure 2: Activation of PAR1 leads to the association of Etk/Bmx with PAR1 C-tail. This association is mediated through Etk/Bmx PH-domain enabling next the binding of Shc. The site of the "signal binding" domain (e.g., Etk/Bmx, as a prime signaling partner) in PAR1 has been identified. Insertion of successive replacement of A residues forming a PAR1 mutant incapable of binding Etk/Bmx showed impaired capabilities of PAR1 induced invasion and migration. This site provides therefore a platform for the development of future therapeutic medicaments in breast cancer.

\[ hPar_1 \] and MCF7/Y397Z hPar1 cells were shown to effectively induce breast tumor growth, proliferation, and angiogenesis, while the MCF7/truncated hPar1 and MCF7/empty vector-expressing cells had no significant effect. This experimental results highlight the significance of PAR1 signaling in PAR1-induced breast cancer progression.

4. Antibody Array for Protein-Protein Interactions Reveals Signaling Candidates

Next, in order to identify specific PAR1 signaling components, the following approach was utilized. To detect the putative mediator(s) linking PAR1 to potential signaling pathway, we examined a custom-made antibody-array membranes. When aggressive breast carcinoma MDA-MB-435 cells (with high \( hPar_1 \) levels) were incubated with the antibody-array membranes before and after PAR1 activation (15 minutes), the following results were obtained. Several activation-dependent proteins which interact with PAR1, including ICAM, c-Yes, Shc, and Etk/Bmx, were identified. Of these proteins, we chose to focus here on Etk/Bmx and Shc.

The epithelial tyrosine kinase (Etk), also known as Bmx, is a nonreceptor tyrosine kinase that is unique by virtue of being able to interact with both tyrosine kinase receptors and GPCRs [28]. This type of interaction is mainly attributed to the pleckstrin homology (PH) which is followed by the Src homology SH3 and SH2 domains and a tyrosine kinase site [29]. Etk/Bmx-PAR1 interactions were characterized by binding of lysates exhibiting various \( hPar_1 \) forms to GST-PH-Etk/Bmx. While Y397Z \( hPar_1 \) and \( wt \) \( hPar_1 \) showed specific association with Etk/Bmx, lysates of truncated \( hPar_1 \) or JAR cells (lacking PAR1) exhibited no binding. A tight association between the PAR1 C-tail and Etk/Bmx was obtained, independent of whether \( wt \) or kinase-inactive Etk/Bmx (KQ) was used [29, 30].

5. Hierarchy of Binding

Next, we wished to determine the chain of events mediating the signaling of PAR1 and the binding of Shc and Etk/Bmx to PAR1 C-tail. Shc is a well-recognized cell signaling adaptor known to associate with tyrosine-phosphorylated residues. To this end, analysis of MCF7 cells that express little to no \( hPar_1 \) were ectopically forced to overexpress \( hPar_1 \) gene. When communoprecipitation with anti-PAR1 antibodies following PAR1 activation was performed, surprisingly, no
Successful PAR1-derived peptides termed “pepducin” were shown to inhibit cancer growth and development. Along this line of evidence, certain PAR receptors are critical targets for therapy in breast cancer. What is the relative efficacy of PAR1 versus PAR2 in breast cancer tumor growth and development is yet an open question and a subject of current evaluation.

**Conflict of Interests**

The authors have declared that no conflict of interests exists.

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