ANXA7 Expression has Prognostic Impact for Patient Survival In Triple Negative Breast Cancers

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Abstract

Triple-negative breast cancers account for 10–17% of all breast carcinomas and there is considerable need for reliable prognostic markers to assist clinicians in making diagnostic and therapeutic management decisions. Altered ANXA7 (a novel pro-apoptotic tumor suppressor gene located on chromosome 10q21) protein levels are associated with a tumor-prone phenotype in knockout mouse model and prognostically challenging aggressive forms of prostate and breast cancer. So far, information is not available regarding the association of patient survival and ANXA7 expression in triple-negative breast cancers. Therefore, we used a retrospective prognostic tumor microarray (TMA) technology in order to evaluate the ANXA7 immunoreactivity as a possible diagnostic and/or prognostic marker of triple-negative breast cancer by immunoperoxidase assay using an ANXA7 monoclonal antibody. We report here that the expression of ANXA7 is significantly enhanced in triple-negative breast cancers and is associated with poor overall patient survival. We conclude that ANXA7 may be a new prognostic markers or a target for improving the treatment efficiency of patients with triple-negative breast cancers.

Keywords: ANXA7; Triple-negative breast cancer; Survival; Prognosis

Introduction

Breast cancer represents a heterogeneous group of tumors that are diverse in behavior, outcome, and response to therapy [1-3]. Currently, breast cancer patients are managed according to algorithms based on the clinical and histopathological parameters in conjunction with assessment of hormone receptor (estrogen and progesterone receptor) status and HER2 overexpression/gene amplification. While effective tailored therapies have been developed for patients with hormone receptor-positive or HER2+ disease, chemotherapy is the only modality of systemic therapy for patients with breast cancers lacking the expression of these markers (triple-negative breast cancers) [4]. Thus, the development of biologically informed systemic therapies and targeted therapies for triple-negative breast cancers is of paramount importance and may prove to be a challenging task, only achievable by understanding the complexity of this heterogeneous group of tumors. Although triple-negative breast cancers are reported to respond to neoadjuvant chemotherapy, survival of patients with such tumors is still poor and their management may therefore require a more aggressive alternative intervention [5]. Therefore, finding a reliable biomarker or a target that could be used to individualize both patient prognostic and therapy is essential for the prevention and cure of triple-negative breast cancers.

The finding of a novel tumor suppressor gene (ANXA7) in a chromosomal region with frequent mutations/deletions in human cancers raised important questions as to its use as a prognostic factor for the triple-negative breast cancer. Biochemically, we found that ANXA7 codes for a membrane-associated, Ca2+-activated GTPase and is involved in exocytotic secretion [6-9]. In our work with the Anxa7 knockout mouse we found that the nullizygous Anxa7 (-/-) mutant is embryonically lethal and the Anxa7(+/-) animals developed profoundly increased frequency of tumors compared to the Anxa7 (+/+) normal littermates control. Tumor frequency is in the range of 20-50% of animals, becoming more accentuated with advancing age [10]. Consistently, using a prostate tissue microarray, we found alterations of ANXA7 protein expression in metastases and hormone insensitive local recurrent cancers. In addition, we found that allelic loss of the ANXA7 gene occurs in over one third of primary carcinoma of the prostate and breast [11,12].

Further studies from our laboratory indicated that altered expression of ANXA7 was associated with metastatic breast cancer with poor patient survival [13]. We have therefore hypothesized that ANXA7 signaling might also play a role in triple-negative breast cancer. To test this hypothesis, we have used breast tissue microarrays containing approximately 71 biopsy specimens to ask whether the levels of expression of ANXA7 might have predictive value for diagnosis and survival of these patients. The present study aims to show the expression of ANXA7 in triple-negative breast cancer tissues and to elucidate its relationship to clinicopathological parameters and its impact on patient prognosis.

Material and Methods

Patient characteristics

In our study, the conditions of 71 patients were diagnosed as triple-negative breast cancer patients and the archival specimens were printed on glass slides. This retrospective prognostic breast cancer tissue microarray consisted of the follow up data with tumor specific survival and treatment information. The age of the patients varied from 33 to 97 years, with a median age of 61 years. They were treated for
primary breast cancer at the University Hospital in Basel (Switzerland),
Womens Hospital Rheinfelden (Germany), and the Kreiskrankenhaus
Lörrach (Germany) between 1985 and 1994. The median follow up
time was 63.0 months (range 1 – 151). Formalin fixed, paraffin
embedded tumor material was available from the Institute of Pathology,
University of Basel. The pathologic stage, tumor diameter, and nodal
status were obtained from the primary pathology reports. All slides
from all tumors were reviewed to define the histologic grade according
to Elston and Ellis (Gusterson et al., 1992) (BRE) and the histologic
tumor type. Stage, grade and nodal status were strongly associated with
tumor specific survival of our patients (p<0.0001 each).

Tissue microarray construction

Tumor samples were arrayed as previously described [14]. Briefly,
H&E-stained sections were made from each selected primary tumor
block (“donor blocks”) to define representative tumor regions. Tissue
cylinders with a diameter of 0.6 mm were then punched from each
“donor” block using a custom-made precision instrument (Beecher
Instruments, Silver spring, MD) and brought into a recipient paraffin
block eventually containing individual samples. The tissue microarray
blocks were constructed in four replicas each containing samples from
different regions of the donor tissues. One of these four samples
was taken from the central part of the tumor and three from different
peripheral areas. Four µm sections of the recipient blocks were then
cut using an adhesive coated slide system (Instrumedics Inc., New
Jersey) supporting the cohesion of the 0.6mm array elements on
glass. One section from each of the four replica arrays was used for
immunohistochemical analysis.

Immunohistochemistry

Three conventional “large” sections from all tumors and three
sections from each of the four different replica tumor tissue microarray
blocks were used for immunostaining. The guidelines from the
package insert were followed for each antibody. Standard indirect
immunoperoxidase procedures (ABC-Elite, Vector Laboratories) in
combination with monoclonal antibodies were used for detection of
ANXA7 (1:1000, DAKO), Her-2 (Hercep test™ DAKO) p53 (DO-7,
prediluted DAKO, Glostrup, Denmark), estrogen receptor (ER ID5,
1:1000, DAKO), and progesterone receptor (NCL-PGR, 1A6, 1:600,
NOVOCASTRA Laboratories Ltd, Newcastle upon Tyne, United
Kingdom). Tumors with known positivity were used as positive
controls. The primary antibody was omitted for negative controls.
These arrays have previously been tested for lack of interaction with
irrelevant monoclonal antibodies. Scoring of the immunohistochemical
staining followed the guidelines in the package insert using an objective
at 10x magnification.

Immunohistochemical Evaluation of ANXA7 Expression

The ANXA7 monoclonal antibody has been shown to
recognize specifically ANXA7 and proved to be a useful reagent for
immunohistochemical studies [11]. Human breast carcinomas with 71
specimens diagnosed as triple-negative breast cancer with follow-up
data were examined for the expression of ANXA7 and their reactivity
compared with normal human breast tissues. Three types of ANXA7
expression were detected in triple-negative breast cancer specimens.
The first group showed weak ANXA7 expression (designated low),
the second group showed moderate ANXA7 expression (designated
medium), and the third group showed strong ANXA7 expression
(designated high). The staining was nuclear and cytoplasmic as
expected for a protein localized to the nucleus and cytoplasm. The

specificity of tissue staining was determined by the demonstration of
negative staining by omitting primary antibody and with an irrelevant
antibody.
Clinical correlation of ANXA7 Expression in triple-negative breast cancer patients

We used a prognostic breast cancer array containing 71 triple-negative breast cancer patient specimens with the retrospective follow-up of 105 months. ANXA7 expression was detected by immunohistochemistry and the presence of ANXA7 in each of these patients was correlated to survival parameters. Kaplan-Meier curves of cumulative survival in patients with low versus medium and high cytoplasmic ANXA7 expression shows a significant separation within 5 years of follow-up. Significantly, there is no change observed in nuclear ANXA7 staining in all the cases. Figure 1 illustrates the cumulative survival of 3 groups from the diagnosis of triple-negative breast cancer. The duration of survival was significantly shorter in patients with strong cytoplasmic ANXA7 expression (group 3) compared with patients with weak ANXA7 expression (group 1) (25% versus 100% in 5 years). Similarly, with the increased ANXA7 expression in groups 1 and 2, the cumulative survival was decreased from 100% to 65%. When considered in a univariate analysis, that the patient group with weak ANXA7 have greater probability of survival, and that high cytoplasmic staining of ANXA7 is associated with lower probability of survival. These results indicate ANXA7 levels have considerable potential to be of practical use in routine assessment of triple-negative breast cancer patients.

Table 1: The relationship between ANXA7 cytoplasmic expression levels and clinicopathological factors in triple negative breast cancer samples.

| Variable                     | Patients | ANX7 | Pearson p | Spearman r (p) |
|------------------------------|----------|------|-----------|----------------|
| Age                          |          |      |           |                |
| <60                          | 41       | 0    | 30        | 11             |
| ≥60                          | 30       | 3    | 24        | 3              |
| pT                           |          |      |           |                |
| 1                            | 19       | 1    | 13        | 5              |
| 2                            | 39       | 2    | 30        | 7              |
| 3                            | 3        | 0    | 3         | 0              |
| 4                            | 9        | 0    | 7         | 2              |
| pN                           |          |      |           |                |
| 0                            | 41       | 2    | 32        | 7              |
| 1                            | 21       | 1    | 14        | 6              |
| 2                            | 5        | 0    | 4         | 1              |
| BRE                          |          |      |           |                |
| 1                            | 2        | 0    | 2         | 0              |
| 2                            | 16       | 2    | 13        | 1              |
| 3                            | 43       | 0    | 32        | 11             |
| PS3                          |          |      |           |                |
| neg                          | 38       | 2    | 28        | 8              |
| pos                          | 33       | 1    | 26        | 6              |
| Vessel infiltration          | Not mentioned | 58 | 46 | 9 | 0.049 | 0.236 (0.048) |
| Lymph node metastasis        | yes | 13 | 0 | 8 | 5 | |
| Node negative                | 40 | 2 | 31 | 7 | 0.420 | 0.102 (0.413) |
| Node positive                | 27 | 1 | 19 | 7 | 1 | |
| 5 year survival              |          |      |           |                |
| Node negative                | 75.1%  | 100% | 77.8%  | 55.6%  |
| Node positive                | 37.9%  | 100% | 46.2%  | 0     |
| all                          | 60.1%  | 100% | 65.9%  | 24.7%  |

Statistical analysis

All data were analyzed by statistics software (SPSS 13.0 for Windows; SPSS, Inc.). Survival time was measured in months from date of surgery until date of death or last follow-up. Survival analysis was performed using the Kaplan-Meier method and compared by the log-rank test. Prognostic relevance was evaluated by multivariate Cox proportional hazards regression analysis. P < 0.05 was considered as significant.

Discussion

Our study focused on the relationship between ANXA7 expression and human triple-negative breast cancer. Triple-negative breast cancer encompasses a heterogeneous group of tumors that show distinctive, but rather heterogeneous, pathological and clinical features and constitutes one of the most challenging groups of breast cancers to treat. Thus, the discovery of novel molecular targets for its diagnosis and treatment has the potential to improve the clinical strategy and outcome of patients with this disease. In this study, we investigated the high-risk group of breast cancer with the triple-negative phenotype that lacks the benefit of specific therapy and identified a central role for ANXA7 involvement in the progression of this aggressive triple-negative breast cancer. To our knowledge, this is the first study to demonstrate the role of ANXA7 expression in triple-negative breast cancer.

The selection of therapies for breast cancer today is based on prognostic features (chemotherapy, radiotherapy), hormone receptor status (hormonal therapy) and Her-2 status (trastuzumab therapy). Her-2 and p53 are tumor related proteins that have the potential to further improve individualization of patient management, by predicting response to chemotherapy, hormonal therapy and radiotherapy. The development of multiple organ hyperplasia and high incidence of spontaneous tumors in ANXA7 (+/-) knockout mice demonstrated that ANXA7 plays an important role in repressing tumor development. A significant relationship emerged between Her-2 and ANXA7. When Her-2 is negative, 66% of the patients with high ANXA7 expression can be distinguished from patients with no ANXA7 expression [12].
Our results in this study indicate that high cytoplasmic expression of ANXA7 is associated with triple-negative breast cancer. In addition, our results show that high cytoplasmic expression of ANXA7 is also associated with poor prognosis. Parallel sections of the same specimens were investigated for alteration in the expression of Her-2, p53, ER and PR. Our studies with a 105 months follow-up demonstrate that early stage patients with low cytoplasmic ANXA7 expression have an excellent prognosis. Importantly with lymph node status with poor outcome, significant number of patients can be identified for high risk on the basis of their ANXA7 expression, thus providing a powerful prognostic tool that can be validated and utilized in all the patients.

In conclusion, the present study shows the possibility of using ANXA7 as both a clinically relevant indicator of disease progression and a prognostic biomarker for survival in the patients with triple-negative breast cancer. Based on the present data we therefore suggest that this new knowledge appears to operationally simplify prognosis for a significant fraction of the breast cancer population. For the triple-negative breast cancer patients so identified as being at particular risk, physicians can be alerted to the necessity of aggressive treatment. We conclude that if these data can be validated in a larger population of patients and in prospective studies with extensive follow-up, high cytoplasmic ANXA7 expression could become an important biomarker for identifying triple-negative breast cancer patients at high risk, and is worthy of further exploration as a prognostic factor in survival. Finally, ANXA7 may serve as a promising target for triple-negative breast cancer therapy.

References

1. Reis-Filho JS, Simpson PT, Gale T, Lakhani SR (2005) The molecular genetics of breast cancer: the contribution of comparative genomic hybridization. Pathol Res Pract 201: 713–725.
2. Lacroix M, Toillon RA, Leclercq G (2004) Stable ‘portrait’ of breast tumors during progression: data from biology, pathology and genetics. Endocr Relat Cancer 11: 497–522.
3. Simpson PT, Reis-Filho JS, Gale T, Lakhani SR (2005) Molecular evolution of breast cancer. J Pathol 205: 248–254.
4. Rouzier R, Perou CM, Symmans WF, Ibrahim N, Cristofanilli M, et al. (2005) Breast cancer molecular subtypes respond differentially to preoperative chemotherapy. Clin Cancer Res 11: 5678–5685.
5. Carey LA, Dees EC, Sawyer L, Gatti L, Moore DT, et al. (2007) The triple negative paradox: primary tumor chemosensitivity of breast cancer subtypes. Clin Cancer Res 13: 2329–2334.
6. Creutz CE, Pazoles CJ, Pollard HB (1978) Identification and Purification of an Adrenal Medullary Protein (Synexin) that Causes Calcium- dependent aggregation of isolated Chromaffin granules. J Biol Chem 253:2858-2866.
7. Creutz CE, Pazoles CJ, Pollard HB (1979) Self-Association of Synexin in the Presence of Calcium. Correlation with Synexin-induced membrane fusion and examination of the Structure of Synexin Aggregates. J Biol Chem 254: 553-558.
8. Raynal P, Pollard HB (1994) Annexins: the problem of assessing the biological role for a gene family of multifunctional calcium-and phospholipid-binding proteins. Biochim Biophys Acta1197: 63-93.
9. Caohuy H, Srivastava M, Pollard HB (1996) Membrane fusion protein synxin (annexin VII) as a Ca++/GTP sensor in exocytotic secretion. Proc Natl Acad Sci USA 93:10797-10802.
10. Srivastava M, Glasmann M, Leighton X, Naga S, Montagna C, et al. (2003) Haplinsufficiency of Anx7 tumor suppressor gene and consequent genomic instability promotes tumorigenesis in the Anx7(+/-) mouse. Proc Natl Acad Sci U S A 100: 14287-14292.
11. Srivastava M, Bubendorf L, Srikantan V, Fossum L, Nolan L, et al. (2001) ANX7, a candidate tumor-suppressor gene for prostate cancer. Proc Natl Acad Sci USA 98:4575–4580.
12. Leighton X, Srikantan V, Pollard HB, Sukumar S, Srivastava M (2004) Significant allelic loss of ANX7 region (10q21) in hormone receptor negative breast carcinomas. Cancer Lett 210: 239-244.
13. Srivastava M, Bubendorf L, Raffeld M, Bucher C, Torhorst J, et al. (2004) Prognostic impact of ANX7-GTPase in metastatic and HER2- negative breast cancer patients. Clin Cancer Res 10:2344-2350.
14. Kononen J, Bubendorf L, Kallioniemi A, Bärfurd M, Schraml P, et al. (1998) Tissue microarrays for high-throughput molecular profiling of tumor specimens. Nat Med 4: 844–847.
15. Torhorst J, Bucher C, Kononen J, Haas P, Zuber M, et al. (2001) Tissue microarrays for rapid linking of molecular changes to clinical endpoints. Am J Pathol. 159: 2249-2256.