ANX7 as a bio-marker in prostate and breast cancer progression

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The ANX7 gene codes for a Ca^{2+}-activated GTPase, which has been implicated in both exocytotic secretion in cells and control of growth. In this review, we summarize information regarding increased tumor frequency in the Anx7 knockout mice, ANX7 growth suppression of human cancer cell lines, and ANX7 expression in human tumor tissue micro-arrays. The loss of ANX7 is significant in metastatic and hormone refractory prostate cancer compared to benign prostatic hyperplasia. In addition, ANX7 expression has prognostic value for predicting survival of breast cancer patients.

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1. Introduction

Long-term survival in cancer currently rests on detection and appropriate therapy at the earliest possible stage. Molecular markers for cancer are therefore being strongly sought after, in hopes of achieving the very earliest detection. Such markers include new tumor suppressor genes (TSG), whose identities are presently only hypothesized on the basis of allelic loss. For example, multiple potential tumor suppressor genes have been hypothesized to exist around the 10q21 locus of chromosome 10. Interestingly, the human ANX7 gene is located on chromosome 10q21, and gradually, we became alert to the possibility that ANX7 might have tumor suppressor gene activity. We found that ANX7 codes for a membrane-associated, Ca^{2+}-activated GTPase, and is involved in exocytotic secretion [8,12,13, 34,38]. ANX7 GTPase activity is sensitive to such critical modulators of conventional G-proteins as Al_2F_6 and mastoparan [8,9]. In studies with cultured cells, ANX7 can be shown to bind and hydrolyze GTP [8]. ANX7 protein also forms Ca^{2+} channels in membranes [33], which can be stabilized in long open states by GTP (Pollard and Arispe, unpublished data). The subcellular distribution of ANX7 protein is predominantly in membranes and to a lesser extent in the nucleus [11, 27].

2. Methods and results

2.1. ANX7 plays a role in growth throughout phylogeny

Early work on the annexin VII gene (anx7 isynexin) has shown that it is expressed in small amounts in nearly every cell, and is found throughout phylogeny as a single copy gene in organisms as diverse as man [36], mouse [44,45], Xenopus [38], and Dictyostelium [15, 19,21]. The first molecular hints as to the possible involvement of the anx7 gene towards growth have come from studies on Dictyostelium. The first anx7

gene disruption mutants were noted to have growth defects [15], and more recent studies have shown that these anx7-knockout mutants lose many properties related to growth, differentiation, motility and chemotaxis, especially in Ca^{2+} limiting conditions [3,16]. It has been reported that the anx7 level is increased during the transition of D. discoideum Ax-2 cells from growth to differentiation. Bonfils et al. [3] have also shown that compared with the differentiated form of Dictyostelium, the proliferating form possesses only 1/5th the amount of anx7-mRNA and only 1/60th the amount of ANX7 protein [3]. Okafugi et al. [31] have discovered that the mechanism involves genesis of a naturally occurring anx7 antisense mRNA which activates growth and proliferation in wildtype Dictyostelium. These latter data have been interpreted as indicating a possible role for anx7 in a signal transduction pathway for growth. In summary, the anx7 gene could seem to control the Dictyostelium cell cycle such that a relative decrease in the anx7 gene activity would appear to enhance growth and proliferation at the expense of Ca^{2+}-dependent differentiated functions.

2.2. ANX7 is phosphorylated by protein kinases and mitogen-stimulated protein kinases

Protein kinase C phosphorylates ANX7 with a 2:1 Pi/Protein molar ratio, both in vitro and in vivo [10]. This result is of possible relevance to ANX7 function in the cell cycle, since many isoforms of PKC have been directly implicated in activating intracellular signalling [30], and in specifically activating mitosis [2,7,26,29] and tumorigenicity [22,28,32]. Quantitative phospho-ANX7 adducts have also been prepared in vitro with EGF (epidermal growth factor) receptor and $pp60^{src}$. In vivo, cells treated with tyrosine kinase activators such as epidermal growth factor (EGF) and platelet derived growth factor (PGDF) also support phosphorylation of endogenous ANX7. These reactions are of as yet unknown biological significance. However, the potential relevance of such reactivity to tumor suppressor gene activity is manifest by reports that splice variants of the prostate and ovarian cancer susceptibility gene BRCA1 contain phosphotyrosine and play a role in cell cycle regulation [14,43,46].

2.3. Anx7 knockout mice have growth anomalies and increased incidence of tumors

We used targeted homologous recombination technology to prepare a knockout mouse for the Anx7 gene.

Fig. 1. Adenocarcinoma of salivary gland. An image is shown, of the gross appearance of the tumor in the anx7 $(+/-)$ mouse.

The null Anx7 $(-/-)$ mouse is lethal, while the heterozygous Anx7 $(+/-)$ mouse has been shown to display defects in growth control, Ca^{2+} signal transduction, endocrine functions and tumor suppression [37, 39]. The fact that the anx7 $(-/-)$ mice does not survive after 10 days of gestation indicates an essential role in the development of the embryo when maternal message is exhausted. The male Anx7 $(+/-)$ mouse begins an extraordinary growth spurt relative to normal littermate controls after postpartum week 4. Growth continues uninterrupted for at least 12 months, leading to 40–60 gram mice. Many internal organs increase in weight, some out of proportion to the weight increment by the mouse itself. Insulin secretion from beta cells in the islets of Langerhans is inefficient as a function of external Ca^{2+}, and IP_3 mediated calcium transients are attenuated in cultured beta cells [37]. Enlargement of the prostate has also been systematically noted in male mutants.

In more recent work, we have become aware of a profoundly increased frequency of tumors, including prostate carcinoma in Anx7 $(+/-)$ animals compared to Anx7 $(+/+)$ normal littermate controls. In general, tumor frequency is in the range of 20–50% of animals, becoming more accentuated with advancing age. An instance of a salivary gland adenocarcinoma was observed clinically as a steadily increasing mass on the right side of the neck region. This is shown in Fig. 1. An example of a Hepatocellular Carcinoma is shown in Fig. 2(B). For comparison, a sample of normal liver from an anx7$(+/+)$ mouse shown in Fig. 2(A).

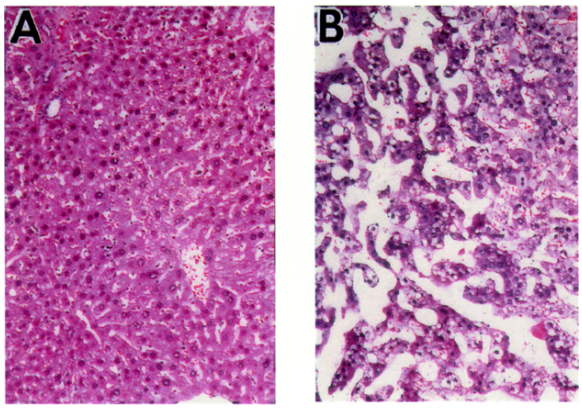

Fig. 2. Hepatocellular Carcinoma: Liver was taken from the anx7 $(+/-)$ mouse and fixed in buffered formalin. Sections were stained with hematoxylin and eosin (H and E). A section from a hepatocellular carcinoma, taken at 100-X magnification, is shown in Fig. 2(B). For comparison a sample of normal liver from an anx7$(+/+)$ mouse is shown in Fig. 2(A).

2.4. ANX7 and other tumor suppressor genes suppress the growth of tumor cell lines

A parallel and highly quantitative method of testing for tumor suppressor gene activity has been to transfect the candidate gene into a tumor cell line, and to determine whether the gene in question suppresses proliferation (e.g. [20]). The basic strategy behind this experiment is to transduce the wildtype tumor suppressor gene and to show that the growth characteristics of the tumor cell are lost in transfected cells. In the case of the ANX7 gene, our data, complete with p53 positive controls, show that the human ANX7 gene also suppresses growth of prostate, breast and osteosarcoma cell lines by a mechanism involving G1 arrest. This is an important result for candidate tumor suppressor gene characterization because certain human prostate tumor cell lines can be suppressed when a mutated Rb gene is supplanted by a wildtype Rb gene [4,23]. Equivalent results have been reported for a human bladder carcinoma cell line [42]. Similar reports have also been made for the p53 gene (e.g. [17,18,24]). Specific examples include suppression of growth of human colorectal cancer cells [1] and human prostate cancer cells lines such as LNCaP and DU145 [41].

2.5. ANX7 expression is completely lost in a high proportion of metatases and hormone-refractory prostate cancers

Human tumor tissue microarray technology allows one to query hundreds of tumors at a time for under- or over-expression of the candidate gene product using immunohistochemical or other techniques [6,25,35]. To efficiently analyze the clinical significance of ANX7, we therefore used a prostate tissue microarray in which we were able to query ANX7 protein expression in hundreds of tumors. The tissue microarray we used was specifically constructed with 376 specimens [5]. These specimens were from across all stages of progression including 25 normal controls, 25 PIN lesions, 150 untreated localized tumors, 135 hormone-refractory local recurrences, and 41 distant metastases. The levels of ANX7 were evaluated by immunohistochemistry using a monoclonal anti-ANX7 antibody.

Table 1
Levels of ANX7 protein expression in a prostate cancer tissue microrray. BPH: Benign prostatic hyperplesia, PIN: high-grade prostatic intraepithelial neoplesia, stage T2: clinically localized primary cancer, stage T3/T4: locally advanced primary cancer, Hr loc re: Hormone refractory local recurrence

Tumor	ANX7		Total	Percent
	positive	negative	samples	positive
BPH	20	2	22	91%
PIN	16	1	17	94%
Stage 2	106	2	108	98%
Stage 3/4	16	2	18	89%
Dist. metastasis	21	14	35	60%
Hr loc rec	70	38	108	65%

As shown in Table 1, we found that ANX7 expression is completely lost in a high proportion of metatases (57%) and in local recurrences of hormone refractory prostate cancer (63%). These data, highly significant, strongly suggest that the ANX7 gene has clinical relevance for prostate cancer in man. By contrast, ANX7 occurs at close to normal levels in benign prostate glands, high grade prostatic intraepithelial neoplasms (PIN), and stage T2 and T3/4 primary tumors (all in the range of 89–96%). Using Ki67 immuno-staining as an index of tumor cell proliferation, we find that a high Ki67 labeling index is positively correlated with lack of ANX7 expression [39]. Thus ANX7 expression is most profoundly reduced in the most prognostically challenging forms of prostate cancer.

2.6. Alterations in ANX7 expression as a function of breast cancer progression

We determined the frequency of ANX7 protein expression in a breast tissue microarray containing 525 tumor specimens from all stages of human breast tumor progression. The levels of ANX7 were evaluated by immunohistochemistry using a monoclonal anti-ANX7 antibody. We find that a significant reduction in ANX7

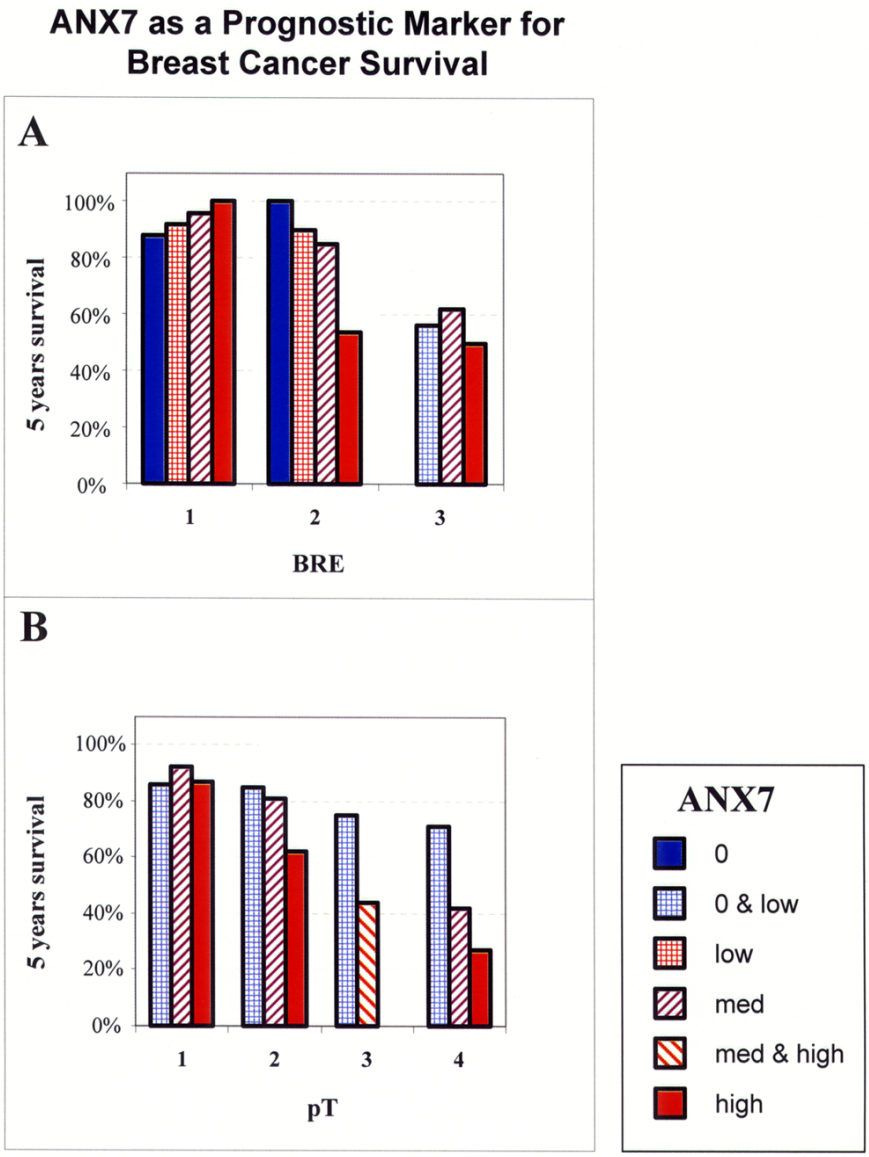

Fig. 3. ANX7 expression in a breast cancer tissue microarray as a function of cumulative survival at the five year time point. A. Survival is measured as a function of clinical stages of breast cancer (BRE). B. Survival is measured as a function of pathological stages of breast cancer (pT). The levels of ANX7 expression in the tissue array are color coded, as shown in the inset.

expression occurs in primary breast cancers. On the other hand the percent of ANX7 positives increase progressively as the tumor progresses (data not shown; and [40]). In a second set of experiments, we used a prognostic breast cancer array containing 303 tumor specimens to detect ANX7 by immunohistochemistry using a mono-clonal anti-ANX7 antibody. The ANX7 levels are classified as 0 (no staining), 1 (low staining), 2 (moderate staining), and 3 (highest staining intensity). Kaplan-Meier curves of disease-free survival in patients with low (0) versus high (3) ANX7 staining shows a significant separation within 5 years of follow-up ($p = 0.0017$). As depicted in the histograms, the high cytoplasmic ANX7 expression predicts that the patient survival is 50% at the clinical BRE grade 2 level (Fig. 3(A)) and 30% at pathological stage, pT4 (Fig. 3(B)). Significantly, there is no change observed in nuclear ANX7 staining in any of the cases. Thus these data strongly support the clinical relevance of the ANX7 gene as a prognostic marker for aggressive

treatment of breast cancer in women.

3. Conclusion

Prostate cancer is the most common cancer detected in American men, for whom metastatic, and hormone refractory prostate cancer are the end-stage, lethal forms of this disease. Of profound relevance to prostate cancer in humans is our finding that both metastatic and hormone refractory prostate cancers in human are associated with a significant loss of ANX7 gene expression. The key role of ANX7 in regulating cell proliferation is reflected in the properties of the Anx7 knockout mouse which we generated in our lab. The phenotype of this mouse includes gigantism and a high incidence of spontaneous tumors, including prostate carcinoma. In addition, overexpression of wild type ANX7 shows potent inhibitory effects on the growth of two metastatic prostate human cancer cell lines. These results thus further lend support to our proposed role for ANX7 in cancer and cell proliferation, not only in the mouse knockout model created by us, but also in breast and prostate cancer specimens that we tested. This is an important insight, because until now the ANX7 gene has not been thought to play such a role [34]. It is of particular importance that ANX7 is located on human chromosome 10q21, where hitherto unidentified potential tumor suppressor genes have been predicted to exist. It is possible that ANX7 may be at least one of the tumor suppressor genes predicted to occur at this locus.

The fact that ANX7 protein expression is significantly reduced in androgen-insensitive metastatic and locally recurrent hormone insensitive prostate cancers suggests that the study of ANX7 gene action will have great potential importance for understanding human prostate cancer progression. Most importantly, in studies of human breast tumors, high cytoplasmic expression of ANX7 was found to be a strong predictor of reduced disease-free survival. As these studies show, the relationship between levels of ANX7 and progression of these different cancers is at a nascent stage of understanding. Nonetheless, we anticipate that further work to elucidate the tumor-specific action of ANX7 will provide a new and useful tools for diagnosis, prognosis and therapy for these different types of cancers.

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