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Minireview

Human kallikrein 10, a predictive marker for breast cancer

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Abstract

Our laboratory is involved in identifying genes that can be used as early diagnostic or prognostic markers in breast cancer. We previously identified a gene (*NES1*) that is expressed in normal but not in transformed mammary epithelial cells (MECs). *NES1* is located on chromosome 19q13.4 within the kallikrein locus and thus was designated as human kallikrein 10 (*hK10*), although we have been unable to detect any protease activity. Importantly, *hK10* expression is decreased in a majority of breast cancer cell lines. Transfection of *hK10* into *hK10*-negative breast cancer cells reduces the tumorigenicity. Using methylation-specific PCR and subsequent sequencing, we demonstrate a strong correlation between hypermethylation of *hK10* and loss of mRNA expression. Further analysis showed that essentially 100% of normal breast specimens had *hK10* expression, whereas 46% of ductal carcinoma *in situ* (DCIS) and the majority of infiltrating ductal carcinoma (IDC) samples lacked the *hK10* mRNA. Importantly, *hK10*-negative DCIS diagnosed at the time of biopsy were subsequently diagnosed as IDC at the time of definitive surgery. It has been shown that hK10 protein expression is regulated by steroids. In addition to breast cancers, hK10 is downregulated in cervical cancer, prostate cancer and acute lymphocytic leukemia, whereas it is upregulated in ovarian cancers. These results point to the paradoxical role of hK10 in human cancers and underscore the importance of further studies of this kallikrein.

Keywords: breast cancer; hK10; hypermethylation; kallikreins; predictive markers; serine protease.

Introduction

Despite significant advances in early diagnosis and treatment, nearly a quarter of women with newly diagnosed breast cancer (211 240 new cases in the year 2005 in the US alone) will not survive the disease (American Cancer Society, 2005). Breast cancer is a heterogeneous disease with multiple genetic alterations that influence tumor growth, progression and metastasis to various organs. However, in contrast to other malignancies, breast cancer is potentially curable given the availability of several effective treatment modalities and favorable clinical and pathobiological tumor features. Thus, the major challenge for physicians is to accurately define risk profiles for individual patients on initial diagnosis (prognostic assessment) and to identify appropriate treatment modalities (prediction of treatment response). Therefore, defining biological markers that can provide prognostic assessment and predict treatment response at the time of diagnosis is of critical importance in breast cancer management. At present, the widely used prognostic and predictive factors include patient characteristics that are disease-independent, such as age, and disease-related, such as tumor size, axillary lymph node status, standardized histological grades and some biological tumor features (Coradini and Daidone, 2004). While a number of markers have been identified for more than 30 years, few have been accepted in the clinical practice guidelines of the American Society of Clinical Oncology as useful biomarkers, such as estrogen receptor (ER), progesterone receptor (PR) and c-ErbB2, mainly as predictors of response to specific therapies (Astrom et al., 1994; Bast et al., 2001). Notably, most investigators have focused on proteins that are upregulated in breast cancers rather than proteins that are downregulated (owing to methylation or other mechanisms, representing potential tumor suppressors). The intrinsic problem of working with proteins that are underexpressed in cancers is that the assay system has to be even more reliable than for proteins that are overexpressed. An emerging set of genes, including estrogen receptor, *p16*, *BRCA1* and *RARβ*, with proven roles in apoptosis, cell cycle, differentiation and senes-

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Regarding a recommendation for future nomenclature of kallikrein gene-derived proteases, see the article 'A comprehensive nomenclature for serine proteases with homology to tissue kallikreins' by Lundwall et al., this issue pp. 637–641.

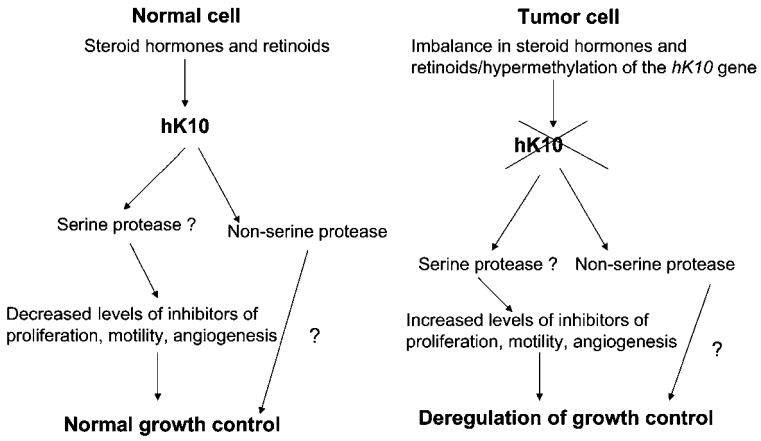

Figure 1 Hypothetical model of the tumor suppressor function of hK10 protein.
In normal cells hK10 is synthesized under the control of steroid hormones and retinoids. Although not yet determined, hK10 may function as a serine protease (or function in an alternate non-serine protease pathway) and decrease levels of inhibitors of proliferation, motility and angiogenesis, thus maintaining normal growth control. In tumor cells, loss of expression of hK10, either by gene hypermethylation or an imbalance in steroid hormone/retinoid production, may lead to increased levels of inhibitors of proliferation, motility and angiogenesis, leading to deregulation of growth control.

cence, fall into this recent category and these genes are likely to become useful in the future management of cancers (Mielnicki et al., 2001).

Identification of NES1/hK10

For approximately 15 years, our laboratory has been involved in identifying the molecular pathways that are involved in breast cell transformation (Dimri et al., 2005). Over the years, we have developed several human mammary epithelial cell (hMEC) immortalization models that have helped us to discover novel and known proteins involved in breast epithelial cell transformation (Dimri et al., 2005). In one such approach, we performed subtractive hybridization between normal hMEC strain 76N and its radiation-transformed tumorigenic derivative 76R-30 (Wazer et al., 1994), and cloned a novel gene that was expressed in normal hMECs but dramatically downregulated in radiation-transformed cells and, most importantly, in a majority of breast cancer cell lines (Liu et al., 1996). Based on its selective expression in normal hMECs but not in fibroblasts, the corresponding gene was designated *NES1* (normal epithelial cell-specific 1). Further work in our laboratory and by other groups revealed that *NES1* cDNA is a novel serine protease with high homology to the glandular kallikrein family (Yousef et al., 2005). Subsequent studies showed the localization of *NES1* gene to chromosome 19q13.4, a locus where most kallikreins are located (Goyal et al., 1998; Luo et al., 1998). Based on these characteristics, the *NES1* gene was subsequently renamed human kallikrein 10 (*hK10*) (Yousef et al., 2005). Furthermore, it was demonstrated that hK10 is a secreted protein and is detected exclusively in the supernatants of cultured cells (Liu et al., 1996). Further studies demonstrated the presence of hK10 in biological fluids (Luo et al., 2001a).

A role for hK10 as novel tumor suppressor

Based on the expression of *hK10* in normal epithelial cells and its downregulation in breast cancer cells, we assessed the effects of its re-expression in a *hK10*-negative MDA-MB-231 breast cancer cell line. These studies demonstrated that hK10 suppressed the oncogenicity, as revealed by the inhibition of anchorage-independent growth and tumor formation in nude mice (Goyal et al., 1998). These studies point towards a possible growth/tumor suppressor role of hK10. A hypothetical model for the possible tumor suppressor role of hK10 is presented in Figure 1.

DNA hypermethylation provides one mechanism for lack of *hK10* expression in breast cancer

To begin to understand the mechanism of downregulation of *hK10* in several human cancers, including breast cancers, we and others have cloned and characterized the active promoter region of *hK10* (Li et al., 2001; Luo et al., 2003a). Using a luciferase reporter system, we demonstrated that most tumor cell lines are able to support full or partial transcription from the *hK10* promoter, suggesting a role for promoter-independent *cis*-acting mechanisms for the loss of *hK10* expression. We showed that hypermethylation of the *hK10* gene represents one such mechanism. Using methylation-specific PCR and sequence analysis of sodium bisulfite-treated genomic DNA, we demonstrated a strong correlation between exon 3 hypermethylation and loss of *hK10* mRNA expression in a panel of breast cancer cell lines and in primary tumors. Treatment of *hK10*-non-expressing cells with a demethylating agent led to the re-expression of *hK10* mRNA, suggesting that hypermethylation is one of the mechanisms for tumor-specific loss of *hK10* gene

expression. Our results also suggested that hypermethylation of the *hK10* gene may serve as a potential marker for breast cancer (Li et al., 2001). Our recent studies and work in other laboratories support this notion (Dhar et al., 2001; Sidiropoulos et al., 2005).

Lack of *hK10* expression in DCIS and infiltrating ductal carcinoma of the breast and its potential as a predictor of invasive carcinoma in DCIS

Given that hK10 is a secreted protein and its expression is lost in breast cancer cells, we developed an *in situ* hybridization (ISH) technique with an anti-sense *hK10* probe to detect *hK10* mRNA in tissue sections in 30 samples each of normal, typical hyperplasia, atypical hyperplasia, ductal carcinoma *in situ* (DCIS) and infiltrating ductal carcinoma (IDC). These analyses showed that all of the 30 normal breast specimens and approximately 75% of typical and atypical breast hyperplasia specimens showed high *hK10* expression, with moderate expression in the remaining 25% of cases. Significantly, approximately half of the DCIS specimens lacked *hK10* expression and the other half showed moderate expression. Finally, approximately 97% of IDC samples lacked *hK10* mRNA, with weak expression in the one remaining sample. These results demonstrate that loss of *hK10* mRNA expression correlates with tumor progression (Dhar et al., 2001). Although the sample size was small, an interesting correlation was observed between the lack of *hK10* expression in DCIS specimens examined at the time of biopsy and IDC identification at the time of surgery (Yunes et al., 2003). These results indicate that lack of *hK10* expression in DCIS at diagnostic biopsy can predict a higher risk of invasive cancer at definitive surgery. Interestingly, the predictive value of *hK10* expression appears to be particularly relevant for low- and intermediate-grade DCIS (Yunes et al., 2003). Analysis of larger data sets should help to determine if lack of *hK10* expression may be a predictor of future recurrence and/or survival and treatment response, and whether it serves as an independent biomarker in breast and other cancers.

hK10 is a steroid-regulated protein

Notably, a number of studies have shown increases in the mRNA levels of various kallikreins, such as hK3, hK5, hK15 and hK10, upon treatment of cells with steroid hormones, followed by analyses of kallikrein mRNA levels using RT-PCR (Cleutjens et al., 1997; Luo et al., 2000, 2003b; Magklara et al., 2002; Yousef et al., 2002, 2003). However, these studies did not investigate the presence of steroid response elements in the promoter region of these kallikreins. To the best of our knowledge, only the *hK3* (PSA) promoter has been analyzed extensively and was shown to contain an androgen-response element (ARE) (Cleutjens et al., 1997). Recent studies showed that *hK10* mRNA levels were induced upon treatment with estradiol, androgens and corticosteroids (Luo et al., 2000, 2003a), although the basis for this induction remains to be elucidated. In our laboratory, we observed that treatment of mammary epithelial cell lines with retinoids led to an increase in *hK10* mRNA and protein levels. Retinoids bind to retinoic acid receptors (RARs) and retinoid X receptors (RXRs), which are members of the steroid/thyroid hormone receptor superfamily. The RAR family is composed of RARα, β and γ and their isoforms, which recognize two natural stereoisomers of RA, all-*trans*-RA and 9-*cis*-RA. The RXR family is composed of RXRα, β and γ and their isoforms, which are activated exclusively by 9-*cis*-RA (Rastinejad, 2001).

Given the retinoid induction of *hK10* mRNA and protein expression, we examined the *hK10* promoter for potential retinoid response elements (RAREs) (Zeng et al., 2006). RAREs are *cis*-acting DNA sequences composed of directly repeated hexameric half-sites with consensus sequences [5′-PuG(G/T)TCA-3′] within the transcriptional regulatory regions of target genes (Rastinejad, 2001). Our analysis of the *hK10* promoter using a website software (TRANSFAC 3.0) for sequences related to the RARE half-site [5′-PuG(G/T)-TCA-3′] uncovered three potential sites: RARE1 at -1041 (TGACCTCGTGATCC), RARE2 at -859 (TGACCTCCTATGA) and RARE3 at -765 (TGACCTCCTGTGA). All of these contained half-sites of canonical sequence (TGACCT; the reverse complementary sequence is AGGTCA). RARE1 was predicted to be DR2-type with a spacer of 2 nt between the half-sites, and the other two RAREs were DR1-type (with a single nucleotide spacer). Electrophoretic mobility shift assays showed that RXR only binds to RARE1, and the activity of the RARE1 site for binding to RXR was in competition with a consensus RARE (DR5 of the *CRABPII* gene) (Astrom et al., 1994). Notably, RARE1 is very similar to a known RARE on the promoter of apolipoprotein, a well-accepted retinoid responsive gene (Ramharack et al., 1998). Importantly, the *hK10* promoter was found to bind to RXR not only in electrophoretic mobility shift assays, but also under native promoter conditions, as demonstrated by ChIP assays. Lastly, we demonstrated that mutation of this site abolished the RA-dependent increase in *hK10* promoter activity. These results are described in detail in the article by Zeng et al. in this issue.

hK10 protein and other cancers

Immunohistochemical analysis of normal tissues showed that literally every tissue in the human body expresses hK10 protein (Petraki et al., 2002). Further analyses showed that in addition to breast cancer, the *hK10* gene is hypermethylated in prostate, cervical, and ovarian cancers and leukemia, and is downregulated in testicular tumors, indicating the general importance of the *hK10* gene in human cancer (Goyal et al., 1998; Li et al., 2001; Luo et al., 2001b; Roman-Gomez et al., 2004; Sidiropoulos et al., 2005). Surprisingly, however, two laboratories reported increased expression of *hK10* in certain types of ovarian cancer (Luo et al., 2001c,d, 2003b; Shvartsman et al., 2003). Using an immunoassay to detect hK10

Table 1 Historical perspective on the identification and characterization of NES1/hK10.

Identification and demonstration of downregulation in breast cancer cell lines and structural similarities with other serine proteases	Liu et al., 1996
Demonstration of its tumor suppressor function and chromosomal localization. Downregulation in prostate cancer cell lines	Goyal et al., 1998
Chromosomal localization and structural characterization	Luo et al., 1998
Demonstration of steroid-regulated gene expression	Luo et al., 2000
Identification in biological fluids	Luo et al., 2001a.
Down-regulation in testicular tumors	Luo et al., 2001b.
CpG methylation as a basis for tumor-specific loss in breast, prostate and cervical cancer cell lines	Li et al., 2001
Use of mRNA *in situ* hybridization to demonstrate decrease mRNA levels in breast DCIS and in IDC	Dhar et al., 2001
Overexpression in ovarian cancer	Luo et al., 2001c
Unfavorable prognostic marker in ovarian cancer	Luo et al., 2001d
Protein expression in normal tissues by immunohistochemistry	Petraki et al., 2002
Identification of single nucleotide polymorphism	Bharaj et al., 2002
Demonstration that hK10 can serve as a predictive marker for breast cancer	Yunes et al., 2003
Overexpression, serum marker for ovarian cancer	Luo et al., 2003b
Downregulation in acute lymphoblastic leukemia by hypermethylation	Roman-Gomez et al., 2004
CpG methylation in breast, ovarian and prostate cancer	Sidiropoulos et al., 2005
Demonstration of retinoid responsive element in the promoter region	Zeng et al., 2006

proteins, one laboratory demonstrated that hK10 levels in the sera of ovarian cancer patients were significantly elevated. Importantly, this laboratory compared *hK10* expression with the well-known serum marker *CA125*, and showed that approximately 35% of *CA125*-negative ovarian cancer patients were *hK10*-positive at 90% specificity. They showed that high serum hK10 was strongly associated with serous epithelial type, late stage, advanced grade, large residual tumor (>1 cm), suboptimal debulking, and lack of response to chemotherapy (all parameters significant at $p<0.001$). These authors concluded that the preoperative serum hK10 concentration is a strong and independent unfavorable prognostic marker for ovarian cancer (Luo et al., 2001d, 2003b). The second study used microarray-based transcriptional profiles of RNA specimens isolated from normal ovarian epithelium, ovarian cancer cell lines, and primary ovarian tumors and confirmed these data using Northern blotting, *in situ* hybridization and Western blot analysis. These authors reported that 32 out of 35 primary serous ovarian carcinoma samples (91.4%) expressed higher levels of hK10 compared to normal ovarian epithelium (Shvartsman et al., 2003; Rosen et al., 2005). Thus, it is likely that hK10 and other kallikreins will serve as useful diagnostic and prognostic markers in patients with ovarian carcinoma. Contradictory to these findings, our data using PA1, SKOV3, SW626, CAOV3, and DUNT ovarian cancer cell lines showed downregulation of *hK10* in all but the SW626 cell line (Zeng and Band, unpublished data). Consistent with our results, a recent paper by the first group cited above also reported hypermethylation of *hK10* in some ovarian cancer cell lines (Sidiropoulos et al., 2005). Notably, we have observed high *hK10* expression in normal ovarian tissue, indicating that hK10 may play an important role in the ovary (Liu et al., 1996). Although the mechanisms and reasons for increased *hK10* expression in ovarian cancers have not yet been determined, it is important to note that several kallikreins that are downregulated in breast cancers are upregulated in ovarian cancers. We speculate that certain cell subtypes in the ovary express high levels of hK10, and tumors arising from these cells may thus express high levels of hK10, although this hypothesis needs to be tested by careful mRNA *in situ* hybridization of normal and tumor ovarian tissue sections. Interestingly, a recent study reported a single nucleotide polymorphism of the *hK10* gene in cancers of the prostate, breast, ovary, and testis. The single nucleotide variation at codon 50 appears to be associated with prostate cancer risk (Bharaj et al., 2002). Future studies should assess the risk of other cancers. The historical perspective of research in the hK10 field is summarized in Table 1.

hK10 is not a functional serine protease

Since the discovery of hK10, it has been of interest to us and others working in this area to determine if hK10 functions as a serine protease, as the predicted hK10 protein showed high homology to serine proteases.

A neural network-based signal peptide and cleavage site prediction program (http://www.cbs.dtu.dk/services/SignalP/) predicted that *hK10* cDNA encodes a polypeptide with a putative signal peptide (amino acids 1–33), with a potential signal peptide cleavage site between amino acid positions 33 and 34 (Ala-Ala). The predicted 33-aa signal peptide is longer than the majority of known serine proteases. A potential trypsin-susceptible cleavage site at position 42 (Arg) was also predicted.

To determine the actual N-terminal sequence of the mature hK10 protein, we subjected hK10 protein purified from the insect cells, as well as from conditioned media of a *hK10*-transfected breast cancer cell line, MDA-MB-231, to amino-terminal sequencing. In both cases, five cycles of sequencing revealed the sequence ALLPQ corresponding to hK10 sequence 34ALLPQ38 that follows the predicted signal peptide. These results directly establish that, similar to other serine proteases, the hydrophobic signal sequence of the hK10 polypeptide is

cleaved at the Ala-Ala signal cleavage site prior to its secretion. Furthermore, the secreted hK10 protein retains the 8-aa inhibitory pro-peptide proceeding the potential cleavage site and hence is expected to be proteolytically inactive. Next, we analyzed the protease activity of purified full-length hK10 and its two serine mutants (S229A and del229) using a universal serine protease substrate, casein resorufin. These studies showed extremely low protease activity for both hK10 and its mutants. Based on the sequence homology of hK10 with other active serine proteases, we predict that hK10 needs to be cleaved at R42 in order for it to be a potentially active protease. For this purpose, we performed limited proteolysis (to remove the pro-peptide, amino acids 34–42) using low concentrations of trypsin or glandular kallikrein, which are known to have arginine-directed specificity. After each of these treatments, we tested the protein on SDS-PAGE and protease activity was measured using casein resorufin as substrate. As expected, very low concentrations of trypsin alone (used as a control) showed high protease activity (data not shown). In contrast, 5 μg each of hK10 and its mutants showed extremely low protease activity. In addition, we also carried out hK10 protease reactions at different pH values (range 2–9) and temperatures, but still failed to observe any protease activity. Next, we examined if hK10 has activity against a chymotrypsin substrate, Suc-Ala-Ala-Pro-Phe-pNA. As expected, low doses of chymotrypsin (400 ng) showed protease activity. In contrast, 5 μg each of hK10 and its mutants showed no protease activity. We also examined a number of synthetic *para*-nitroanilide substrates that are known to be specifically cleaved by plasmin (H-D-Val-Leu-Lys-pNA), trypsin (H-D-Ile-Pro-Arg-pNA), tissue plasminogen activator (H-D-Val-Phe-Lys-pNA), glandular kallikrein (H-D-Val-Leu-Arg-pNA), plasma kallikrein (H-D-Pro-Phe-Arg-pNA), elastase (<Glu-Pro-Val-pNA), urokinase (pyro-Glu-Gly-Arg-pNA) and chymotrypsin (MeO-Suc-Arg-Pro-Tyr-pNa). Untreated hK10 or treated with trypsin or glandular kallikrein displayed no detectable hydrolytic activity against any of the synthetic substrates listed above (data not shown). Each of these substrates was clearly cleaved by their respective protease (data not shown). These data suggest that either hK10 has no protease activity or that hK10 is unable to be cleaved of its pro-sequences and therefore is inactive. Next, we purified endogenous hK10 protein from normal breast cells and tested its *in vitro* activity, but still failed to observe any protease activity.

Although the above-mentioned experiments clearly showed that hK10 lacks protease activity, one possibility remained that the above treatment to activate Pro-hK10 may not have activated hK10. As the difference between the predicted activated hK10 and pro-hK10 sequences is only eight amino acids, we could not separate these two forms on SDS-PAGE gels for sequencing. We therefore engineered an enterokinase (EK) site at R42 using a PCR approach, by introducing an EK-susceptible peptide Asp-Asp-Asp-Asp-Lys into hK10 between the pro-peptide and the N-terminus of mature hK10 and a FLAG epitope tag (Asp-Tyr-Lys-Asp-Asp-Asp-Asp-Lys) at the C-terminus (to facilitate purification). EK is a highly specific enzyme that recognizes the Asp-Asp-Asp-Asp-Lys motif. These proteins were expressed in insect and mammalian cells, and recombinant Pro-EK-hK10 and Pro-EK-hK10-Flag were recovered in the conditioned media as soluble proteins and purified as above or by one-step affinity chromatography using an anti-FLAG monoclonal antibody for FLAG-tagged protein.

Interestingly, when assessed by SDS-PAGE, both recombinant proteins decreased in size by approx. 8 kDa after treatment with EK, although the expected size difference should be approximately 1 kDa. A possible explanation is the presence of a potential glycosylation site at N39 expected to be removed upon EK treatment. Amino acid sequence analysis revealed that both recombinant proteins possessed an N-terminal sequence of LDPEAY after treatment with EK, which is identical to that of mature NES1, as deduced from its cDNA. These preparations were then examined for protease activity as described above. It was anticipated that the secreted recombinant hK10 could be activated by EK treatment.

Despite extensive efforts, we could not demonstrate any protease activity in mature hK10. A probable cause may be the unusual N-terminus LDPEAY of hK10 that is left after the removal of pro-peptide sequences. In contrast to hK10, the N-terminus of most active serine proteases starts with isoleucine (most common amino terminus is IVGG), which is important in making a salt bridge after removal of the pro-peptide and assembly of the catalytic triad, rendering the protease active. Salt bridge formation between Ile-Asp residues is considered the primary switch mechanism for activating proteases (Stryer, 1988). Thus, our present evidence suggests that hK10 is not an active protease. However, it remains possible that we have yet not identified the relevant protein that can facilitate the conversion of hK10 into its active form or that the substrates used in our studies are not the biological substrates for hK10 protein (Goyal and Band, unpublished data).

Conclusions

Kallikreins, including hK10, are an emerging family of serine proteases that have the potential to serve as diagnostic and prognostic markers in various cancers. As these proteins are secreted, their quantification may provide potential serum markers of the presence or progression of cancer. hK3, another member of the family that is also known as PSA, is a well-known marker for prostate cancer. Interestingly, some of the kallikreins are upregulated, whereas some are downregulated in cancers. It is somewhat paradoxical that some kallikreins such as hK10 are downregulated in several cancers, whereas they are upregulated in ovarian cancers. Similar findings are also reported for hK3, with upregulation in prostate cancers but downregulation in breast cancers. This dichotomy of kallikrein expression patterns in relation to different cancers presents a challenge for investigators to examine their potential as markers in cancers and define their functions. It is also important to point out that many of the kallikrein genes are regulated by steroid hormones. Obviously, more studies are needed to clearly understand how these proteins are regulated in

normal cells, how their expression is changed during cancer progression, and what biological roles they play in the oncogenic process.

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