Review

Alternative splicing and the progesterone receptor in breast cancer

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Abstract

Progesterone receptor status is a marker for hormone responsiveness and disease prognosis in breast cancer. Progesterone receptor negative tumours have generally been shown to have a poorer prognosis than progesterone receptor positive tumours. The observed loss of progesterone receptor could be through a range of mechanisms, including the generation of alternatively spliced progesterone receptor variants that are not detectable by current screening methods. Many progesterone receptor mRNA variants have been described with deletions of various whole, multiple or partial exons that encode differing protein functional domains. These variants may alter the progestin responsiveness of a tissue and contribute to the abnormal growth associated with breast cancer. Absence of specific functional domains from these spliced variants may also make them undetectable or indistinguishable from full length progesterone receptor by conventional antibodies. A comprehensive investigation into the expression profile and activity of progesterone receptor spliced variants in breast cancer is required to advance our understanding of tumour hormone receptor status. This, in turn, may aid the development of new biomarkers of disease prognosis and improve adjuvant treatment decisions.

Introduction

Breast cancer is the most commonly diagnosed cancer in the UK, with 44,659 new cases diagnosed in 2004 [1]. Survival among women diagnosed with the disease is relatively good; however, 12,509 breast cancer related deaths in 2005 [1] demonstrate the potential for improving both diagnostic and therapeutic procedures.

After menarche, the cyclical release of the steroid hormones oestrogen and progesterone stimulates the primitive ducts of the immature breast to differentiate into terminal end buds, which branch into alveolar buds. During pregnancy, high circulating steroid hormone levels stimulate increased lobular differentiation to the mature secretory gland. Upon withdrawal of sex steroid hormones at menopause, involution to an undifferentiated morphology occurs [2].

Normal mammary ducts consist of a layer of luminal epithelial cells, surrounded by a layer of myoepithelial cells, encased in delimiting fibroblasts set in a fatty, collagenous stroma [3]. Most breast carcinomas are of epithelial origin and are either localised to the duct/lobule of origin or capable of invading surrounding stroma [4]. Whilst age is the biggest contributory risk factor for developing breast cancer, use of hormone replacement therapy (HRT) and oral contraceptives have also been implicated, implicating hormone signalling in breast cancer development [1,4]. A further increased risk with combined oestrogen-progesterone HRT compared to oestrogen alone also bears out the significant role of progesterone signalling through progesterone receptor (PR) in breast cancer development [5-7].

PR, along with oestrogen receptor (ER), status is routinely measured in breast cancer specimens. PR+ patients are more likely to respond to hormonal therapies and survive disease [8]. Approximately 40% of breast tumours are ER+/PR+ and these patients are considered to have the best prognosis and are most likely to respond to hormonal therapies [4,9]. These cancers are associated with a lower rate of cell proliferation and well differentiated tumours, leading to a better prospect of overall survival [9]. Since PR is an oestrogen responsive gene, PR positivity indicates not only ER being present but also functional [8]. However, a small proportion of tumours are ER-/PR+ and still respond more favourably to hormonal therapies than ER-/PR- tumours [9,10], demonstrating the independent importance of PR in breast cancer development and treatment, not just as an indicator of ER function.

AF = transactivation domain; ASV = alternatively spliced variant; DBD = DNA binding domain; DD = dimerisation domain; ER = oestrogen receptor; HRT = hormone replacement therapy; LBD = ligand binding domain; NLS = nuclear location signal; PR = progesterone receptor; PRE = progesterone response element.

Potential mechanisms for PR loss or down-regulation in response to growth factor signalling have been described. In some tumours PR- status appears to correlate with upregulation of epidermal growth factor receptor; it has been proposed that cross-talk between ER and epidermal growth factor receptor pathways may account for the resistance to selective oestrogen receptor modulators (SERMS; for example, Tamoxifen) of PR- tumours, which can still respond to removal of oestrogen signal by aromatase inhibitors [10,11]. Another study has shown that insulin-like growth factor-1 inhibits transcription of PR in breast cancer cells, via the PI3K/Akt/mTOR signalling pathway, which appears to be independent of ER levels or activity [12]. Loss of PR also occurs by a phosphorylation-dependent process in which ligand binding activates PR phosphorylation by mitogen-activated protein kinases, which in turn targets the receptors to the ubiquitin-proteosome for degradation [13]; these researchers demonstrate that growth factors can paradoxically activate transcription of PR yet rapidly down-regulate PR protein. It is plausible, therefore, that PR variants may be difficult to detect at the protein level because they have relatively short half-lives and rapid turnover.

In addition to down-regulation of PR by growth factor-oestrogen receptor cross-talk, other potential mechanisms for the development of PR- tumours include: low circulating oestrogen levels in post menopausal women being insufficient to generate detectable levels of PR, consistent with the higher incidence of ER+/PR- tumours in older women; loss of heterozygosity at the PR gene; or hypermethylation of the PR promoters, common in many cancer cell types, including breast cancer, causing transcriptional silencing [11,14,15]. One further possibility is that alternative splicing of PR pre-mRNA might generate cancer specific PR variants that are not detectable by current pathological screening methods, thus leading to potential misdiagnosis of a tumour as PR-.

This review will describe the structure and function of PR, and outline the mechanisms of alternative splicing, their implications in cancer and evidence for the existence of alternatively spliced PR isoforms. The potential gain or loss of function associated with each PR variant and their significance in breast cancer will be discussed.

Progesterone receptor structure and function

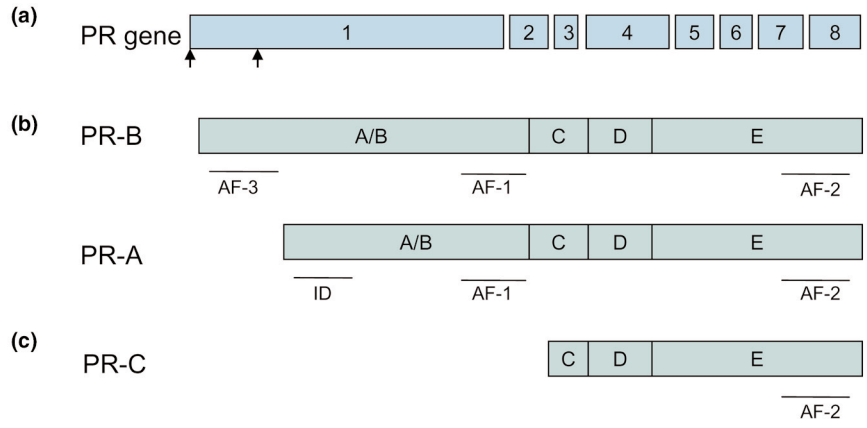
The human PR gene consists of eight coding exons separated by seven non-coding introns [16]. The two main nuclear isoforms, PR-A and PR-B [17-20], are independently regulated from defined promoter regions within the PR gene [18,21]. PR-A is a truncated form of PR-B, lacking the amino-terminal 164 amino acids that form the third transactivation domain (AF-3) [18,20]. Other than this, the two forms are structurally identical (Figure 1).

PR is a member of the steroid/thyroid hormone-retinoid receptor superfamily of ligand activated nuclear transcription factors [18,22]. The mechanisms by which PR regulates hormone-response genes are complex. Progesterone binds PR, inducing a conformational change in PR causing its nuclear translocation, dimerisation and interaction with specific DNA progesterone response elements (PREs) present in the promoter regions of target genes. However, the presence of a PRE does not necessarily predict progestin responsiveness. PR can also mediate its effect independently of PREs, through the protein-protein interactions of PR with other sequence-specific transcription factors [23]. Protein products from PR target genes are involved in a diverse range of cellular activities, including transcription, steroid and lipid metabolism, cell growth and apoptosis, protein and nucleic acid processing and membrane associated signalling, indicating a broad range of potential progesterone effects [24]. Some of these proteins are associated with mammary gland and breast cancer development, including the transcription factors STAT5A and C/EBPβ.

In breast cancer cells expressing either PR-B or PR-A, most PR regulated genes are reported to be controlled by PR-B [24], less by PR-A and only a minority by both isoforms. PR-A has been shown to act as a trans-dominant repressor of PR-B controlled transcription in a cell and promoter specific manner [24,25]. The balance of PR isoform expression is also important in breast cancer management. Overexpression of PR-A protein compared to PR-B is common in breast cancer [17,26], changing progestin responsiveness of cells [26]. Predominant PR-A protein expression signifies a poorer outcome of hormonal therapies [27], and predominance of PR-B poorer outcome of chemotherapy. Predominance of one isoform is also seen in women at high risk of breast cancer, for example, women with a *BRCA1* or *BRCA2* mutation commonly exhibit a lack of PR-B [26]. Therefore, not only could PR variants lead to incorrect assessment of hormone receptor status, but could also alter the progestin responsiveness of tissue, providing potential new indicators of efficacy and targets for therapeutics.

In addition to the nuclear hormone receptors PR-A and PR-B, a truncated and predominantly cytoplasmic PR protein has also been described. PR-C is a 60 kDa protein, first detected in the T47-D breast cancer cell line, resulting from initiation at a methionine at position 595 [28] and is capable of forming heterodimers and modulating the activity of PR-A and PR-B *in vitro* [28,29]. The PR-C protein lacks a full DNA binding domain (DBD) [30] as well as the first two transactivation domains (AF-3 and AF-1), so although potentially able to bind hormone, it would not directly bind PRE (Figure 1c). However, PR-C may still influence transcription events since it has the capacity to dimerise and also interact with common nuclear co-factors. A potential physiological role for PR-C in human pregnancy has been proposed [31]; this study shows that PR-C mRNA and protein levels significantly increase within the fundus of uterus of women in labour. The authors suggest that the observed increase in the ratio of PR-C to

Figure 1



Progesterone receptor (PR) gene and main isoforms. **(a)** Exon organisation map of the PR gene, showing PR-A and PR-B promoters (indicated by arrows). **(b)** PR-B and PR-A protein structures. The A/B region is encoded by exon 1 and part of exon 2, and contains the PR-B specific transactivation domain AF-3, AF-1, common to PR-B and PR-A, and the PR-A specific inhibitory domain (ID). The C region forms the DNA binding domain (DBD); each of exons 2 and 3 encodes one zinc finger. The D region is encoded by exon 4 and part of exon 3, and forms the hinge region responsible for the nuclear location signal (NLS). The E region contains AF-2, common to PR-B and PR-A, and the hormone (ligand) binding domain (LBD), encoded by exons 4 to 8. **(c)** PR-C, an amino-terminally deleted PR protein predicted to result from alternative translation initiation at a methionine at position 595. PR-C lacks a complete DBD and the first two transactivation domains (AF-3 and AF-1).

PR-B may influence functional progesterone withdrawal by blocking the activity of PR-B to maintain uterine quiescence in pregnancy [31].

Alternative splicing

Alternative pre-mRNA splicing is a vital mechanism for generating protein diversity from a relatively small number of genes. In excess of 60% of all human genes undergo alternative splicing. Splicing occurs at sites determined by the presence of a 5' donor splice sequence (usually ending -GU), a branch point adenosine and a 3' acceptor splice sequence (usually ending -AG) within pre-mRNA sequences [32,33]. The proximity of *cis*-regulatory sequences, either enhancers or silencers of exon or intron splicing, influences the binding of different *trans*-splicing factors to the splice sites (Figure 2a) and aids assembly of the spliceosome multi-protein complex, resulting in cleavage and ligation of introns and exons, respectively [32,33]. At least five basic types of alternative splicing have been described [32-34] (Figure 2b).

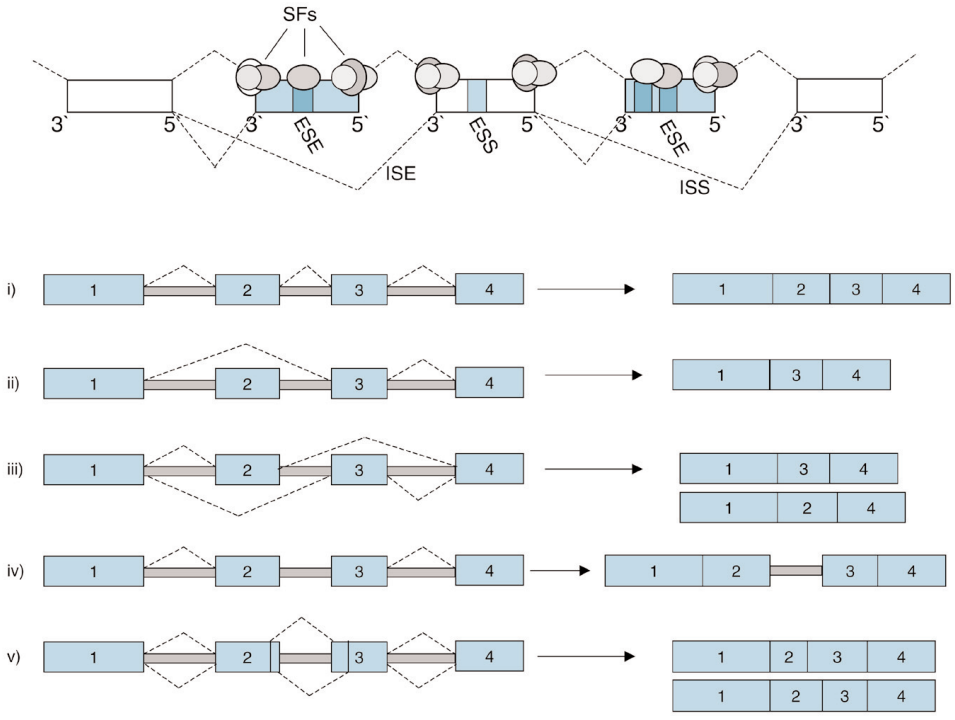
A wide range of alternatively spliced genes have been described in cancers [33,35], affecting disease initiation, resistance to apoptosis, invasion and angiogenesis [36]. Alternative splicing appears to be increased in cancer, possibly due to mutations in *trans*-splicing or *cis*-regulatory elements allowing increased competition from cryptic splice sites and misdirected spliceosome formation [34]. Alternatively spliced genes involved in breast cancer have been observed; *BRCA1* variants [35,37] as well as variants of other members of the steroid receptor superfamily such as an androgen receptor variant lacking exon 3 and several ER variants with differing expression in normal and tumourigenic breast tissues [38-40].

Alternatively spliced progesterone receptor isoforms

As well as PR-A, PR-B and PR-C, several other smaller isoforms encoded by the PR gene have also been described [28,41-44] (Figure 3). Screening of a testicular cDNA library revealed a PR mRNA isoform containing a novel sequence before exon 4 termed exon S [41]; mRNA for this isoform was found to be present in ejaculated spermatozoon and the uterine endometrium. A protein encoded from this mRNA would lack the entire DBD but would most likely possess nearly the entire sequence for the progesterone-binding domain and be capable of dimerisation. This isoform, PR-S, and another isoform, PR-T, resulting from retention of another intronic sequence before exon 4 (exon T) [42], could give rise to identical proteins. Neither exon S nor exon T encode a translation initiation site, so translation is likely to start at the first methionine in exons 4 to 8 [42]. Exons S and T are located between exons 3 and 4 in the PR gene [45]. Since both PR-S and PR-T proteins lack the ability to bind DNA, they may be important in mediating non-genomic PR mediated responses or recruiting co-factors to PR hetero-dimers.

A study of endometrial carcinomas revealed a variant PR mRNA that contained a 252 base-pair insertion between exons 4 and 5 [44]. This was termed PR-i45 and the insertion is the result of transcription of two intronic sequences termed exons i45a and i45b. The protein translated from PR-i45

Figure 2



Regulation and different patterns of alternative splicing. **(a)** Alternative pre-mRNA splicing involves a complex interplay of *trans*-acting splicing factors with numerous *cis*-acting regulatory elements within the precursor mRNA sequences of eukaryotic genes. *Trans*-acting factors recognise and interact with different *cis*-acting RNA motifs present within the pre-mRNA sequence of genes; these include 5' donor and 3' acceptor sites at exon-intron boundaries; exon and intron enhancer elements (ESE, ISE), which can promote the use of specific splice sites; and exon and intron silencer elements (ESS, ISS), which when bound by proteins can repress the use of specific splice sites (adapted from [59]). **(b)** Different patterns of alternative splicing include: (i) normal pattern of splicing - removing introns 1, 2 and 3; (ii) whole exon exclusion - introns 1, 2 and 3, and entire exon 2 are removed; (iii) mutually exclusive exons - either exon 2 or exon 3 is removed and both exons are never retained together; (iv) intron retention - intron 2 is retained in the mRNA; and (v) cryptic splice sites - exon 2 contains a 3' cryptic splice site and exon 3 contains a 5' cryptic splice site. These compete with the native splice site to generate mRNA lacking part of an exon.

mRNA would have the normal amino-terminal domain of genomic PR, the DBD and nuclear location signal (NLS). A change in reading frame due to the insertion would cause truncation of the ligand binding domain (LBD) [44].

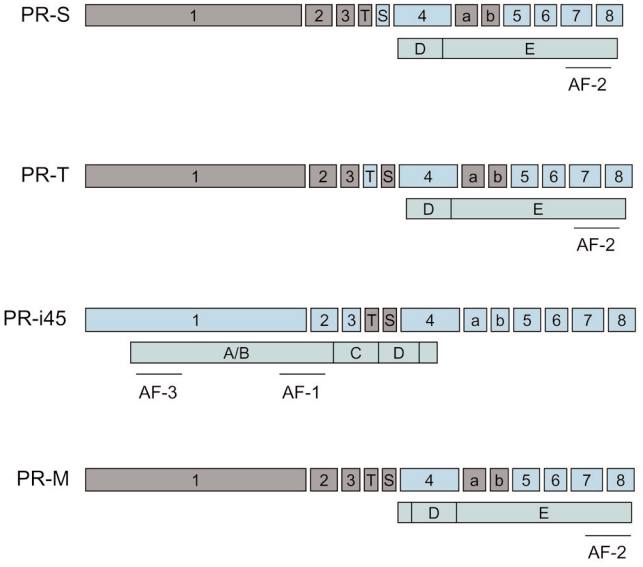
PR-M mRNA was discovered in the T47D breast cancer cell line that contained a 5'-untranslated region consistent with use of a promoter within intron 3 [43]. This encoded a short 5' signal sequence of hydrophobic amino acids, suggesting that the protein may be processed for either expression on the cell membrane or secretion. PR-M contains the full LBD and dimerisation domain (DD) so is likely to be physiologically active despite lacking the DBD [43], consistent with a role on the plasma membrane or as a secreted protein. PR-M was later detected in the PR- T47D-Y cell line by immunofluorescent antibody staining. Cells that overexpressed PR-M demonstrated a decreased rate of calcium influx consistent with PR-M functioning as a membrane bound protein associated with non-genomic responses to progesterone [46].

Other progesterone receptor spliced variants

The initial observation of a 78 kDa protein detected by anti-PR antibodies [17,47] prompted the analysis of alternatively spliced variant (ASV) PR mRNAs by reverse transcription PCR. Due to the predicted size of proteins encoded by PR variant mRNAs, a deletion of exon 3 or part of exon 4 was suggested [47]. If this was a PR protein lacking either of these exons, it could still be transcriptionally active as it was capable of binding progesterone [47] and, therefore, may be able to elicit a response or modulate the activity of other PR isoforms.

Many alternatively spliced, whole exon deleted, PR variant mRNAs have since been described. Exon 2 deleted PRΔ2 was found in breast tumour tissue and in two PR+ breast cancer cell lines MCF-7 and $T47D_{CO}$ [47,48]. A protein encoded from this mRNA would lack the first zinc finger of the DBD, making DNA binding doubtful, and a change in reading frame would lead to the insertion of a stop codon in exon 3 so it would also lack a LBD [48]. It is probable, therefore, that

Figure 3



Truncated progesterone receptor (PR) isoforms. The upper part of each diagram shows the PR gene; darker shading indicates regions removed by splicing, lighter regions those retained in mRNA. The lower part of each diagram shows the predicted protein structure. PR-S: retention of intronic sequence termed exon S and initiation of transcription within exons 4 to 8. PR-T: retention of intronic sequence termed exon T and initiation of transcription within exons 4 to 8. PR-S and PR-T mRNA are likely to give rise to identical proteins that are amino-terminally truncated, lacking AF-1, AF-2 and the DNA binding domain. PR-i45: retention of two intronic sequences termed exons i45a and i45b leads to a change in reading frame and truncation of the protein E region so the PR-i45 protein lacks a functional ligand-binding domain and dimerisation domain. PR-M: inclusion of an intronic 5' untranslated region sequence causes amino-terminal truncation and leads to encoding of a protein with a 5' signal sequence and complete ligand-binding domain and dimerisation domain, consistent with a function as a membrane bound receptor.

were this protein translated it would be unable to bind DNA or hormone so not be functional as a PR. Exon 3 deleted PRΔ3 mRNA was seen at low levels in most breast tumour samples examined and in T47D cells [47,49]. A protein from this ASV would lack the second zinc finger of the DBD but might still be active if it were able to bind progesterone and dimerise with other PR forms. Exon 4 deleted PRΔ4 mRNA was detected in both normal and tumour tissue from the breast and in T47-D cells [47,48,50]. This variant was also found in normal endometrial, normal ovarian and ovarian endometriotic tissues [51] as well as in vascular smooth muscle [52]. The putative protein from this mRNA would lack the hinge region, so be segregated from the nucleus and would lack the proximal end of the LBD, though the rest of the LBD would be intact. It has been demonstrated that PRΔ4 is unable to bind PRE and is not responsive to the progestin R5020 [48]. It is unlikely, therefore, to function as a PR, but may compete for dimerisation with other PR rendering them inactive, or compete for co-factor binding.

Exon 6 deleted PRΔ6 mRNA has been detected in normal and tumour samples from the breast [50] and has since been detected more frequently in tumour than normal breast tissue, and at a higher rate in PR- tumours than PR+ [53]. PRΔ6 mRNA has also been detected in tissue samples from vascular smooth muscle [52], normal ovaries, ovarian endometriosis and normal endometrium [51]. The protein encoded from PRΔ6 mRNA lacks a functional LBD but possesses the NLS, ability to dimerise and bind DNA and has been shown to be a dominant negative inhibitor of wild-type PR-A and PR-B function [48]. Therefore, PRΔ6 protein expression, which may be elevated in breast cancer, could be an important regulator of PR function in the disease. It has further been demonstrated that PRΔ6 is able to bind PRE independent of progestin activation and suggested that this constitutive binding of transcriptionally inactive PRΔ6 to PRE may block wild-type PR binding [48]. The protein encoded by PR-i45 mRNA is likely to be similar in structure to a PRΔ6 protein, so may have a similar ability to inhibit PR-A and PR-B [45]. Interestingly, the expression of PRΔ6 and PRΔ4, relative to wild-type PR, in vascular smooth muscle appears to be decreased in post-menopausal women [52]. If this pattern was also true in breast tissue, then perhaps the associated change in progesterone responsiveness could relate to the increased risk of developing breast cancer after menopause. However, in this very small study, three of the four post-menopausal subjects were receiving oestrogen only HRT [52]. Since PR is, in part, an oestrogen responsive gene, the use of exogenous oestrogens could alter PR expression. Perhaps the increased risk of breast cancer associated with HRT could reflect a change in PR variant expression.

As well as these single exon deleted mRNAs, many variants exist in which multiple exons have been deleted; PRΔ2+3 [47,49], PRΔ3+4 [54], PRΔ3+6 [50], PRΔ4+6 [51], PRΔ5+6 [50], PRΔ4+5+6 [51], PRΔ3+4+5+6 [54]. Some of these would be unlikely to encode functional PR since they would lack too many of the protein domains; for example, PRΔ4+6 would lack the nuclear location signal and LBD but has been shown to be present more frequently in malignant than 'normal' breast tissue [55], and is perhaps indicative of a breakdown in normal splicing control mechanisms. Furthermore, any transcriptionally inactive PR variants expressed may be able to compete with wild-type receptors for co-factor binding ('squelching'), thereby interfering with normal progestin responsiveness. In this context, PR mutants deleted of their LBD have been shown to be constitutively active and capable of binding PRE [56]. In addition, ASV such as PRΔ5+6, would encode a protein with a complete DBD and NLS as well as being able to dimerise and possessing both AF-1 and AF-2. This protein has been demonstrated to be a dominant repressor of wild-type PR function, but is not itself progestin responsive or able to bind PRE [48], and its distribution in a wide range of normal and pathological tissue types [48,50,51] suggests an important physiological role. Another ASV with the potential to encode a functional protein

is PRΔ2+3. This putative variant protein would lack the entire DBD but could still modulate PR activity by binding progesterone, dimerising and migrating to the nucleus. Some of the variants, although unlikely to encode functional PR, seem to be tissue specific. PRΔ3+6 has to date only been seen in breast tumour tissue, and PRΔ4+5+6 only in endometrial tissue, suggesting that any protein encoded from these mRNA could have an important role in a specific progesterone target tissue. Alternatively, the presence of the mRNA could be indicative of a breakdown in splicing control mechanisms in cancer.

Other ASV mRNAs are seen that lack a specific part of an exon rather than being whole exon deleted. PRΔ4/2 mRNA, in which the first 126 base-pairs of exon 4 have been deleted, was detected frequently but at a low level in breast tumour tissue [47,49]. This deletion could be due to a donor splice sequence in intron 3 and an acceptor splice sequence within exon 4 forming a cryptic splice site and acting in competition to the normal exon 4 splice site in some transcripts [49]. PRΔ4/2 has since been observed in normal endometrial tissue [57] and would encode a protein lacking the NLS but probably able to dimerise and bind progesterone so could well be able to modulate PR function. A second partial exon deleted PR variant mRNA is PRΔ6/2 [58] in which a 52 base-pair section of exon 6 has been deleted. This mRNA has been detected at a far greater frequency in tumour compared to normal breast tissue, suggesting that the protein could be functional in cancer and may be a result of a cancer associated breakdown in splicing control. The protein would lack a fully functional LBD but would retain all of the other functional domains required to act as a transcription factor. Two further variants lacking part of exon 6, along with either exon 3 (PRΔ3+6/2) or exon 4 (PRΔ4+6/2) [58], have been observed infrequently, but appear specific to breast cancer tissue. Neither of these variants is likely to encode functional hormone-responsive receptors but again their presence in breast cancer tissue suggests mis-splicing leading to a change in PR expression.

Conclusion

Discrepancies between ER/PR status and response to hormone therapies could be due to the presence of these variant PRs (or variant ERs) that are either not detected (amino-terminally truncated variants) or not distinguishable from full-length receptor (functional domain deleted variants) by current screening methods that use antibodies directed against the PR-A/B common amino-terminal region. It has already been demonstrated that differences in the ratio of PR-A:PR-B can affect the outcome of breast cancer treatment [27]. The discovery of truncated PR isoforms adds another level of complexity to the study of PR in breast cancer. This is further complicated by reports of many exon deleted ASV PR mRNA species in a variety of cell lines, as well as normal and malignant human tissues. If translated, the proteins encoded by theses ASV mRNAs may have transcriptional activities that differ from the full length PR-A or PR-B to which they are related, or could interact with the full-length PR to modulate its activity, as is seen with PR-A repression of PR-B [25]. Moreover, PR spliced variants may also modulate PR-A and PR-B activity indirectly, by competitively binding to common nuclear co-regulatory proteins.

The expression of any isoform or variant proteins could modify progesterone responsiveness of a target tissue, for example, providing a mechanism for the differing responses to the same dose of progesterone in the uterus (proliferative) and breast (anti-proliferative) [48]. Variant PR expression breast cancer could, therefore, provide a mechanism for abnormal epithelial proliferation. Alternatively, the presence of variant mRNAs that are not translated to protein could be indicative of a loss of normal PR expression and thus normal response to progesterone.

Although the expression of many of these variants is yet to be reported in breast tissue at the protein level, the possibility that shorter forms of PR may be functional and perhaps not detected by conventional screening methods could lead to discrepancies between the reported PR status of a tumour and the progression of the disease or response to endocrine therapies. Therefore, detailed and accurate characterisation of PR variant expression in breast cancer could generate novel and more efficient biomarkers of disease prognosis or targets for therapeutics.

Competing interests

The authors declare that they have no competing interests.

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