Endokrynologia Polska/Polish Journal of Endocrinology
Tom/Volume 62; Numer/Number 2/2011
ISSN 0423–104X

Alternative splicing and its role in pathologies of the endocrine system

Różnicowe składanie pierwotnego transkryptu w patologiach układu endokrynnego

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Abstract

Alternative splicing of pre-mRNA is a process in which noncoding regions of primary transcript are removed and coding regions are joined in different manners to produce mRNA molecules of different sequences. Alternative splicing affects nearly all human genes and is a key source of diversity of proteins coded by a relatively small number of genes. Since alternative splicing is of crucial importance for the proper functioning of cells, including those involved in hormonal signalling, aberrations of alternative splicing can lead to disruption of cellular mechanisms and in consequence result in serious endocrine pathologies. Disturbances of alternative splicing include mutations of consensus splice regulatory sites and improprieties in the action of splicing factors, the proteins involved in regulating the process. In consequence of disturbed alternative splicing, improperly spliced mRNA and protein isoforms can be produced which can lead to disruption of function of their wild type counterparts.

This review aims to discuss the role of alternative splicing in pathologies of the endocrine system and gives examples that highlight the importance of this process in the proper functioning of hormones, hormone receptors and other factors involved in hormonal regulation. The examples given include endocrine-related tumours (pituitary tumours, cancers of the thyroid, prostate, ovary and breast, and insulinoma), isolated growth hormone deficiency, and Frasier syndrome. Non-endocrine pathologies in which aberrant alternative splicing of transcripts of genes involved in hormonal signalling have been detected are also described. Finally, we discuss future perspectives on the possible usage of alternative splicing in diagnostics and therapy.

(Pol J Endocrinol 2011; 62 (2): 160–170)

Key words: *alternative splicing, endocrine-related tumours, isolated growth hormone deficiency, Frasier syndrome*

Streszczenie

Różnicowe składanie pierwotnego transkryptu (*splicing alternatywny pre-mRNA*) jest procesem polegającym na usuwaniu rejonów, które nie kodują dojrzałej cząsteczki mRNA (intronów) i łączeniu rejonów kodujących (eksonów) w różnych konfiguracjach, dzięki czemu powstają cząsteczki mRNA o różnej sekwencji nukleotydów. Różnicowemu składaniu podlegają transkrypty prawie wszystkich kodujących białka genów człowieka, przyczyniając się do powstawania wielkiej różnorodności izoform białek kodowanych przez stosunkowo niewielką liczbę genów. Proces splicingu alternatywnego w istotny sposób przyczynia się do prawidłowego funkcjonowania komórek, również tych biorących udział w sygnalizacji hormonalnej. Zaburzenia różnicowego składania pre-mRNA prowadzą do nieprawidłowości w procesach komórkowych i w konsekwencji mogą prowadzić do zaburzeń endokrynnych. Zaburzenia różnicowego składania pre-mRNA wynikają z mutacji w tak zwanych miejscach splicingowych bądź z nieprawidłowego działania czynników splicingowych, czyli białek zaangażowanych w regulację tego procesu.

Niniejsza praca ma na celu przedstawienie roli różnicowego składania pre-mRNA w patologiach układu endokrynnego, takich jak nowotwory endokrynne (guzy przysadki, rak tarczycy, prostaty, jajnika, piersi, *insulinoma*), izolowany niedobór hormonu wzrostu, dysgenezja gonad. Omówione zostały także przykłady patologii niezwiązanych bezpośrednio z układem endokrynnym, w których wykryto zaburzenia alternatywnego splicingu transkryptów genów biorących udział w sygnalizacji hormonalnej. Artykuł kończy przegląd możliwości diagnostycznych i terapeutycznych w chorobach endokrynnych, wykorzystujących proces różnicowego składania pre-mRNA.

(Endokrynol Pol 2011; 62 (2): 160–170)

Słowa kluczowe: *różnicowe składanie pierwotnego transkryptu, nowotwory endokrynne, izolowany niedobór hormonu wzrostu, zespół Frasiera*

The work supported by Polish State Committee for Scientific Research grant NN 401 071 939 (to Alicja Nauman).

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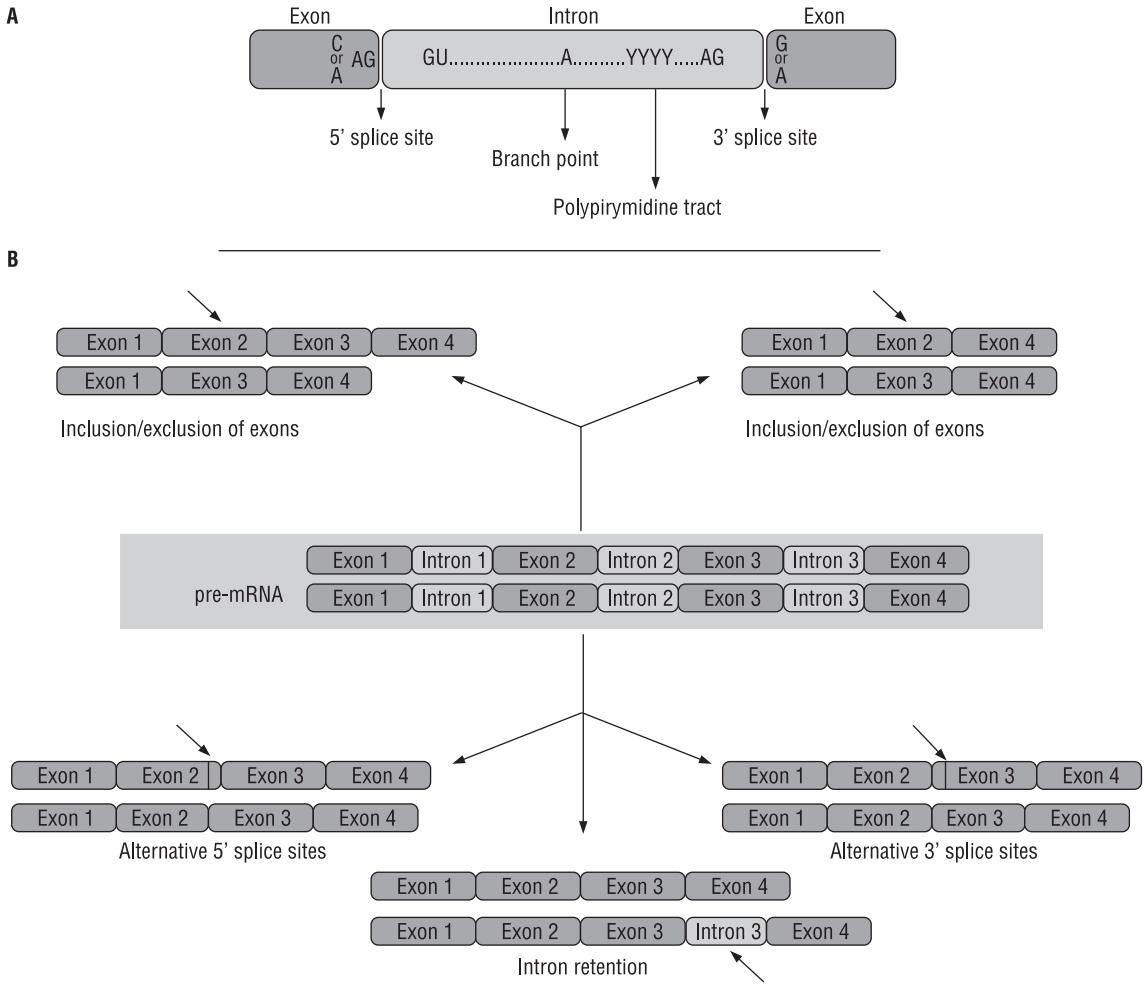

Figure 1. *Basis of alternative splicing.* ***A.*** *Consensus sequences participating in alternative splicing include 5′ and 3′ splice sites, branch point, and polypirymidine tract.* ***B****. Types of alternative splicing (starting from left, clockwise): inclusion/exclusion of exons (here: of exon 2, shown with an arrow); mutually exclusive exons (here: exons 2 and 3); usage of alternative 3′ splice sites (shown with an arrow); retention of an intron (shown with an arrow); usage of alternative 5′ splice site (shown with an arrow)*

Rycina 1. *Różnicowe składanie pierwotnego transkryptu.* ***A.*** *Sekwencje nukleotydów biorące udział w składaniu pre-mRNA: miejsca splicingowe 5' i 3', punkt rozgałęzienia (*branch point*) i trakt polipirymidynowy.* ***B.*** *Rodzaje różnicowego składania pre-mRNA (rozpoczynając od lewej, zgodnie z ruchem wskazówek zegara): włączanie/wyłączanie eksonów (tutaj: eksonu 2, wskazanego strzałką); wzajemnie wykluczające się eksony (tutaj: eksony 2 i 3); wykorzystanie alternatywnych miejsc 3' splicingowych (miejsce wskazano strzałką); zatrzymanie intronu (wskazany strzałką); wykorzystanie alternatywnych miejsc 5' splicingowych (miejsce wskazano strzałką)*

Alternative splicing: basis of the process

The majority of human genes encoding proteins consist of regions which do not code for the mature transcript (introns) and regions of coding sequences (exons).

During the synthesis of RNA, both kinds of sequences are transcribed into the primary transcript (pre-mRNA). Splicing is the process in which introns are removed and exons are joined to produce an mRNA molecule which can be further translated. Exons, however, can be joined in different ways. As a result of this process, multiple mRNA and protein isoforms are produced from a relatively small number of genes (in human genome: ~25,000). Alternative splicing affects the vast majority of human genes (more than 90%) [1] and is one of the main sources of protein diversity, allowing for the synthesis of multiple isoforms of receptors, adhesion molecules, protein hormones etc.

Alternative splicing is a complicated process, involving specific consensus sequences in exons and introns of primary transcript and multiple regulatory proteins. The sequences include 3′ splice site (3′ss), polypyrymidine tract, branch point sequence (BPS), and 5′ splice site (5′ss) (Fig. 1A.). Splicing reactions result in different types of mRNA isoforms due to differential usage of splice sites, exclusion/inclusion of exons or even intron retention (Fig. 1B.). The process of splice site selection during alternative splicing is supported by auxiliary

sequences of exonic and intronic splicing ehancers (ESEs and ISEs, respectively), and exonic and intronic splicing silencers (ESSs and ISSs, respectively).

Splicing reactions are catalysed by spliceosome, a large complex formed by five small ribonucleoproteins (snRNAs: U1, U2, U4, U5, and U6) and more than 300 other proteins. Alternative splicing is strictly regulated by a number of splicing factors, including SR proteins (serine-arginine rich proteins) [2] and hnRNPs (heterogeneous nuclear ribonucleoproteins) [3].

Binding of these regulatory proteins to ESE/ISE sequences (in the case of SR proteins) or to ESS/ISS (in the case of hnRNPs) defines selection of specific splice sites and thus selection of exons included in the spliced mRNA [4]. The selection of splice sites is also influenced by relative levels of specific splicing factors binding to the regulatory sequences. In consequence, the final result of alternative splicing is an effect of the concerted action of antagonistically acting splicing factors.

Disturbances of alternative splicing are frequently observed in different types of pathologies, including neurological disorders [5], inherited metabolic diseases [6], and cancer [7, 8]. Improperly spliced mRNA isoforms, if translated, may lead to synthesis of proteins of altered sequence, length, and structure. This may lead to malfunction of proteins, disturbances of cellular physiology, and finally to disease. Disturbances of alternative splicing may result from mutations in splice sites or cis regulatory elements, or from altered function of spliceosome elements.

Mutations in splice sites and other consensus sequences lead to missplicing, exon skipping, activation of cryptic splice sites or retention of intron. Mutations in splicing silencers or enhancers change the repertoire of splicing factors recognising the regulatory sequence and in this way influence splice site selection, leading to synthesis of improperly spliced mRNAs.

Since relative levels of antagonistic splicing factors influence the selection of splice sites, disturbances of expression of splicing factors play a significant role in pathologies associated with impaired splicing. An example is splicing factor SF2/ASF, a member of the SR protein family, whose overexpression is often observed in cancers. SF2/ASF regulates alternative splicing of tumour suppressor Bin1. Inclusion of exon 12A into Bin1 transcript, caused by overexpressed SF2/ASF, leads to loss of its tumour suppressive activity [9].

Alternative splicing is a process affecting genes involved in all human physiological systems. The role of alternative splicing in the physiology of the endocrine system was set out more than a decade ago in two excellent reviews [10, 11].

This review aims to discuss the role of alternative splicing in pathologies of the endocrine system and gives several examples that highlight the importance of this process in the proper functioning of hormones, hormone receptors and other factors involved in hormonal regulation.

Endocrine-related tumours

Disturbances of alternative splicing are especially frequently observed in cancers [7]. Improperly spliced mRNA isoforms and aberrantly acting splicing factors are therefore also found in endocrine-related tumours.

Pituitary tumours

Thyrotropin-secreting pituitary adenomas (TSH-omas) are rare pituitary tumours accounting for less than 2% of pituitary tumour cases [12]. These tumours lead to disruption of the negative feedback system of the hypothalamus-pituitary-thyroid (HPT) axis, resulting in elevated free T3 and T4 in serum with concurrent high levels of serum TSH. The molecular basis of TSH-omas etiology is not well understood, although some observations of chromosomal and genetic aberrations have been described [12]. Since one of the features of TSH-omas is the block of thyroid hormone mediated inhibition of TSH secretion in the pituitary, several studies have tried to analyse possible alterations in the function of thyroid hormone receptors (TRs). Those studies led to identification of an improperly alternatively spliced variant of thyroid hormone receptor beta 2 (TRβ2spl) in TSH-oma [13]. TRβ2spl lacked more than 40 amino acids in the ligand binding domain of the receptor, substituted with single amino acid isoleucine, resulting in complete loss of T3 binding, as well as the ability to interact with co-repressors and coactivators. Moreover, as DNA binding domain of the receptor was not changed, TRβ2spl exerted a dominant negative activity. Those functional improprieties resulted in loss of T3-dependent regulation of TSH expression and secretion. In the THRB gene coding the receptor, no mutations were detected, suggesting that abnormal alternative splicing was a result of malfunction of splicing factors.

The agonists of dopamine receptors (DRs) are used for the treatment of pituitary tumours to achieve reduction of hormone release and tumour size [14]. However, a significant number of patients do not respond to this treatment. Several studies have suggested that this lack of response may be, at least partially, connected with the expression of alternatively spliced isoforms of DR2 receptor: DR2S and DR2L. DR2S is the shorter isoform which is devoid of exon 5 encoding 29 amino acids in the third cytoplasmic loop involved in G protein coupling [14]. The expression of the two variants is heterogenous in pituitary adenomas, ranging from the equal level of DR2S and DR2L [15], or different ratios of

DR2L to DR2S [16–18] to a complete loss of DR2 receptor [16, 17]. The specific ratios of DR2 isoforms may influence patient response to dopamine agonists. Studies on nonfunctioning adenoma-derived cell lines revealed that treatment with the dopamine agonist, bromocriptine, led to inhibition of proliferation in cells expressing only DR2S or in cells in which the expression of DR2S was predominant [16]. Those initial *ex vivo* experiments were subsequently confirmed by studies on pituitary tumour samples. High expression of DR2S was associated with better response to treatment with bromocriptine in prolactinomas [19] and with cabergoline in NFPA tumours [17]. This beneficial effect of DR2S variant may result from differential induction of intracellular signalling when compared to DR2L. This hypothesis is supported by the observation that while stimulation of both receptors leads to an increase of free calcium in cytoplasm, DR2S is more effective in inhibition of cAMP accumulation than DR2L [20]. Moreover, the two isoforms differ in inhibitory effects on ERK1/2 signaling [21].

Thyroid cancer

Considering the frequency of disturbed alternative splicing events in cancers, surprisingly few papers have reported alterations of this process in thyroid neoplasia. Papillary thyroid carcinomas (PTCs) are characterized by high frequency of mutations in BRAF oncogene [22]. Baitei et al. [23] identified in PTCs, follicular variants of PTCs (FVPTCs), and in one case of anaplastic thyroid carcinoma (ATC) splice variants of BRAF which coded for a variant of B-Raf protein which was devoid of N-terminal auto-inhibitory domain but contained C-terminal kinase domain and caused constitutive activation of B-Raf. Those splice variants, when expressed in cell lines, resulted in activation of MAP kinase signalling pathway, tumoural transformation of cells and induction of tumours in nude mice [23] suggesting that disturbance in alternative splicing of BRAF may directly contribute to development of PTC tumours.

Differences in expression of splice variants can serve as markers for differentiation between malignant and benign nodules as shown in the study analysing telomerase reverse transcriptase (hTERT) [24]. Malignant thyroid tumours, irreversibly of the histopathological type (with the exception of follicular variants of papillary thyroid cancer), were characterized by higher expression of active full-length hTERT transcript than of inactive splice variants which were devoid of regions a and/or β.

In another report, in 18 per cent of analysed PTC samples, expression of MDM4-211 splice variant of MDM4 protein, an inactivator of p53 tumour suppressor, was detected [25]. MDM4-211 was not expressed in non-tumourous paired controls. Since it was shown that MDM4-211 exerts oncogenic potential [26], its overexpression may contribute to tumoural transformation in certain cases of PTC.

The causes of aberrant alternative splicing of thyroid cancers are usually unknown. The exception is a study of a family with hereditary goitrous hypothyroidism and metastatic thyroid carcinoma in which aberrant splicing of thyroglobulin (Tg) was detected [27]. The patients had an autosomal recessive mutation in the splice donor site of intron 5 of Tg gene which resulted in the frame shift and a premature stop codon at position 147. The authors suggested that the malignant transformation could possibly result from prolonged TSH stimulation. In cases when no splice disturbing mutations are known, a possible cause could be aberrant expression of splicing factors such as SF2/ASF whose overexpression was found in follicular thyroid carcinoma [9]. However, it is clear that more studies on thyroid tumours are needed to reveal to what level disturbances of alternative splicing contribute to pathology of this type of neoplasia.

Prostate and ovarian cancers

Krüppel-like factors (KLFs) are transcription factors whose dysregulated activity was suggested to be linked with endocrine-related malignancies [28]. Specific splice isoforms of one of the members of this family, KLF6, play a direct role in carcinogenesis. Splice variant KLF6-SV1 is an oncogenic isoform which antagonises tumour-suppressive properties of KLF6 variant [29]. The isoforms differ in their structure: KLF6-SV1 lacks zing finger DNA binding domain, while in KLF6 this domain is present. In a study involving more than 3,400 men it was found that a germline single nucleotide polymorphism (SNP) in KLF6 is associated with an increased risk of prostate cancer [30] (Fig. 2A). This SNP resulted in the generation of a novel binding site for splicing factor SRp40 which shifted splicing reaction into increased synthesis of three KLF6 spliced isoforms, including KLF6-SV1. In hormone-refractory metastatic prostate cancer, the levels of KLF6-SV1 were upregulated. In human prostate cancer cell lines overexpression of KLF6-SV1 led to increased proliferation due to downregulation of cyclin-dependent kinase inhibitor p21 and upregulation of antiapoptotic Bcl-2, and of oncogenic c-myc. Overexpression of KLF6-SV1 was also followed by and enhanced expression of metalloproteinase MMP9 which is involved in tumour cell migration and invasion [30]. Those observations were completed by mouse models of metastatic prostate cancer in which overexpression of KLF6-SV1 led to more rapid metastasis and dissemination to lymph nodes, bone, and brain. Similar effects were observed in ovarian cancer cells [31] in which overexpressed KLF6-SV1 bound a proapoptotic protein NOXA and induced its degradation

through HDM2 ubiquitin ligase mediated mechanism. NOXA acts as a binding partner for Mcl-1, which is a member of an antiapoptotic Bcl-2 family. Binding of Mcl-1 by NOXA inhibits its antiapoptotic activity. KLF6-SV1-induced degradation of NOXA leads to upregulation of Mcl-1 and this way contributes to survival of cancer cells (Fig. 2A).

Results of studies on prostate and ovarian cancer suggest that KLF6-SV1 targeting offers an approach for treatment of these cancers. RNAi mediated downregulation of KLF6-SV1 leads to spontaneous apoptosis in cultured prostate cancer cell lines and suppresses tumour growth in mice [32, 33]. Silencing of KLF6-SV1 in ovarian cancer cells induces spontaneous apoptosis, restores cisplatin sensitivity, decreases tumour progression, and improves survival in mice [31]. Since disturbed alternative splicing of KLF6 has been observed in many other cancers, including pancreatic [34], gastric [35], liver [36], and other [37, 38, 39], targeting of KLF6 alternative splicing may be a more generalised strategy to fight cancer.

Insulinoma

Alternative splicing can lead to synthesis of different mRNA isoforms which are translated into proteins of identical amino acid sequence. An example is alternative splicing of proinsulin whose alternatively spliced variants NAT and SPV differ in their 5′ untranslated regions (5′UTRs) [40] (Fig. 2B). UTRs are regulatory regions of mRNA molecules involved in the regulation of mRNA stability, cellular localization and translational efficiency [41]. Variant SPV of proinsulin is synthesised due to retention of intron 1 which leads to change of 5′UTR sequence but leaves the coding region of mRNA unchanged (Fig. 2B). This splice variant accounts for less than 1% of native insulin mRNA in normal pancreatic islets [40]. The change in 5′UTR of SPV variant results in doubling the efficiency of translation, yielding more (pre)proinsulin protein. In insulinoma tumours, disturbed alternative splicing of proinsulin leads to a dramatic, greater than 50-fold, increase of SPV : NAT ratio [42]. This in turn results in enhanced synthesis and secretion of insulin, leading to hyperinsulinemia.

Breast cancer

In breast cancers disturbed alternative splicing of multiple endocrine system related genes have been detected, including altered splicing of oestrogen, thyroid hormone, and progesterone receptors (ER, TR, and PR, respectively). All these receptors belong to the nuclear receptor family and act as ligand dependent transcription factors. Several studies have suggested that the expression of oestrogen receptor splice variant ERβcx may influence disease progression in breast cancer patients [43]. Loss of ERβcx was correlated with more aggressive phenotype, increased risk of vascular invasion [44], higher number of affected lymph nodes, and increased risk of developing metastatic breast cancer [45]. ERβcx expression was associated with response to endocrine therapy and/or survival in breast cancer patients [46–49]. ERβcx (also known as ERβ2) differs from the ERβ1 variant by the sequence of amino acids in AF-2 (activation function) domain which is essential for ligand-dependent transcriptional activation. ERβcx is devoid of ligand binding ability but binds to DNA in heterodimers with ERα or ERβ1 isoforms. Binding of ERβcx in complex with ERα leads to an inhibition of its transcriptional activity, while binding with ERβ1 does not influence the activity of the latter. Thus, ERβcx acts as a dominant negative inhibitor of ERα. Therefore, expression of ERβcx in ERα-positive breast cancers has the potential to modulate the response to antioestrogen therapy. As suggested by Palmieri et al. [46], ERβcx mediated blockade of ERα activity could synergise with actions of antioestrogens such as tamoxifen, used in anticancer therapy, and thus may provide a beneficial effect for patients [46]. Other studies on the role of ERβcx in breast cancer, however, have provided conflicting results. For instance, in one study no connection between ERβcx protein status and patient survival was found [50]. In other studies, cytoplasmic ERβcx expression correlated with poor prognosis [49, 51], while nuclear positivity of ERβcx was shown to correlate with overall survival and disease-free survival [49]. In other reports, ERβcx expression did not have the predictive value on tamoxifen resistance [52] or correlated with poor response to this drug [44]. On the other hand, in the study by Vinayagam et al. [53], a positive correlation between tamoxifen-therapy outcome and ERβcx mRNA but not protein level was found, and especially good results of the therapy were achieved in patients who expressed high levels of both protein and mRNA of ERβcx. These results suggest that ERβcx impact on breast cancer progression may be post-transcriptionally controlled. To explain the contradictory results of the ERβcx significance in prognosis prediction, the following possible causes have been proposed: ethnic differences, cellular localization of ERβcx [54], and cutoff values used for defining ERβcx positivity and type of statistical analysis [46]. Clearly, further studies using carefully standardised methods are needed to elucidate the role of ERβcx in breast cancer pathology.

Thyroid hormone receptors (TRs) are encoded by two genes, THRA and THRB. Due to alternative splicing and different transcription start sites usage, several TRα and TRβ protein isoforms are produced [55]. Disturbances in expression and functioning of TRs have been identified in many types of cancer [56, 57]. In breast cancer, apart from mutations, promoter hypermethy-

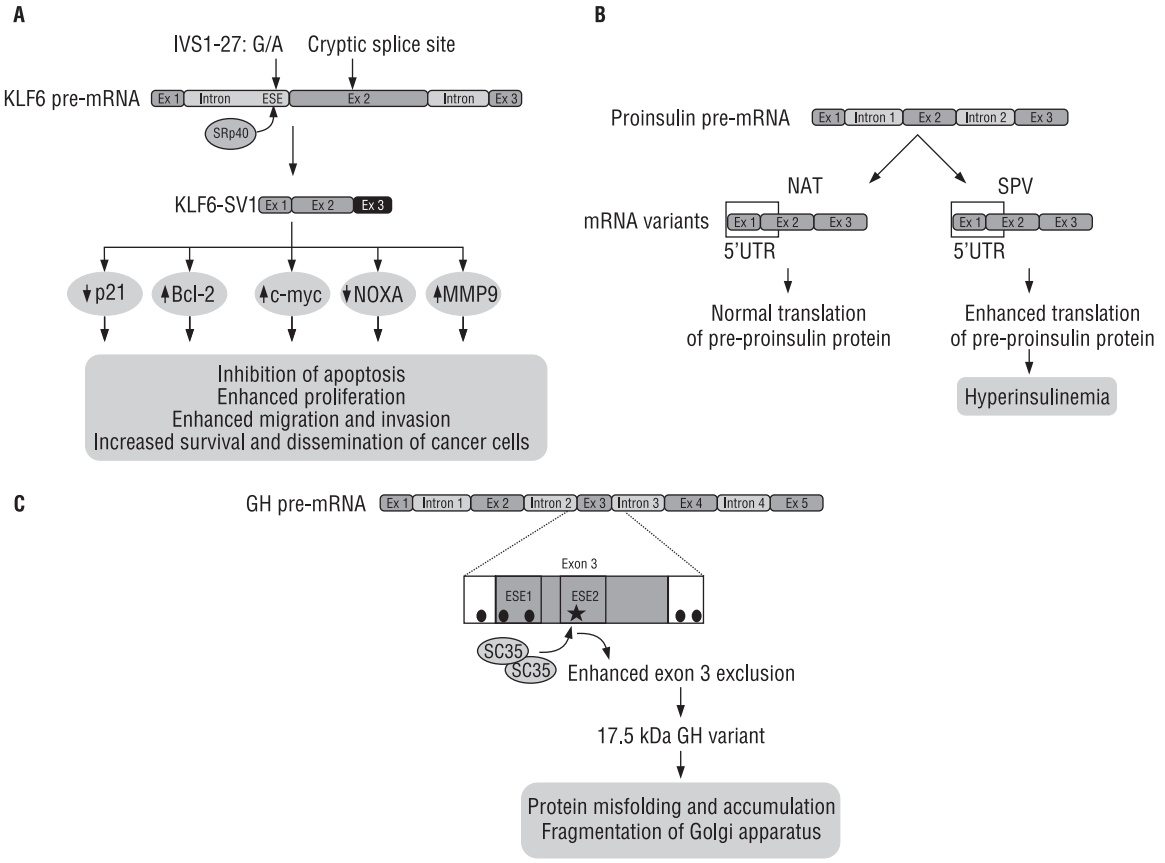

Figure 2. *Examples of disturbed alternative splicing in endocrine pathologies.* ***A.*** *Disturbed alternative splicing of KLF6 in prostate and ovarian cancers. A IVS1-27: G/A polymorphism in intronic region of KLF6 gene leads to creation of an additional ESE binding site specifically recognized by splicing factor SRp40. Binding of SRp40 results in activation of cryptic splice site located in exon 2 and synthesis of alternatively spliced variant KLF6-SV1 with shortend exon 2 and out-of-frame exon 3 (dark). KLF6-SV1 activity leads to downregulation of cyclin dependent kinase inhibitor p21, upregulation of antiapoptotic Bcl-2, and oncogenic c-myc, enhanced expression of metalloproteinase MMP9, and degradation of proapoptotic protein NOXA. These changes result in increased survival and dissemination of cancer cells.* ***B.*** *Disturbed alternative splicing of proinsulin in insulinoma [42]. NAT and SPV: alternatively spliced variants. Retention of fragment of intron 1 in SPV variant results in changed 5'UTR resulting in enhanced translational efficiency, increased synthesis of insulin protein and hyperinsulinemia.* ***C.*** *Alternative splicing of growth hormone in IGHD II (according to [66]). Mutation in exon 3 creates an additional binding site for SC35 and leads to enhanced skipping of exon 3. As a result, a short, 17.5 kDa GH protein isoform is synthesized which is misfolded and accumulates in endoplasmic reticulum and finally leads to fragmentation of Golgi apparatus.*

Rycina 2. *Przykłady zaburzeń różnicowego składania pierwotnego transkryptu w patologiach endokrynnych.* ***A.*** *Zaburzony splicing alternatywny transkryptu genu KLF6 w raku prostaty i jajnika. Polimorfizm IVS1-27: G/A w intronie genu KLF6 prowadzi do powstania dodatkowego miejsca ESE wiążacego czynnik splicingowy SRp40. Wiązanie tego czynnika powoduje aktywację dodatkowego miejsca splicingowego w eksonie 2 i powstanie alternatywnego wariantu KLF6-SV1 ze skróconym eksonem 2 i eksonem 3 z przesuniętą ramką odczytu (zaznaczone ciemnym kolorem). KLF6-SV1 powoduje obniżenie ekspresji inhibitora p21 kinazy zależnej od cykliny, podwyższenie ekspresji antyapoptotycznego białka Bcl-2, onkogenu c-myc i metaloproteinazy MMP9 oraz degradację proapoptotycznego białka NOXA. W rezultacie prowadzi to do zwiększonej przeżywalności komórek nowotworowych i ich rozsiewu.* ***B.*** *Zaburzenie alternatywnego splicingu proinsuliny w guzach insulinoma [42]. NAT i SPV: warianty splicingowe proinsuliny. Zatrzymanie fragmentu intronu 1 w wariancie SPV powoduje zmianę sekwencji w rejonie 5'UTR, co skutkuje zwiększoną wydajnością translacji, nasiloną syntezą białka insulinowego i hiperinsulinemią.* ***C.*** *Alternatywny splicing hormonu wzrostu w IGHDII (według [66]). Mutacja w eksonie 3 prowadzi do powstania dodatkowego miejsca wiążącego czynnik splicingowy SC35, co skutkuje nasilonym wyłączaniem eksonu 3. W rezultacie powstaje krótki wariant cząsteczki hormonu wzrostu, o wielkości 17,5 kDa i nieprawidłowej strukturze. Wariant ten gromadzi się w retikulum endoplazmatycznym i prowadzi do fragmentacji aparatu Golgiego*

lation and disturbed expression [58–60], impaired alternative splicing of TRs have been detected [61]. Altered TRβ1 transcripts had broad deletions in regions coding DNA and ligand binding domains, and in the hinge region involved in transcriptional co-repressor recruitment. Such transcripts, if translated, would yield TRβ1 proteins with seriously disturbed function.

Progesterone receptor gene PGR is transcribed into two main isoforms, PR-A and PR-B, which are the result of the presence of two different functional promot-

er regions [62]. PR-B protein is the longer one, and PR-A protein lacks 164 amino acids of AF-3 (activation function-3) domain at the N-terminus. The primary transcript of PGR gene undergoes intensive alternative splicing processing which results in additional PR variants whose function is largely unknown. Hisatomi et al. [63] identified a novel PR delta6/2 variant that was lacking 52 bp in exon 6, a region coding for ligand binding domain. PRdelta6/2 was expressed statistically significantly more frequently in breast cancer tissues than in non-cancerous paired controls. In other studies, multiple PR variants with randomly deleted exons were found. However, their expression was not compared among normal and cancerous tissues [64]. Hence, the role of PR splice isoforms in breast cancer pathology is unknown and future studies are needed to reveal their potential contribution to tumour initiation and/or progression.

Isolated growth hormone deficiency type II

Isolated growth hormone deficiency type II (IGHD II) is an autosomal dominant growth hormone deficiency [65] which results mainly from aberrant alternative splicing of pre-mRNA encoded by growth hormone gene GH1. Alternative splicing of GH1 primary transcript leads to synthesis of at least five protein isoforms. The major product is a 22 kDa isoform exerting full biological activity of growth hormone. The use of cryptic splice site located in exon 3 leads to synthesis of a shorter, 20 kDa variant, which retains the activity of a normal growth hormone molecule. When exon 3 is skipped during alternative splicing, 17.5 kDa variant is produced which acts as a dominant negative mutant and blocks secretion of full-length protein. The residual two 11.3 kDa and 7.4 kDa variants are produced in trace amounts. Splice mutations identified in patients with IGHD II result in increased production of 17.5 kDa GH variant (Fig. 2C). These mutations affect splice sites at borders of exon 3 or two ESEs located in exon 3. Skipping of exon 3 is regulated by two splicing factors, SF2/ASF and SC35, which bind to ESE2. These two factors act antagonistically: binding of SF2/ASF prevents skipping of the exon, while binding of SC35 leads to exclusion of exon 3 during splicing. The mechanism of enhanced skipping of exon 3 in IGHD II was elucidated by Solis et al. [66] who found that mutation in exon 3 creates an additional functional binding site for SC35 and thus leads to enhanced skipping of the exon and synthesis of 17.5 kDa variant of growth hormone (Fig. 2C). This protein is misfolded and accumulates in endoplasmic reticulum, and subsequently leads to fragmentation of Golgi apparatus, and in consequence, to disturbed trafficking of GH and of other proteins [67].

Gonadal dysgenesis

Frasier syndrome (FS) is a rare disease characterized by pseudohermaphroditism and progressive glomerulopathy. The phenotype includes gonadal dysgenesis and normal female external genitalia in spite of XY karyotype. Child patients suffer from proteinuria which increases with age leading to nephrotic syndrome and development of end-stage renal failure in the second or third decade of life. FS is associated with a high risk of gonadoblastoma [68, 69]. FS is caused by disturbed alternative splicing of WT1 gene transcript resulting from mutations in splice site in exon 9 [70].

WT1 gene is located in 11p13 chromosome region and encodes multifunctional protein which acts as a transcription factor, and influences translation and alternative splicing [71]. WT1, initially identified as Wilms tumour suppressor gene, participates in multiple stages of kidney development. WT1 gene comprises ten exons, of which exons 7–10 encode DNA-binding domain composed of four zinc finger motifs. Alternative splicing of WT1 primary transcript leads to synthesis of four WT1 isoforms due to inclusion or skipping of exons 5 and 9. Inclusion of exon 9 results in incorporation of a motif consisting of three amino acids, lysine-threonine-serine (KTS), between the third and fourth zinc fingers and leads to synthesis of +KTS isoform, in contrast to KTS variant which lacks the motif. In normal human tissues the ratio of +KTS/–KTS isoforms is within the range 1.10–1.49. In FS patients alternative splicing of WT1 is disturbed due to heterozygous mutations in donor splice site located in intron 9 which result in the loss of +KTS isoform expression from one allele [70, 72]. As a result, the ratio of +KTS/–KTS isoforms decreases to 0.5. Recently, Bradford et al. proposed a model by which disturbed alternative splicing of WT1 leads to development of Frasier syndrome [73]. Accordingly this model, +KTS isoforms are regulators of expression of Sry gene which is the sex-determining gene located on the Y chromosome [74]. SRY protein is a transcription factor which triggers differentiation of Sertoli cells. These actions of SRY involve interactions with testis-specific enhancer of Sox9 (TES) and lead to activation of Sox9 transcription factor expression [75]. Using knockout +KTS$^{-/-}$ mice, Bradford et al. [73] proposed that +KTS isoform controls the expression of SRY which subsequently triggers upregulation of Sox9. This in turn induces expression of fibroblast growth factor 9 (FGF9) which finally leads to differentiation of Sertoli cells. FGF9 regulates differentiation of Sertoli cells via two mechanisms. In pre-Sertoli cells in a feedback mechanism FGF9 upregulates expression of Sox9 and this way initiates differentiation. FGF9 induces also male-specific

proliferation in the coelomic epithelium. Improper ratio of +KTS/–KTS isoforms in Frasier syndrome patients inhibits this pathway and leads to development of small gonads with a number of pre-Sertoli cells insufficient to ensure the proper development of testes.

Other pathologies

Since hormonal regulation is involved in wide physiological processes, disturbed alternative splicing of transcripts encoded by genes involved in endocrine regulation is also found in non-endocrine diseases. This concerns, for instance, the signalling pathway of thyroid hormones which regulate key cellular processes including proliferation, differentiation, apoptosis and metabolism [76]. The human thyroid gland secretes hormones: 3,5,3',5'-tetraiodothyronine (thyroxine, T4) and 3,3',5-triiodothyronine (T3) in a proportion defined by the T4/T3 ratio = 11:1 [77]. The majority of an active form of thyroid hormone, T3, is synthesized in extrathyroidal tissues from T4 due to deiodination at the position 5′ of phenolic ring. Deiodination reactions are catalysed by enzymes called iodothyronine deiodinases. There are three types of these enzymes. Type 1 deiodinase (Dio1) is the only enzyme catalysing two types of deiodination (5′ and 5) and is involved both in activation (synthesis of T3) and inactivation (deiodination of T4 and T3) of TH.

Type 2 (Dio2) deiodinase is an activating enzyme and catalyses deiodination at the position 5′ resulting in synthesis of T3 from T4.

Type 3 deiodinase (Dio3) is an inactivating enzyme which removes iodine at the position 5 of phenolic ring of iodothyronines and is involved in deiodination of T4 (yielding rT3) and T3 (yielding T2, diiodothyronine). Type 3 deiodinases is encoded by an intronless gene and therefore its primary transcript is not spliced. Type 1 and type 2 deiodinases are alternatively spliced; however, only one protein product of each gene is known and the physiological role of their mRNA isoforms remains unknown.

Variant disturbances of thyroid hormone receptors and iodothyronine deiodinases are often found in different types of cancers [56, 57, 78–93]. These disturbances concern also alternative splicing and can lead to severe pathological conditions, as in the aforementioned case of disturbed alternative splicing of TRβ2 leading to thyroid hormone resistance in TSH-oma [13]. In kidney cancer, disturbances of alternative splicing of untranslated region (UTR) of TRβ1 and of type 1 iodothyronine deiodinase have been identified [78, 94]. UTR variants of TRβ1 differed in their predicted secondary structure, translational efficiency and the presence or absence of microRNA binding sites. In case of type 1 iodothyronine deiodinase, three splice isoforms were cloned exclusively from tumour samples, suggesting their potential use as cancer markers. The disturbances of alternative splicing consisted of changes in ratios between specific mRNA isoforms. A growing body of evidence suggests that the ratios between splice isoforms contribute significantly to individual phenotype variability and influence cellular homeostasis [95, 96]. A possible cause of alterations observed in kidney cancer can be improper expression of splicing factors whose disturbed mRNA and protein level have been identified in tumours [78, 97].

Oestrogen receptors, ERα and ERβ, are encoded by two genes, ESR1 and ESR2, whose primary transcripts undergo multiple alternative splicing events leading to the synthesis of several splice variants [98]. Disturbed alternative splicing of oestrogen receptors has been linked to pathogenesis of Alzheimer's disease (AD) and schizophrenia. Ishunina and Swaab mapped the expression of ERα splice variants in the human brain and found that distribution of mRNA isoforms was brain area- and case-specific [99]. Moreover, in AD patients, the number of ERα splice variants per brain area was significantly diminished and the ratios between specific splice variants were changed. This difference was more prominent in women than in men [99–101). The most commonly detected was variant Δ7. This variant is devoid of exon 7 which encodes a substantial part of the ligand binding domain. Variant Δ7 acts as a dominant negative ERα isoform. Several papers have suggested that decreased oestrogen levels in the menopausal period may be involved in the development of AD. Moreover, it has been shown that oestrogen therapy during menopausal transition is linked to a decreased risk of AD and delayed development of the disease. However, trials with oestrogen therapy in women with diagnosed AD failed to show benefits on cognitive functions [102]. Ishunina and Swaab suggested that high expression of Δ7 in elderly healthy controls and AD patients may possibly explain reduced effects of oestrogen on cognitive functions [99]. Interestingly, this same variant seems to be involved in the development of major unipolar depression. In patients suffering from this disease, the expression of Δ7 was 1.7 times more frequent than in normal controls [103]. Since Δ7 variant inhibits oestrogen signalling mediated by the wild type ERα, enhanced expression of Δ7 may change brain oestrogen response in depression patients. These findings are in agreement with observations that circulating levels of oestrogen correlate with symptoms of depression [103].

In schizophrenia patients, multiple disturbances of ERα at genomic and transcriptomic level have been found, including altered frequency of splice variants and detection of an abnormally spliced transcript with re-

tained intronic sequence [103]. It was shown that the transcriptional activity of ERα is modulated by a ErbB4--ICD protein, belonging to the leading schizophrenia susceptibility pathway [104]. ErbB4 is a receptor with tyrosine kinase activity whose activation leads to its cleavage and release of the transcriptionally active intraplasmic domain (ErbB4-ICD). ErbB4-ICD enhances ERα mediated transcriptional activation. However, this effect is inhibited by the presence of Δ7 splice variant, suggesting that Δ7 functions as a dominant negative variant that suppresses ERα mediated oestrogen signalling [104].

Alternative splicing in diagnostics and therapy

Evidence of the disturbed alternative splicing in different pathologies raised an idea of using specific splice variants as diagnostic markers or targets for therapeutic actions. The most efficient method for searching for specific splicing markers is global transcriptomic analysis followed by verification using reverse transcription PCR. Klinck et al. [105] proposed a new approach which they called layered and integrated system for splicing annotation (LISA) based on direct high-throughput reverse transcription-PCR validation of alternative splicing events. Using this method, they analyzed splicing of 600 cancer-related genes in ovarian cancer samples and discovered 48 highly cancer-specific splicing events that created a molecular signature of epithelial ovarian cancer. The same approach used for analysis of breast cancer samples allowed for identification of cancer tissues with 96% accuracy and ordering of cancer samples according to histopathological grade [106]. New data on cancer-specific splicing markers can be identified using mouse models as shown by Menon et al. [107]. Using mass spectra derived from the plasma proteome of mouse model of human pancreatic ductal adenocarcinoma, they identified a pattern of splice isoforms, including novel and differentially expressed variants that allowed for differentiating tumour-bearing and control mice. Thus, the study provided a basis for a noninvasive assay of candidate biomarkers for pancreatic cancer.

Pro- or antiproliferative activity of different splicing variants offers an opportunity for manipulations aiming for therapeutic effects. For instance, antisense oligonucleotide induced shift in alternative splicing of Bcl-x pre-mRNA resulted in increased expression of proapoptotic Bcl-xS isoform, sensitized breast and prostate cancer cells to several antineoplastic agents and radiation, and promoted apoptosis of multidrug-resistant MCF-7/ADR breast cancer cell line [108].

Since alternative splicing events are tightly regulated by splicing factors and elements of spliceosome, there is growing interest in compounds targeting these proteins to achieve antiproliferative effects. Some of these compounds, such as pladienolides, are currently being tested in clinical trials in Europe and the United States [109, 110]. Pladienolide is an antitumour macrolide produced by *Streptomyces platenensis* that targets SF3b splicing factor complex, and which leads to accumulation of unspliced or incompletely spliced primary transcripts [2007]. The effects of modulated expression of splicing factors in cancer cells can be especially promising in case of proteins that exert oncogenic properties. This was shown for SF2/ASF splicing factor which belongs to the serine-arginin rich family of proteins. Karni et al. proved that overexpression of SF2/ASF results in transformation of immortal rodent fibroblasts which form sarcomas in nude mice. Conversely, knockdown of SF2/ASF led to reduction of tumour volume [9].

Summary

Alternative splicing is a process of crucial importance for the proper function of cells, including those involved in hormonal signalling. Future studies using global analysis of transcript variants and splicing regulatory proteins would certainly provide further data for understanding the molecular basis of multiple endocrine pathologies. Knowledge of specific mechanisms initiated by improperly spliced transcripts will offer opportunities for new diagnostic and therapeutic approaches. The most challenging seems to discriminate between individually-specific splicing variances and changes resulting from disease. Therefore, apart from employing tools for global analysis at transcriptomic and proteomic level, studies on large numbers of samples from patients are needed.

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