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Transcriptional and post-transcriptional regulation of the adaptor/scaffold protein gene *ITSN1*

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ITSN1 adaptor/scaffold protein takes part in a variety of physiological and pathological cellular processes. It has a complex expression regulation and many protein partners. **Aim.** Characterization of the *ITSN1* functioning and expression control is important for understanding its role in cell. **Methods.** Bioinformatic analysis, semi-quantitative expression analysis by RT-PCR, immunoprecipitation. **Results.** We have described and analyzed the *ITSN1* promoter regions, detected *ITSN1* alternatively spliced isoforms at mRNA and protein levels in different cancer specimens. Using different bioinformatic servers, we have identified the sites for miRNA binding and analyzed the sites for serine, threonine and tyrosine phosphorylation of the *ITSN1* protein. **Conclusions.** We have obtained new data on the *ITSN1* expression in pathology. We have also shown the possibility of *ITSN1* expression regulation by miRNA and phosphorylation of serine, threonine and tyrosine.

**Keywords:** *ITSN1*, bidirectional promoter, alternative splicing, miRs, phosphorylation.

**Introduction**

*ITSN1* is a multifunctional scaffold protein that is implicated in many cellular processes such as clathrin- and caveolin-mediated endocytosis, exocytosis, MAP kinase signaling, actin cytoskeleton rearrangements, membrane remodeling, etc. The *ITSN1* gene is localized at chromosome 21. It was shown that *ITSN1* is involved in such neurodegenerative pathologies as the Down’s syndrome, Alzheimer’s and Huntington’s diseases [1–3]. *ITSN1* takes part in migration and invasion of human glioma cells and is localized in invadopodia, actin-rich protrusions that drive invasion in cancer cells [4, 5]. Three main *ITSN1* isoforms are produced in human: long isoform *ITSN1*-L expressed mostly in nervous system, short isoform *ITSN1*-S and *ITSN1*-22a isoform which are ubiquitously expressed. *ITSN1* is an adaptor protein with unique multidomain structure. *ITSN1*-L consists of two N-terminal EH domains (EH1 and EH2), coiled-coil region (CCR), five Src homology 3 domains (SH3A-E), Dbl homology (DH), pleckstrin homology (PH), and C2 domain. EH domains, CCR and SH3 domains interact with specific protein motifs: EH domains bind NPF motifs; α-helix regions form dimers; SH3 domains bind proline-rich motifs (PxxP). DH domain is a guanine nucleotide exchange factor which activates a small GTPase Cdc42 from the Rho family. The main function of PH domain is considered to be the regulation of the DH domain and membrane localization of the protein via phosphoinositides binding [6]. However, the *ITSN1* PH domain binds phosphoinositides but it neither influences guanine nucleotide exchange by...
DH nor, neither determines the subcellular localization of the protein [6, 7]. The C2 domain is thought to take part in Ca²⁺-dependent phospholipid binding [8].

Due to the multiple alternative splicing events all other ITSN1 isoforms lack some domains or several amino acid residues. For instance, the short isoform ITSN1-S lacks the DH, PH and C2 domains and therefore does not have any enzymatic activity.

The majority of ITSN1 functions is performed by binding to its protein partners. At the moment, more than thirty ITSN1 partners are discovered, and this list is permanently increasing as well as the list of ITSN1-related processes. Regarding the complexity of the ITSN1 interactome, the regulation of ITSN1 gene expression and functioning is quite sophisticated and is fulfilled with the variety of mechanisms both at transcriptional and post-transcriptional levels [9, 10].

Materials and Methods

Bioinformatical analysis

For the Bioinformatical analysis we used GeneBank (http://www.ncbi.nlm.nih.gov/) and PhosphoSitePlus (http://www.phosphosite.org/homeAction.action) databases and the following servers and online utilities: UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly (http://genome.ucsc.edu/cgi-bin/hgTracks?db=hg38&lastVirtModeType=default&lastVirtModeExtraState=&virtModeType=default&virtMode=0&nonVirtPosition=&position=chr21%3A33641275-33643785&hgsid=497171239_NC3k6OaFOrwOdeBEWs9RxAJn2qj8), RepeatMasker (http://www.repeatmasker.org/), TargetScan (http://www.targetscan.org/vert_71/), MicroRNA.org (http://www.microrna.org/microrna/getGeneForm.do), RegRNA 2.0 (http://regrna2.mbc.nctu.edu.tw/index.html).

RNA isolation and RT-PCR

Total RNA from human tissues was isolated as described previously [11]. cDNA was synthesized from 1 to 5 μg of total RNA using oligo(dT₁₈) primer and RevertAid H Minus Reverse Transcriptase («Thermo Scientific», USA). Five percent of the cDNA obtained was used as a template for PCR as described previously [11]. The following oligonucleotides were used for the expression analysis of transcripts generated from an alternative promoter:

20 exon frw 2566-agtcaggttaaaggggaatgg-2587;
23 exon rev 3166-aactgagtttgagactctctatg-3184;
23 exon frw 3112-tggtggttttgagacttcaagg-3135;
22 exon frw 2583-gaagaagttgaacacaggg-2606;
22a exon rev 2891-aactgagtttgagacttcaagg-2868;
27 exon rev 3530-tggtggttttgagacttcaagg-3509;
33 exon frw 4160-gsgetagctgcagagagatg-4182;
34 exon frw 4394-tgaagctgtaagaggttaaag-4416;
39 exon rev 5122-tggtggttttgagacttcaagg-5100;
41 exon rev 5307-atccctgcagcctaaatg-5285;
frw GAPDH 5'-tggtggttttgagacttcaagg-3';
rev GAPDH 5'-catgtgggccatggtcaccaccc-3';
frw β-actin 5'-gaactgctgctgaatgctg-3';
rev β-actin 5'-gcgtgctgctgctgctg-3'.

Nucleotide positions for oligonucleotides are based on the ITSN1-L, ITSN1-S and ITSN1-22a cDNAs (GenBank accession numbers NM_003024.2, NM_001001132.1 and DQ386455).

Cell culture, transfection and immunoprecipitation

MDA-MB-231 cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10 % fetal bovine serum, 50 U/ml penicillin and 100 μg/ml streptomycin. The cells were transiently transfected with PEI (polyethyleneimine) according to the manufacturer’s instructions and processed 24 h after transfection. For immunoprecipitation (IP), the cells were lysed in IP buffer (20mM Tris-HCl pH 7.4, 150mM NaCl, 0.5 % Nonidet P40, 10 % glycerol, 2 mM EDTA and protease inhibitor cocktail). The lysates were incubated with antibodies and protein A/G PLUS-Agarose («Santa Cruz Biotechnology», USA) prewashed in IP buffer. After incubation for 3 h at +4 °C, the beads were washed three times with IP buffer. Bound proteins were eluted by boiling in Laemmli sample buffer and then analyzed by SDS-PAGE and Western blotting.
Antibodies

The mouse polyclonal antibodies against the DH-PH domains of human ITSN1 (anti-ITSN1-DH) were produced in mouse immunized with recombinant His-tagged protein comprising amino acid residues 1240-1575 of human ITSN1-L. The rabbit polyclonal antibodies against the EH2 domain of human ITSN1 (anti-ITSN1-EH2) were described previously [12]. The secondary horseradish peroxidase-labeled anti-rabbit antibodies were purchased from Promega.

Results and Discussion

Promoter regions of human ITSN1 gene

The ITSN1 gene has 246826 bp (NG_029504.1) and is localized on chromosome 21 locus q22.1-q22.2. The gene is comprised of 42 exons which influence the amino acid composition of the protein molecule. The expression regulation of ITSN1 at the transcriptional level is performed by transcription from two different promoters and the generation of a vast majority of alternatively spliced mRNA isoforms. The main ITSN1 promoter, which gives rise to the most mRNAs, is a bidirectional one. These promoters are present in head-to-head class of genes whose TSS (Transcription Start Site) are located at the distance from 0 to 2000 bp. About 10% of protein-coding genes are bidirectional in mammals. Furthermore, it was observed that many genes that are transcribed from bidirectional promoters possess functions or are involved in the same processes such as DNA reparation, chromatin stability etc. [13]. Recently, Yang et al. have explored the biological implication of the control of genome stability by bidirectional promoters and showed a high enrichment of bidirectional promoters in genes strongly associated with breast and ovarian cancer [14]. Trinklein et al. analyzed TSS of 23752 human genes and revealed that TSS of 1352 gene pairs (11%) were localized less than 1 kb from each other. Besides, the 5’-ends of 315 pairs (23%) were partially overlapped, whereas in the majority of bidirectional promoters (67%) which did not overlap the distance between TSS was less than 300 bp. After the functional analysis in different cell lines it was shown that 90% of the analyzed promoters were active in both directions, and only 10% were active in only one direction. Most bidirectional promoters that were analyzed in the study of Trinklein et al. are CpG-rich, 77% of them have CpG islands and only 8% have TATA-sequence compared to 38% and 28% of unidirectional promoters respectively [15]. Bidirectional promoters containing TATA boxes consistently show an asymmetrical distribution favoring one TSS over the other [16]. The presence of DPE, Inr and BRE elements does not significantly differ in bidirectional and unidirectional promoters [15–18]. According to another study, the CpG islands were found to be more prevalent in bidirectional (90%) promoters as opposed to unidirectional ones (45%). Moreover, it was shown that CpG islands of bidirectional promoters differ from those of unidirectional promoters and are more functional [16]. Neuron-specific genes are poor in bidirectional promoters, however, specific bidirectional promoters were found for nervous tissue (NBiPs). Novel bidirectional promoters showing divergent transcription of the novel and potentially brain-specific lncRNAs (long non-coding RNAs), are highly enriched in neuronal genes [19]. Previously, several authors have shown that bidirectional promoters are the major source of gene activation-associated non-coding RNAs in different cells in mammals [20].

The distance between the TSS of ITSN1 and CRYZL1, which are annotated in GeneBank, is 623 bp (Fig. 1). Thus, these genes are in the head-to-head position at the distance less than 2000 bp that meet the requirements of the bidirectional promoter. Trinklein et al. in their work [15] also defined the promoter of the gene pair ITSN1/CRYZL1 as a bidirectional, but the DNA fragment they explored corresponds to the incomplete promoter region of ITSN1/CRYZL1, and does not cover the part of CRYZL1 gene. The explored fragment was 201 bp long and located 10 bp to the annotated TSS of ITSN1 gene (Fig. 1) [15].

The ITSN1/CRYZL1 promoter is GC-rich and has no TATA-boxes. Using the UCSC Genome Browser
on Human Dec. 2013 (GRCh38/hg38) Assembly we have identified a CpG island which comprises 196 bp of the exon 1 and 773 bp of the intron 1 of ITSN1 gene. CpG-island association is characteristic for bi-directional promoters. CRYZL1 also has a CpG-island which comprises exon 1 and a part of intron 1 of CRYZL1. The characteristics of the above-mentioned CpG-islands are specified in Table 1. ITSN1/CRYZL1 promoter analysis by RepeatMasker server has shown only one low complexity repeat of the G-rich subclass that comprises 71 bp before the annotated ITSN1 TSS.

Despite all these data the statement about the bidirectionality of the ITSN1/CRYZL1 promoter requires further experimental confirmation.

The ITSN1-S isoform is expressed in all tissues. No significant difference was detected in different tissues or pathological states. The CRYZL1 gene is poorly investigated and there is no data about its expression at the moment. However, if the bidirectionality of the ITSN1/CRYZL1 promoter is confirmed, we may suppose that the CRYZL1 product is also ubiquitously expressed and is implicated in clathrin-mediated endocytosis, cell signaling etc., as the genes transcribed from bidirectional promoters are often involved in the same cellular processes.

Besides the main ITSN1 promoter, one more alternative promoter is identified in intron 5. The nucleotide sequence, which promoter activity was investigated, is GC-rich. It has several consensuses that resemble TATA-box but all of them are non-functional which is confirmed by the luciferase test with the deletion mutants of this region as well as by the presence of three TSS found by the 5'-RACE [21], that does not correlate with the presence of the functional TATA-box [22]. The expression from the alternative promoter was detected in most analyzed tissues, however, at the much lower level comparing to the main promoter [21]. The analysis of this region by the RepeatMasker server revealed LINE1-repeat of the L1MC5 subclass, that is almost entirely located at the additional 5'-sequence before the exon 6 which is crucial for the promoter activity and three SINE/Alu repeats (Table 2).

Table 1. CpG islands of ITSN1/CRYZL1 promoter

<table>
<thead>
<tr>
<th>Gene</th>
<th>CRYZL1</th>
<th>ITSN1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position*</td>
<td>33641571-33641951</td>
<td>33936694-33937662</td>
</tr>
<tr>
<td>Band</td>
<td>21q22.11</td>
<td>21q22.11</td>
</tr>
<tr>
<td>Size CpG Island</td>
<td>381</td>
<td>969</td>
</tr>
<tr>
<td>CpG count</td>
<td>33</td>
<td>105</td>
</tr>
<tr>
<td>C count plus G count</td>
<td>246</td>
<td>624</td>
</tr>
<tr>
<td>Percentage CpG</td>
<td>17.3 %</td>
<td>21.7 %</td>
</tr>
<tr>
<td>Percentage C or G</td>
<td>64.6 %</td>
<td>64.4 %</td>
</tr>
<tr>
<td>Ratio of observed to expected CpG</td>
<td>0.85</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* - the positions are according to the UCSC Genome Browser on Human Dec. 2013 (GRCh38/hg38) Assembly site.

The ITSN1 gene is highly conservative. The bioinformatics analysis of the ITSN1 intron 5 of seven organisms (Macaca mulatta, Pan troglodytes, Bos taurus, Canis lupus familiaris, Rattus norvegicus, Mus musculus, Gallus gallus) for the repeat presence by the RepeatMasker server has shown that all of them have LINE1 repeat at the end of the intron 5. Moreover, only in chicken this repeat belongs to a CR1-H subclass repeat whereas in other organisms the repeats belong to L1MC5 was present in all others.
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Alternative splicing of the ITSN1 mRNA

According to the recent data, human genome comprises about 25 thousand genes [23], which encode several hundred thousands of protein molecules with the specific function. This is implemented by many mechanisms the main of which are alternative splicing and alternative transcription. Alternative splicing is the process by which different pairs of splice sites are selected to produce multiple transcripts from a single gene. It is controlled by the concerted action of multiple cis-acting motifs and cognate trans-acting factors that promote or repress the assembly of productive splicing complexes (spliceosomes) at splice sites [24].

The EST analysis together with other bioinformatics computational methods revealed that minimum 70% of human genes have at least one alternatively spliced mRNA, and this proportion tends to increase [25].

ITSN1 gene has three main isoforms produced by alternative splicing with the attachment of three alternative 3’UTR (Fig. 2). Thus, as a result of the sequential connection of 41 exons (excluding the exon 30) the long isoform is produced which has a role in the specialized neuronal cell function and is expressed at high levels in these cells [26, 27, 28]. Its protein product comprises all eleven domains. As a result of the exon 30 splicing, a short isoform is formed, which is ubiquitously expressed and lacks three C-terminal domains (DH, PH, and C2).

Recently described ITSN1-22a isoform is produced by the exon 22a splicing. It is expressed in all analyzed tissues. ITSN1-22a is the shortest isoform as it lacks seven C-terminal domains but includes 116 aa of disordered sequence instead. This sequence is responsible for the AMPH1 SH3 domain binding, allows formation of homodimers and dimers with ITSN1-S, and regulates ITSN1 interaction with the ubiquitin ligase CBL [29].

The ITSN1 expression from the above-mentioned alternative promoter leads to the formation of the isoforms lacking the first EH domain. However, the existence of these isoforms at the protein level has not been proved yet.

Besides the alternative splicing of the 5’- and 3’-ends, five in-frame splicing events are described for ITSN1, which change the amino acid composition of the protein (Fig. 2). The exon 20 splicing, which adds 15 bp coding 5 aa (VKGEW) in SH3A domain, is neuron-specific, and is controlled by nSR100/SRRM4 (vertebrate- and neural-specific Ser/Arg repeat-related protein of 100 kDa) splicing regulator [30]. The exon 20 splicing is the most studied from these ITSN1 in-frame splicing events. It is known that the amount of its transcripts is gradually increased during embryonic development [31]. It is also shown that in mouse neurons both (+20 exon) and (–20 exon) isoforms are expressed. It was demonstrated that inclusion of five amino acids increases N-SRC loop of the SH3A domain, which in turn regulates the interaction strength of SH3A with DNM1, CdgAP, SOS1 and c-CBL. Previously, the human glioblastoma cell line U-251 MG has been tested by RT-PCR for the presence of the exon 20 transcripts, but only isoforms lacking the exon 20 were detected [32]. Its expression in other cell lines and tumors was not investigated. We decided to check the presence of these isoforms in cancer tissues of different types. Interestingly, we have revealed that the amount of the exon 20-containing

<table>
<thead>
<tr>
<th>№</th>
<th>Position at the intron 5*</th>
<th>DNA strand</th>
<th>Repeat name</th>
<th>Repeat family</th>
<th>Position at the repeat</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>13354 13686</td>
<td>+</td>
<td>AluJo</td>
<td>SINE/Alu</td>
<td>1 305</td>
</tr>
<tr>
<td>2</td>
<td>13778 14084</td>
<td>–</td>
<td>AluJr4</td>
<td>SINE/Alu</td>
<td>2 306</td>
</tr>
<tr>
<td>3</td>
<td>14157 14465</td>
<td>+</td>
<td>AluSx1</td>
<td>SINE/Alu</td>
<td>1 310</td>
</tr>
<tr>
<td>4</td>
<td>14484 14772</td>
<td>–</td>
<td>L1MC5</td>
<td>LINE/L1</td>
<td>7108 6843</td>
</tr>
</tbody>
</table>

* - position is represented according to the numeration from 1 to 14938 of the intron 5 nucleotides.
transcripts is decreased at different stages in the brain cancer specimen which could be explained by the decrease of neuron number in tumors. We have not detected the $ITSN1$ exon 20 containing isoforms either in renal or in ovarian tissues (Fig. 3 A-I, B).

Four more in-frame alternative splicing events described for $ITSN1$ are the following (Fig. 2):
1. 5'-truncation of exon 6 which leads to the 37 aa shortening of the spacer between EH1 and EH2;
2. excision of the exons 25 and 26 which leads to the loss of the SH3C domain;
3. excision of exon 35 ($ITSN1-L∆35$) which results in 25 aa truncation of the DH domain and shortening of the linker between DH and PH domains;
4. excision of exon 36 which leads to the PH domain loss.

The functional consequences of these alternative splicing events are currently unknown. We may suppose that the shortening of EH1-EH2 spacer and the SH3C domain loss lead to the conformational changes which serve to regulate the interaction of EH and SH3 domains with their partners; whereas the excision of the exons 35 and 36 most likely regulate the guanine-nucleotide exchange activity of the DH domain. The combination of all the above-mentioned alternative splicing events seems to be sporadic. Thus, for the isoform $ITSN1-S$ all the reading frame combinations were cloned. For the $ITSN1-L$ and alternative promoter isoforms only a few variants were obtained by cloning but this can be explained by cloning difficulties due to the big size of the products and/or their low expression. The expression of different transcripts significantly varies. For instance, the transcripts with the exon 6 truncation and the exon 36 excision make 3–5 % of total transcripts, whereas the transcripts with the exons 25–26 or the exon 35 excision make 20 % and 10 % respectively [11].

The expression analysis of $ITSN1-L$ and $ITSN1-L∆35$ isoform in different cancer types has shown the reduced transcripts number in brain tumors which is consistent with the results for exon 20 and can be explained by the same reasons (Fig. 3 A-II, C). Interestingly, these isoforms, previously considered as ‘neuron-specific’, were also detected in the non-neuronal tumors. Thus, the $ITSN1-L$ and $ITSN1-L∆35$ isoform transcripts were found in pheochromocytome, breast and prostate cancer samples (Fig. 4 A-I). Moreover, we have observed that in several samples of various glioblastoma stages, as well as pheochromocytome, breast and prostate cancers the ratio between $ITSN1-L$ and $ITSN1-L∆35$ transcripts was changed with the preference for the latter (Fig. 3 D, Fig. 4 C, F, I). However, there were no significant changes in isoform 22a and transcripts with or without the exons 25–26 exons expression (Fig. 3 A-III, IV). Thus, we can assume that cancer interferes with the alternative splicing mechanisms, which in particular lead to the changes in $ITSN1$ isoforms ratio.
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Previously we have demonstrated the expression of ITSN1-L in the cancer cell lines MDA-MB-231 and MCF-7 [5, 33]. Here we have shown by immunoprecipitation that ITSN1-L was expressed at the protein level in MDA-MB-231, a cancer cell line with invasive phenotype. We have used specific antibodies to the ITSN1 DH domain which bind only ITSN1-L isoform. ITSN1-L was detected with anti-ITSN1-EH antibodies in the precipitates obtained with anti-ITSN1-DH antibodies from the lysate of MDA-MB-231 cells (Fig. 5).

Besides the above-mentioned alternative splicing events which lead to the amino acid changes in ITSN1 composition, a variety of alternative splicing events causing the frame shift with the early stop-codon appearance were described. Such transcripts are most likely degraded via nonsense-mediated mRNA decay (NMD) [11, 31].

To date there is no evidence on regulation of ITSN1 at mRNA level by changes in RNA transcription from promoters in various tissues and under various physiological states. Considering the previous data and our results we can suppose that the regulation of ITSN1 alternative isoforms quantity is probably performed via different splicing factors together with the mRNA stability control.

Fig. 3. Analysis of ITSN1 isoforms expression: A: I – isoforms ITSN1 containing the exon 20 using primers specific for the exons 20 (forward 2566-2587) and 23 (reverse 3184-3166); II – ITSN1-L and ITSN1-L∆35 using primers specific for the exons 34 (forward 4394-4416) and 39 (reverse 5100-5122); III – isoforms ITSN1 with or without the exons 25-26 using primers specific for the exons 23 (forward 3112-3135) and 27 (reverse 3509-3530); IV – containing the exon 22a using primers specific for the exons 22 (forward 2583-2606) and 22a (reverse 2868-2891); V – analysis of expression of control gene β-actin. B: relative expression level of isoforms containing the exon 20; C: relative expression level of ITSN1-L and ITSN1-L∆35 isoforms; D: portion of ITSN1-L∆35 from total ITSN1-L isoforms expression. Densitometric measurements were performed using ImageJ software. NB – normal brain, GB – glioblastoma, A – astrocytoma, RT – renal tumor, NK – normal kidney, OV – ovary tumor.
Despite the ITSN1 importance for endocytic process there is a limited information about its regulation at the post-transcriptional level by microRNAs (miRs). MicroRNAs are small non-coding RNA molecules 18–25 nt long that regulate post-transcriptionally more than 1/3 of known genes [34]. In animals and

**Fig. 4.** Analysis of expression of ITSN1-L and ITSN1-L∆35 isoforms in breast cancer, prostate cancer and pheochromocytoma: A, D, G: I – ITSN1-L and ITSN1-L∆35 using primers specific for the exons 33 (forward 4160-4182) and 41 (reverse 5307-5285); II – analysis of expression of control gene GAPDH – 983 bp or β-actin – 500 bp. B, E, H: relative expression level of ITSN1-L and ITSN1-L∆35 isoforms; C, F, I: portion of ITSN1-L∆35 from total ITSN1-L isoforms expression. Densitometric measurements were performed using ImageJ software. NT – normal tumor, T – tumor.

**Prediction of microRNA target sites in 3′ UTR of ITSN1**

Despite the ITSN1 importance for endocytic process there is a limited information about its regulation at
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Humans mature miRs bind to the complementary sites in 3′-untranslated regions (3′UTRs) of their target mRNAs by 6–8 nt “seed” region on miRs 5′-end thus negatively regulating gene expression [35]. Such mechanism is considered as main miR action. However, there is growing evidence of the positive regulation of gene expression as a result of miRs binding to the 5′UTRs or even protein coding regions of target mRNAs [36].

As described above, human ITSN1 has three main isoforms with alternative 3′UTRs — ITSN1-L, ITSN1-S and ITSN1-22a, with 3′UTRs length of 11566 nt, 1510 nt и 71 nt, respectively. [11, 29]. Despite the ITSN1 importance for endocytic process there is limited information about its regulation on post-transcriptional level by microRNAs. Currently, only one clear evidence of the ITSN1 post-transcriptional regulation expression is known: Lin et al showed negative changes in the ITSN1-S expression by miR-194 [37]. According to the comparatively long 3′UTRs of main ITSN1 isoforms and their involvement into several cellular processes it would have been intriguing if ITSN1 contains any other potential microRNA sites. To study this we used bioinformatical prediction methods as a fast way to analyze 3′UTR and narrow our search. TargetScan service is based on the search of target sites, which match the seed region of miRNAs, according to the site context and conservation, and known as one of the most accurate predictive servers [38, 39]. Thus, we used TargetScan v7.0 (August 2015 release) [39] as the main service for microRNA sites prediction in 3′UTRs of ITSN1 isoforms. We took into account only highly conservative microRNA target sites based on the prediction for several species including human, mouse, rat, chicken, and tropical frog.

The computational prediction of target sites in 3′UTR of ITSN1-S mRNA (GenBank ID NM_001001132, Ensembl ID ENST00000399352.1) revealed the unique conservative sites for more than 20 different microRNA families (Table 3). Among them, miR-19 family of microRNAs predicted to interact with two sites in 3′UTR of ITSN1-S located at 304–311 nt and 752–759 nt downstream from the start of 3′UTR, respectively; miR-181/4262 family microRNAs could bind two different sites located at 1013–1019 nt and 1405–1412 nt downstream from the start of 3′UTR. The only confirmed site for miR-194 is predicted as well. To refine our search we used MicroRNA.org, which is another web-service for the microRNA target sites prediction [40, 41]. MicroRNA.org confirmed most of the sites predicted by TargetScan (Table 3). MicroRNA sites predicted by MicroRNA.org but not identified by TargetScan may have less conservation thereby classified by TargetScan as less conservative and are excluded from our initial search. The obtained results indicate the factual existence of sites predicted above by TargetScan although further experimental verification is still needed.

On the contrary to ITSN1-S with 3′UTR of 1510 nt and 19 predicted different sites for more than 20 miR families, the brain-specific ITSN1-L isoform is characterized by an extremely long 3′UTR of 11561 nt [11] with only few predicted microRNA target sites. Bioinformatical analysis by TargetScan v7.0 identified only five conserved sites for microRNAs including site for the miR-30 family, which is the only common site for both main ITSN1 isoforms (Table 4). MicroRNA.org did not find any of the above-mentioned microRNAs while some other sites were identified. This could be explained by using ITSN1-L iso-

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**Fig. 5.** Endogenous ITSN1-L expression in invasive cancer cells. Lysates of MDA-MB-231 cells were subjected to immunoprecipitation using anti-ITSN1-DH antibodies and mouse IgG as a control. The precipitated proteins were eluted with Laemmli sample buffer and analyzed by Western blotting (WB). ITSN1-L was detected using anti-ITSN1-EH antibodies.
Table 3. MicroRNA target sites predicted for *ITSN1-S* (NM_001001132; ENST00000399352.1) by TargetScan v.7.0 and MicroRNA.org

<table>
<thead>
<tr>
<th>MicroRNA Target Sites</th>
<th>Position of target sites in 3'UTR <em>ITSN1-S</em> according to TargetScan v. 7.0§</th>
<th>Position of target site in 3'UTR <em>ITSN1-S</em> according to MicroRNA.org§</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-193a-5p</td>
<td>46–52; 411–418</td>
<td>–</td>
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<tr>
<td>miR-190a-5p/190b</td>
<td>59–65</td>
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<td>miR-34ac-5p/449a/449b-5p</td>
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<tr>
<td>miR-224-5p</td>
<td>104–110***</td>
<td>106–113; 382–388</td>
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<td>miR-202-3p</td>
<td>164–170****</td>
<td>164–170</td>
</tr>
<tr>
<td>miR-371-5p</td>
<td>199–205***</td>
<td>199–205</td>
</tr>
<tr>
<td>miR-10ab-5p</td>
<td>246–253</td>
<td>246–253</td>
</tr>
<tr>
<td>miR-19ab-3p</td>
<td>304–311; 752–759</td>
<td>304–311; 752–759</td>
</tr>
<tr>
<td>miR-590-3p</td>
<td>230–236; 329–336****</td>
<td>329–336</td>
</tr>
<tr>
<td>miR-320a-d</td>
<td>–</td>
<td>355–361</td>
</tr>
<tr>
<td>miR-103a-3p/107</td>
<td>429–436</td>
<td>428–435</td>
</tr>
<tr>
<td>miR-26ab/1297</td>
<td>–</td>
<td>477–483</td>
</tr>
<tr>
<td>miR-141-3p/200a-3p</td>
<td>502–509</td>
<td>502–508</td>
</tr>
<tr>
<td>miR-487b</td>
<td>717–723***</td>
<td>716–724</td>
</tr>
<tr>
<td>miR-194-5p</td>
<td>937–944</td>
<td>937–944</td>
</tr>
<tr>
<td>miR-375</td>
<td>–</td>
<td>987–993</td>
</tr>
<tr>
<td>miR-181a-d-5p/4262</td>
<td>1013–1019; 1405–1412</td>
<td>1013–1019; 1404–1412</td>
</tr>
<tr>
<td>miR-92ab</td>
<td>–</td>
<td>1032–1038</td>
</tr>
<tr>
<td>miR-534</td>
<td>–</td>
<td>1046–1052</td>
</tr>
<tr>
<td>miR-30a-e-5p</td>
<td>1048–1055</td>
<td>1046–1054</td>
</tr>
<tr>
<td>miR-128</td>
<td>–</td>
<td>1069–1074</td>
</tr>
<tr>
<td>miR-140-3p.1</td>
<td>1069–1075</td>
<td>–</td>
</tr>
<tr>
<td>miR-136</td>
<td>1096–1102**</td>
<td>1095–1103</td>
</tr>
<tr>
<td>miR-214</td>
<td>1189–1195****</td>
<td>1187–1196</td>
</tr>
<tr>
<td>miR-15ab/16/195/424/427</td>
<td>1191–1197*</td>
<td>1191–1198</td>
</tr>
<tr>
<td>miR-218-5p</td>
<td>1230–1237</td>
<td>–</td>
</tr>
<tr>
<td>miR-488</td>
<td>316–322; 1335–1341***</td>
<td>1335–1342</td>
</tr>
<tr>
<td>miR-130ab/301ab/454</td>
<td>1380–1386*</td>
<td>1382–1389</td>
</tr>
<tr>
<td>miR-200bc-3p/429</td>
<td>1388–1394</td>
<td>1388–1395</td>
</tr>
<tr>
<td>miR-101-3p.2</td>
<td>1447–1453</td>
<td>–</td>
</tr>
</tbody>
</table>

The sites with high conservation predicted by both servers are bolded. Conservation was indicated according to TargetScan v.7.0. For sites predicted by MicroRNA.org levels of conservation were not mentioned. Positions for sites complementary to seed regions of respective microRNAs are indicated. § – positions of predicted binding sites are showed according to nucleotide numbering from the 3'UTR first nucleotide of *ITSN1-S* from GenBank database, * – sites for poorly conserved miRNA families broadly conserved among vertebrates, ** – sites for conserved miRNA families conserved among mammals, *** – sites for poorly conserved miRNA families conserved among mammals, **** – sites for poorly conserved miRNA families.
Transcriptional and post-transcriptional regulation of the adaptor/scaffold protein gene ITSN1

### Table 4. MicroRNA target sites predicted for ITSN1-L (NM_003024; ENST00000381318.3) by TargetScan v.7.0 and MicroRNA.org

<table>
<thead>
<tr>
<th>MicroRNA Target Sites</th>
<th>Position of target sites in 3’UTR ITSN1-L according to TargetScan v. 7.0</th>
<th>Position of target site in 3’UTR ITSN1-L according to MicroRNA.org</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR-125a-5p/125-b</td>
<td>–</td>
<td>17–24</td>
</tr>
<tr>
<td>miR-134</td>
<td>99–106***</td>
<td>100–107</td>
</tr>
<tr>
<td>miR-28-5p/708</td>
<td>139–146***</td>
<td>139–146</td>
</tr>
<tr>
<td>miR-384</td>
<td>144–150; 6744–6750***</td>
<td>145–152</td>
</tr>
<tr>
<td>miR-219-5p</td>
<td>159–165*</td>
<td>161–168</td>
</tr>
<tr>
<td>miR-876-5p</td>
<td>259–265***</td>
<td>261–268</td>
</tr>
<tr>
<td>miR-340</td>
<td>345–351; 3697–3703***</td>
<td>345–351</td>
</tr>
<tr>
<td>miR-33ab</td>
<td>351–357; 4998–5004*</td>
<td>351–357</td>
</tr>
<tr>
<td>miR-25/32/92ab/363/367</td>
<td>3779–3785*</td>
<td>353–359</td>
</tr>
<tr>
<td>miR-342-3p</td>
<td>451–457***</td>
<td>451–458</td>
</tr>
<tr>
<td>miR-185</td>
<td>502–508; 3338–3345***</td>
<td>504–511</td>
</tr>
<tr>
<td>miR-103/107</td>
<td>518–524*</td>
<td>517–525</td>
</tr>
<tr>
<td>miR-300/381</td>
<td>643–649; 2258–2264; 3502–3509; 4298–4303***</td>
<td>642–650</td>
</tr>
<tr>
<td>miR-199ab-5p</td>
<td>670–676; 784–790*</td>
<td>670–677</td>
</tr>
<tr>
<td>miR-615-3p</td>
<td>856–863***</td>
<td>856–863</td>
</tr>
<tr>
<td>miR-181a-d</td>
<td>915–921; 1514–1520; 4905–4911; 10967–10973*</td>
<td>914–922</td>
</tr>
<tr>
<td><strong>miR-203-3p.1</strong></td>
<td>944–951*; 1605–1612</td>
<td><strong>944–952</strong></td>
</tr>
<tr>
<td>miR-653</td>
<td>948–954; 10444–10450****</td>
<td>949–957</td>
</tr>
<tr>
<td>miR-135ab</td>
<td>979–985; 6011–6017*</td>
<td>976–986</td>
</tr>
<tr>
<td>miR-30-5p</td>
<td>1601–1607</td>
<td>–</td>
</tr>
<tr>
<td>miR-137</td>
<td>8527–8534</td>
<td>–</td>
</tr>
<tr>
<td>miR-216-5p</td>
<td>9417–9424</td>
<td>–</td>
</tr>
<tr>
<td>miR-182-5p</td>
<td>11118–11125</td>
<td>–</td>
</tr>
</tbody>
</table>

The sites with high conservation predicted by both servers are bolded. Conservation was indicated according to TargetScan v.7.0. For sites predicted by MicroRNA.org levels of conservation were not mentioned. Positions for sites complementary to seed regions of respective microRNAs are indicated. § – positions of predicted binding sites are showed according to nucleotide numbering from the 3’UTR first nucleotide of ITSN1-L from GenBank database, * – sites for poorly conserved miRNA families broadly conserved among vertebrates, ** – sites for conserved miRNA families conserved among mammals, *** – sites for poorly conserved miRNA families conserved among mammals, **** – sites for poorly conserved miRNA families.

### Table 5. Sites for regulatory elements in canonical and alternative 5’UTRs of ITSN1, predicted by RegRNA 2.0 web server

<table>
<thead>
<tr>
<th>Canonical 5’UTR</th>
<th>Alternative 5’UTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type of Regulatory Element</td>
<td>Position of predicted sites for regulatory elements in 5’UTR according to RegRNA 2.0</td>
</tr>
<tr>
<td>u1056_DISC2-RNA</td>
<td>5–33</td>
</tr>
</tbody>
</table>

§ – positions of predicted binding sites are shown according to the nucleotide numbering of canonical 5’UTR from GenBank database; * – positions of predicted binding sites are shown according to the nucleotide numbering of alternative 5’UTR.
form with incomplete 3’UTR of 1005 nt (GenBank ID NM_003024) instead of *ITSN1-L* with fully annotated 3’UTR (Ensembl ID ENST00000381318.3).

Table 6. Phosphorylation sites in human *ITSN1* that were identified experimentally

<table>
<thead>
<tr>
<th>Residue</th>
<th>Location</th>
<th>Tissue (Condition)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ser203</td>
<td>EH1-EH2 linker</td>
<td>HeLa cells [52], Breast and ovarian cancer [53], HMLER cells [54], Jurkat cells [55], K562 cells [45], Breast cancer cells [56], Muscle [57], A498 cells [58]</td>
</tr>
<tr>
<td>Ser313</td>
<td>EH2-CCR linker</td>
<td>HeLa cells [52], Non-small lung cancer cells [56], Liver [59], Jurkat cells [60]</td>
</tr>
<tr>
<td>Ser315</td>
<td>EH2-CCR linker</td>
<td>HeLa cells [52], Breast and ovarian cancer (Regulated by cold ischemia) [53], HMLER cells [54], Jurkat cells [60], Lung tumor [61], 293E cells [62], HeLa cells [45], 293 cells [63]</td>
</tr>
<tr>
<td>Ser318</td>
<td>EH2-CCR linker</td>
<td>Liver [64]</td>
</tr>
<tr>
<td>Ser324</td>
<td>EH2-CCR linker</td>
<td>Liver [64]</td>
</tr>
<tr>
<td>Thr349</td>
<td>EH2-CCR linker</td>
<td><em>In vitro</em> (Ca²⁺/calmodulin-dependent) [42]</td>
</tr>
<tr>
<td>Ser559</td>
<td>CCR</td>
<td>HeLa cells [52], Breast and ovarian cancer (Regulated by cold ischemia) [53], Non-small lung cancer cells [56] [55], 293 cells [65], HeLa cells [66]</td>
</tr>
<tr>
<td>Ser564</td>
<td>CCR</td>
<td>Liver [64]</td>
</tr>
<tr>
<td>Thr567</td>
<td>CCR</td>
<td><em>In vitro</em> (Ca²⁺/calmodulin-dependent) [42]</td>
</tr>
</tbody>
</table>

At the same time, TargetScan could not predict any site found by MicroRNA.org at the region of 1005 nt downstream from the start of *ITSN1-L* 3’UTR. Such
<table>
<thead>
<tr>
<th>Residue</th>
<th>Location</th>
<th>Tissue (Condition)</th>
</tr>
</thead>
</table>
| Ser904  | SH3A-SH3B linker | In vitro (Ca\(^{2+}\)/calmodulin-dependent) [42]  
HeLa cells (G1 and M-phase) [45]  
Liver [64]  
HeLa cells (Cytosolic) [71]  
HeLa cells [52]  
ovarian cancer [53]  
Non-small lung cancer cells, breast cancer cells [56]  
Muscle [57]  
A498 cells (LPA regulated) [58]  
Jurkat cells [60]  
Lung tumor [61]  
293 cells [65]  
HeLa cells [66]  
Jurkat cells [55]  
K562 cells [45]  
SKBr3 cells [69]  
293E cells [62]  
HeLa cells [67]  
Brain [72]  
293 cells (AT1aR transfection) [73]  
Liver [59]  
U2OS cells [74]  
HUES-7 cells [70]  
hESCs [75]  
293 cells [63]  
WM115 cells [76] |
| Tyr922  | SH3B     | 293 cells (AT1aR transfection) [73] |
| Ser970  | SH3B     | Luminal breast cancer [53] |
| Ser976  | SH3B-SH3C linker | HeLa [52]  
Breast and ovarian cancer (Regulated by cold ischemia) [53] |
| Thr977  | SH3B-SH3C linker | HeLa cells [52]  
Non-small lung cancer cells [61] |
| Ser978  | SH3B-SH3C linker | In vitro (Ca\(^{2+}\)/calmodulin-dependent) [42] |

<table>
<thead>
<tr>
<th>Residue</th>
<th>Location</th>
<th>Tissue (Condition)</th>
</tr>
</thead>
</table>
| Ser981  | SH3B-SH3C linker | Liver [64]  
HeLa cells [67] |
| Ser983  | SH3B-SH3C linker | Lung tumor [61] |
| Ser984  | SH3B-SH3C linker | HeLa cells [52] |
| Ser986  | SH3B-SH3C linker | HeLa cells (M-phase) [45]  
HeLa cells [52]  
Breast and ovarian cancer (Regulated by cold ischemia) [53]  
HMMLER cells [54]  
Non-small lung cancer cells, breast cancer cells [56]  
HeLa cells [67]  
293 cells [63]  
K562 cells [45] |
| Ser989  | SH3B-SH3C linker | HeLa cells [52]  
Luminal breast cancer [53]  
Non-small lung cancer cells [56]  
HeLa cells [67] |
| Ser995  | SH3B-SH3C linker | Liver [64] |
| Ser1137 | SH3D     | HeLa cells [52]  
Breast and ovarian cancer [53]  
Jurkat cells [55]  
HeLa cells [45]  
Brain [72] |
| Thr1144 | SH3D-SH3E linker | Liver [64]  
HeLa cells [52]  
Luminal breast cancer [53]  
Jurkat cells [55] |
| Tyr1208 | SH3E     | HeLa cells [52] |
results suggest an insufficient conservation of sites predicted by MicroRNA.org combined with somewhat different algorithms of prediction utilized by TargetScan and MicroRNA.org, which can be critical for predicting less probable sites.

In addition to the canonical impact of microRNAs on their targets as gene expression inhibition after binding to 3'UTRs of target mRNA, there is growing evidence of identifying functional microRNA target sites in coding regions as well as 5'UTRs of certain mRNAs that could enhance translation of target genes [36]. To predict any putative target sites for microRNAs and other regulatory elements in 5'UTRs of human ITSN1 we used web server RegRNA 2.0 for finding different types of regulatory elements. 5'UTRs of ITSN1-S and ITSN1-L are formed by first and second exons and each has the length of 268 nt. Since 5'UTRs of ITSN1-S and ITSN1-L are identical, the obtained data are applicable to both ITSN1 isoforms. The computational analysis revealed two types of regulatory elements: the site for u1056_DISC2-RNA (sites for hybridization with different types of non-coding RNAs except microRNAs) and one site for the human microRNA hsa-miR-149-3p (without indicating the level of conservation for microRNA target site) (Table 5).

The ITSN1 isoforms transcribed from the alternative promoter have an alternative 400 nt 5'UTR [11]. To check if there are any different regulatory elements in this 5'UTR, we also used RegRNA 2.0, which predicted the sites for u3712_snoRNA and U1995_snoRNA, but did not find any microRNA target sites (Table 5).

In addition to the above-mentioned alternative 5'UTR, ITSN1 is known to have alternative splicing in the exons 1a and 1b leading to the insertions of 62 or 125 bp in canonical 5'UTR (187 bp in sum). The isoforms with these two exons and their combinations are expressed in the majority of analyzing tissues [11]. For 187 bp insertion of the exons 1a and 1b, the computational analysis by RegRNA 2.0 showed no differences compared to the prediction for canonical 5'UTR of ITSN1. This suggests that, according to RegRNA 2.0, 187 bp insertion added no new sites for the regulatory elements in intact 5'UTR sequence.

### Phosphorylation of ITSN1

Post-translational modifications are the common way of regulation or modulation of the protein functions. Such modifications in the scaffold proteins can facilitate or inhibit the interaction with particular proteins, providing a mechanism for the control on molecular complex composition.

Large-scale studies of proteomes from different human tissues revealed a number of the ITSN1 post-translational modifications, the vast majority of which is phosphorylation of serine or threonine residues (data from PhosphositePlus database). In addition, our group have recently identified 5 sites of the Ca²⁺/calmodulin-dependent serine/threonine phosphorylation within ITSN1 molecule [42]. However, either the functional roles of these phosphorylation events or the kinases, responsible for these modifications, have not been studied yet. The available data on phosphorylation of ITSN1 is summarized in Table 6.

It is known that the phosphorylation sites are predominantly located in the intrinsically disordered regions of proteins [43, 44]. As can be seen from the Table 6, in general, this is true for ITSN1 as well. However, the phosphorylated residues within the coiled-coil region, DH, PH and SH3 domains have also been detected. In addition, the difference in the nature of serine/threonine and tyrosine phosphorylation can be observed. Whereas the majority of serine/threonine phosphorylation sites are located within the interdomain linkers, all the identified phosphorylated tyrosines belong exclusively to the SH3 domains. These differences reflect a distinct distribution of these amino acid residues in ITSN1 molecule. As can be seen from the frequency plots, the serine/threonine residues are more abundant and tend to form serine/threonine-rich clusters in the interdomain linkers, which are especially prominent in the EH2-CCR, SH3A-SH3B and SH3B-SH3C linkers (Fig. 6, middle panel). In contrast, the tyrosine residues are mostly individual and located within the structural domains (Fig. 6, lower panel). These differences can reflect distinct functional roles of the tyrosine versus serine/threonine phosphorylation in the protein functions.
It is not clear whether the identified ITSN1 phosphorylation sites are phosphorylated permanently or in response to some conditions. We have shown that some kinases can phosphorylate ITSN1 in vitro in a Ca^{2+}/calmodulin-dependent manner, but it is currently not clear if this occurs in living cells as well [42]. Also, several sites were shown to be phosphorylated in HeLa cells in specific phases of the cell cycle, indicating their possible regulation by the cell cycle-dependent enzymes [45]. In another study, ITSN1 was shown to undergo the tyrosine phosphorylation in 293 cells in response to overexpression of tyrosine kinase Syk and Epstein-Barr virus protein LMP2A (Latent Membrane Protein 2A), but the sites of this phosphorylation were not identified [46]. On the other hand, the ITSN1 tyrosine phosphorylation in response to EGF stimulation in several human cell lines has not been detected, in contrast to its paralog ITSN2 [33].

It is predicted that not all of the phosphorylated sites identified in the large-scale proteomic studies are functional [47]. Therefore, since no functional role was reported for any of the identified ITSN1 phosphorylation sites, their functionality remains questionable. However, some possible outcomes from these phosphorylation events can be proposed. Several phosphorylation sites were identified within the coiled-coil region of ITSN1. It is known that the phosphorylation of threonine residues within coiled-coils can destabilize helices, while the phosphorylation of serine residues can both stabilize or destabilize them depending on the position [48, 49]. It can be expected that modifications in the coiled-coil regions can affect their ability to oligomerize or to form complexes with similar domains in other proteins. In support of this, it has been shown that the phosphorylation of coiled-coil region of the CRN2 protein affects its interaction with actin regulatory proteins, leading to the alterations of actin architecture and inhibition of the cell migration [50].

Most of the phosphorylation sites in the ITSN1 molecule are located in the interdomain linkers that are suggested to be intrinsically disordered. For today, the functions of ITSN1 interdomain linkers remain mostly unknown, although the linker between SH3A and SH3B domains was shown to interact with the α- and β-subunits of endocytic adapter protein AP2 [28]. Phosphorylation of the linkers indicates other potential functions of them. One possibility is

Fig. 6. Phosphorylation of human ITSN1. Upper panel – schematic representation of domain structure of ITSN1 molecule. Middle panel – distribution of serine/threonine residues in ITSN1 molecule. Each value of Ser/Thr frequency was calculated as frequency of serine/threonine per frame of 10 amino acid residues. Ser/Thr phosphorylation sites are marked by triangles. Lower panel – distribution of tyrosine (Tyr) residues in ITSN1 molecule (presented in the same way as serine/threonine distribution).
that the phosphorylation of interdomain linkers can modify their structure and alter mutual orientation of ITSN1 domains, thereby affecting a higher order structure of the molecular complexes formed by ITSN1. Alternatively, the phosphorylation of interdomain linkers can create new interfaces for the protein-protein interactions. In support of this, the ELM server predicts that the Ser978 phosphorylation creates a motif for interaction with the 14-3-3 proteins and the WW domains of Pin proteins. Intriguingly, two large clusters of serine/threonine residues directly surround the SH3B domain, thereby, it is likely that these regions could regulate the SH3B binding properties. This domain interacts with endocytic protein endophilin in a manner that is different from a canonical SH3 domain binding mode [51]. A potential role of phosphorylation in regulation of this and other interactions is a topic for further investigations.

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**REFERENCES**


Transcriptional and post-transcriptional regulation of the adaptor/scaffold protein gene ITSN1


Ми описали та проаналізували промоторні регіони ITSN1, детектували альтернативно сплайсовані ізоформи ITSN1 на рівні mРНК та білка в різних зразках раку. За допомогою різноманітних біоінформатичних серверів ми виявили сайти зв’язування з мікроРНК, а також ми проаналізували сайти серин-, треонин- та тирозин-фосфорилування білка ITSN1.

Висновки. Ми отримали нові дані про експресію ITSN1 при патологіях. Крім того, ми показали можливість регуляції експресії ITSN1 за допомогою мікроРНК та ролі фосфорилування серину, треонину і тирозину в регуляції взаємодії ITSN1 з білками партнерами.

Ключові слова: ITSN1, двонаправлений промотор, альтернативний сплайсинг, мікроРНК, фосфорилування.