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ORIGINAL ARTICLE

Evaluation of Arylsulfatase D (ARSD) and long noncoding RNA ARSD-AS1 gene expression in breast cancer patients and their association with oncogenic transcription factors

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Summary

Purpose: *Long non-coding RNAs (LncRNAs) are thought as tumorigenic factors in cancer progression. We investigated the clinical significance of arylsulfatase D (ARSD) and ARSD antisense in breast cancer patients.*

Methods: *Eighty breast cancer tumors were obtained from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital. The expression level of ARSD and ARSD-AS1 were examined in breast tumors in comparison to the margin of normal tissues using quantitative real-time PCR. Demographic information and the clinicopathologic characteristics including tumor grade, presence of cell receptors, lymph node and vascular invasion were also evaluated. Bioinformatics databases were used for identification of ARSD and ARSD-AS1 molecular targets and their association with cancer.*

Results: *Significant up-regulation of ARSD was observed in tumor tissues in comparison with its antisense ($p<0.05$). Both ARSD and ARSD-AS1 expression in tumor specimens were notably lower than those in adjacent normal tissue. High expression of ARSD was associated to lower tumor grade ($p<0.05$). Bioinformatics results revealed the interaction of ARSD with STS and SUMF1 proteins was attributed to the inhibiting of sulfates activity. Also, ARSD co-expressed genes were associated with oncogenic transcription factors, MAF and GATA. TP53 transcription factor site was identified as a target of ARSD-AS1 mRNA. The interaction of this antisense with microRNA (miR-618) could explain its participation in tumor cell proliferation.*

Conclusion: *Low expression of ARSD was associated with higher tumor grade. The evidence from this study enhance our understanding of ARSD and ARSD-AS1 function in cancer gene therapy. Accordingly, they could be introduced as great potential targets for breast cancer treatment.*

Key words: *ARSD, ARSD-AS1, long non-coding RNAs, breast cancer*

Introduction

Breast cancer is known as a heterogeneous disease representing a major health problem for women worldwide [1]. In Iran, its occurrence is more than 502,000 cases annually [2]. In the past two decades, several reports have been published addressing the association between variants in candidate genes and breast cancer risk [3]. Long non-coding RNA (LncRNA) has become a key instrument in cancer gene therapy through their regulatory activity in transcription and post-transcription pathways [4].

Recently, there has been an increasing interest in lncRNA-mediated cancer therapy as their tumorigenic function was attributed to the breast

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cancer occurrence through processes such as cell growth, metastasis and differentiation [5,6]. For instance, HOTAIR, SPRY4-IT1, and UCA1 lncRNAs participated in breast cancer cell proliferation and progression through invasion and metastasis processes [7-9]. Also, lncRNAs implicated in accurate regulation of protein-coding genes and maintained their amount in the cell [10]. Abnormal rate of sphingolipids was indicated in cancer tissues [11,12] and a key factor in their metabolism is arylsulfatase D (ARSD) gene [13].

ARSD gene (NM_001669) is a member of sulfates family that is essential in bone and cartilage matrix. This gene is located within a cluster of similar arylsulfatase genes on chromosome X. A related pseudogene has been identified in the pseudoautosomal region of chromosome Y [12]. The encoded protein is post-translationally glycosylated and localized to the lysosomes. This protein was introduced as a non-lysosome enzyme that is present in the endoplasmic reticulum and Golgi apparatus. It has been demonstrated that it participates in the post-translational changes of some proteins that possess a critical role in cancer progression [13].

The expression profile of ARSD gene is significantly reduced across breast tumor samples in comparison to paired normal tissues, according to the GEPIA database [14] (Figure 1A).

Previous research reported the up-regulation of ARSD in leukemia only, which was introduced as a new marker [13]. However, little is known about ARSD biological function and it is not clear what factors are affected on its regulation. There has been little debate on the regulatory role of its antisense. As antisense regulates the expression of sense element, we considered the expression of ARSD antisense which is affiliated with ARSD-AS1.

The ARSD-AS1 (NR_144459.1) is a non-coding RNA (816 bp) which is composed of two exons placed at the same chromosome. It is anticipated that the ARSD-AS1 can interfere with various cancers including cervix, ovary, colon, lung, and uterus according to the OncoLnc database (http://www.oncolnc.org/). Low expression level of ARSD-AS1 was also demonstrated in breast carcinoma in comparison to the normal tissue (Figure 1B) which has been significantly correlated with ARSD gene expression (Pearson R=0.1; P=9.2e-13) (Figure 1C).

This is the first study to undertake the expression of ARSD and its neighboring lncRNA gene in breast cancer patients. The purpose of this investigation was to explore the association between ARSD and ARSD-AS1 with clinicopathological factors in the context of breast cancer.

Methods

Clinical samples collection

Eighty tumor tissue samples and margin of normal tissues of breast cancer were collected from patients who underwent surgery without preoperative chemotherapy or radiotherapy between 2015 and 2016 from the Tumor Bank of Cancer Institute, Imam Khomeini Hospital, Tehran, Iran. According to the principles of tumor bank, signed informed consent was provided from the patients before surgery. The fresh tissue specimens were immediately frozen in liquid nitrogen and stored at -80°C for further experiments. The mean age of patients was about 48 years (range: 29-81). To evaluate the association between ARSD and its antisense expression with clinicopathological features, some characteristics

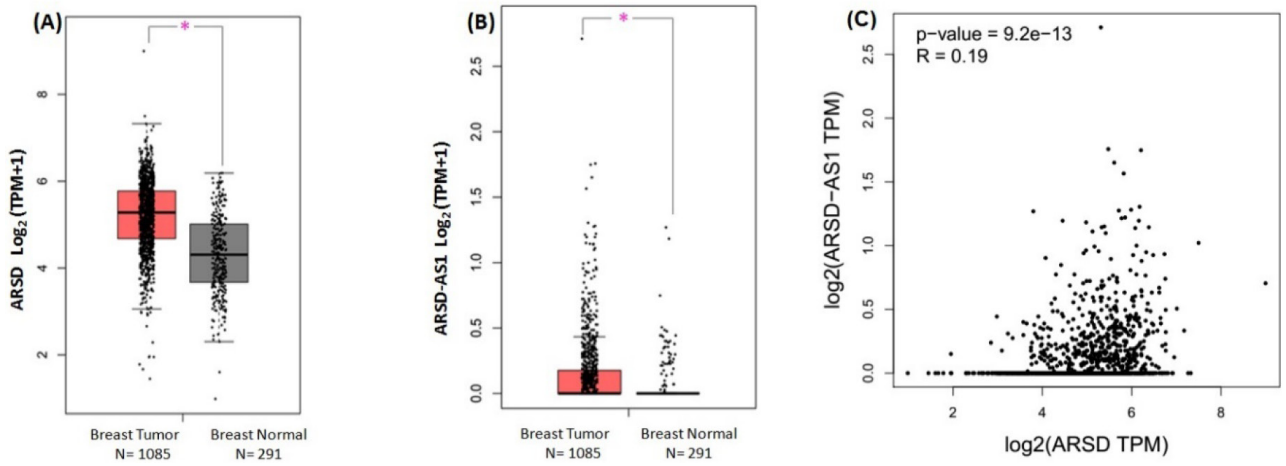

Figure 1. ARSD and ARSD-AS1 gene expression in breast cancer. **A:** ARSD expression is decreased in breast cancer in comparison to the normal tissue. **B:** The expression of ARSD-AS1 is down regulated in breast tumor tissue in comparison to normal tissue. **C:** Pearson correlation of ARSD and ARSD-AS1 gene expression profile in breast cancer. Data adopted from the Gene Expression Profiling Interactive Analysis (GEPIA) resource. TPM: Transcripts Per Million (*p<0.05).

such as cell surface receptors, tumor grade, lymph node metastasis and vascular invasion were considered. The samples were categorized into four groups according to their immunohistochemistry (IHC) classification: ER+/ PR+/ HER2+ group (triple positive; TP), ER/ PR/ HER2 group (triple negative; TN), ER+/ PR+/ HER2- group (PPN) and ER-/ PR-/ HER2+ group (NNP). This study was approved by the Research Ethics committee of the (no.ir.goums.rec 1396.274).

RNA isolation and cDNA synthesis

Total RNA was extracted from the frozen tissues using TRIZOL (Invitrogen, Life Technology, Carlsbud, USA) according to the manufacturer's instructions. The quantity and quality of the RNAs were assessed by PicoDrop (PicoDrop Technologies, Germany). The RNA integrity of samples was further checked by running RNA on 1.5% agarose gel. The 260/280 absorbance ratio of approximately 2.0 was considered as an optimum yield of RNA. All isolated RNA samples were then treated by DNase I enzyme. The complementary DNA (cDNA) of samples (150 ng) was synthesized by cDNA synthesis kit (Thermo Fisher USA) with random hexamer primers. Briefly, the reaction was conducted by incubation according to the protocol of the kit for 5 min at 25°C followed by 60 min at 42°C and 70°C for 5 min.

Quantitative real-time PCR

The quantitative RT- PCR was performed using SYBER Green PCR master mix kit (TAKARA, BIO, Otsu, Japan) to quantify the expression level of ARSD and ARSD-AS1. The first strand needed to certify cDNA synthesis products was subjected to 30s at 95°C followed by 35 cycles of 5s at 95°C for denaturing, 34s at 60°C for annealing/extension steps. The sequences of primers are listed in Table 1. PCR products were quantified and normalized using GAPDH as an internal standard. Subsequently, the fold change of expression was figured out by the Livak method [15].

Finally, gene ontology (GO), STRING and REACTOME bioinformatics tools were applied to evaluate the molecular mechanism underlying ARSD gene function.

System biology analysis

In order to find the association of ARSD and ARSD-AS1 expression level with the survival of patients with breast cancer, the Oncolnc database was used. This database provides a Kaplan-Meier plot. Moreover, the functional annotation of ARSD protein was obtained from the STRING tool in ExPASy database (V. 8.2) [16] using a high confidence score ≥0.7 as threshold. The partner of ARSD was retrieved based on the text mining, co-expression, experimental, and databases. Moreover, to identify the link of ARSD with other key cancer genes TP53, MYC, MAPK1, BCL-2, VEGF, and HIF1A were added to the STRING network analysis. Other bioinformatics resources such as GeneMANIA [17] and Enrichr [18] were also used for the identification of co-expressed genes with ARSD and their involved transcription factors, respectively.

Statistics

The statistical software SPSS version 20, was used for data analysis. One-Way ANOVA statistical package was performed to identify the association of ARSD and ARSD-AS1 expression with tumor grade and cell surface receptor characteristics, while the T-test was used to analyze the relationship between ARSD and ARSD-AS1 expression and two-categorized variables including age, tumor size, vascular invasion, lymph node metastasis. All data were presented as mean ± SD. P values <0.05 showed statistical significance. Overall survival was assessed by Kaplan–Meier analysis and the Cox proportional hazards model to examine whether ARSD expression levels were associated with prognosis in survival analysis.

Results

Expression pattern of ARSD gene in breast cancer patients

The quantification of gene expression process was indicated as a fold-change of expression levels which was calculated by the $2^{-\Delta\Delta Ct}$ equation. The mRNA level of ARSD gene was normalized to GAPDH gene as an internal control, which was performed in the same run. The amplification curve analysis and melting temperature for each gene confirmed the specificity of the amplified products (data not shown).

The relative quantification analysis revealed that the breast tumor cells expressed ARSD 0.74-fold lower than the adjacent normal breast tissues (Figure 2, p<0.05). On the other hand, the mRNA expression of ARSD was suppressed by 26% in the cancer tissues.

Table 1. The primer and amplicon characteristic applied for expression analysis in QRT-PCR

Primers	*Sequences 5′→3′*	*Amplicon length (bp)*	*Tm -°C*
Forward ARSD	5′-TCATGGCGGATGATCTAGGCA-3′	187	61.10
Reverse ARSD	5′-TCCATGCCTGATCTGAAGGAA-3′	187	58.80
Forward ARSD-AS1	5′-CTGAGGTCTGAAGGTGGCTC-3′	191	57.73
Reverse ARSD-AS1	5′-ATACTGGGGATGGGAGGACC-3′	191	59.96
Forward GAPDH	5′-GGTGGTCTCCTCTGACTTCAACA-3′	127	61.82
Reverse GAPDH	5′-GTTGCTGTAGCCAAATTCGTTGT-3′	127	60.55

Expression pattern of ARSD-AS1 in breast cancer patients

The analysis of real-time PCR regarding to the expression of ARSD-AS1 demonstrated that the breast tumors transcribed lower amount of this lncRNA than normal tissues. The expression of ARSD-AS1 was 0.40-fold lower than normal breast tissues (Figure 2, p<0.05). On the other hand, the mRNA level of ARSD-AS1 was reduced approximately 60% in the breast cancer tissues. Based on these results, we estimated that the ARSD-AS1 expression in tumor specimens was approximately 20% lower than ARSD expression.

Association of ARSD expression with the clinicopathological features of breast cancer

The mean expression of ARSD and ARSD-AS1 genes were measured according to the different clinicopathological aspects of the patients (Table 2). It was found that the ARSD expression level was significantly altered with differentiation in tumor grading (p<0.05). In fact, high ARSD expression was perceived in tumor grade I and low expression in grade III. On the other hand, the ARSD expression was significantly lessened by tumor progression. However, its expression was not associated with other clinicopathological features of breast cancer patients including age, tumor size, vascular invasion and lymph nodes metastasis.

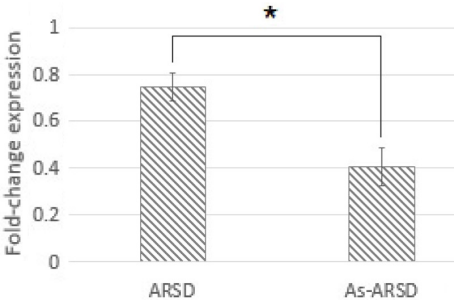

Figure 2. Expression alteration of ARSD and ARSD-AS1 in breast tumor samples. The relative expression levels of ARSD and ARSD-AS1 are represented in breast tumors vs. their matched non-tumor samples obtained from the same patients. A significant down-regulation of ARSD and ARSD-AS1 in breast tumor vs. non-tumor samples is evident (*p<0.05). The lncRNA ARSD expression was significantly lower in breast cancer tissues compared to the ARSD gene expression level.

Table 2. Association between ARSD and ARSD-AS1 expression level and clinicopathologic status of breast cancer

Clinicopathologic variable	*ARSD expression*	ARSD-AS expression
Age, years		
< 50	0.35 ± 0.28	0.69 ± 0.77
>50	0.70 ± 0.77	0.74 ± 0.99
P value	0.13	0.89
Tumor size, cm		
< 2.5	0.84 ± 1.14	0.26 ± 0.17
> 2.5	0.44 ± 0.41	0.80 ± 0.91
P value	0.21	0.25
Cell surface receptor		
Triple negative	0.37 ± 0.28	0.63 ± 0.70
PPN	0.42 ± 0.45	0.99 ± 0.26
NNP	0.89 ± 0.96	0.25 ± 0.29
P value	0.44	0.51
Tumor grade		
I	1.02^{b} ± 0.11 (5)	0.68 ± 0.90
II	0.45^{ab} ± 0.03 (10)	1.02 ± 1.13
III	0.31^{a} ± 0.01 (10)	0.42 ± 0.34
P value	0.05*	0.29
Vascular invasion		
Yes	0.50 ± 0.42	0.71 ± 0.83
No	0.51 ± 0.80	0.71 ± 0.95
P value	0.98	0.99
Lymph nodes metastasis		
Yes/No	0.50 ± 0.41	0.82 ± 0.74
No	0.53 ± 0.85	0.49 ± 0.91
P value	0.92	0.39

*Statistically significant. Note that there is a significant down-regulation for ARSD gene in high-grade tumors of breast cancer. The superscript letters (a,b) represent statistically significant differences.

Moreover, one of the main characteristics of breast tumor tissue is the cell surface receptor, which can be categorized based on the presence or absence of each receptor. It is interesting to note that lower expression level of ARSD and its antisense were observed in triple negative and NNP tissue samples, respectively (p>0.05). It can be concluded that the absence of estrogen and progesterone receptors may be associated with the lower expression of ARSD and ARSD-AS1.

Role of ARSD and ARSD-AS1 in breast cancer patient survival

To analyze the correlations of ARSD and ARSD-AS1 expression level with survival of breast cancer patients, a Kaplan-Meier plot was obtained from Oncolnc database. As shown in Figure 3A, approximately 20-year overall survival of high ARSD expression group was higher than that of the low expression group in some part (p>0.05). The same survival was observed for ARSD-AS1 (Figure 3B). On the other hand, Kaplan-Meier survival analysis showed that patients with high ARSD and ARSD-AS1 expression had poorer survival.

ARSD protein interaction analysis

The molecular action of ARSD protein was demonstrated by STRING (Figure 4A). Across the mentioned proteins, sulfates modifying factor (SUMF) inhibits the activation of sulfates by SUMF1. Moreo-

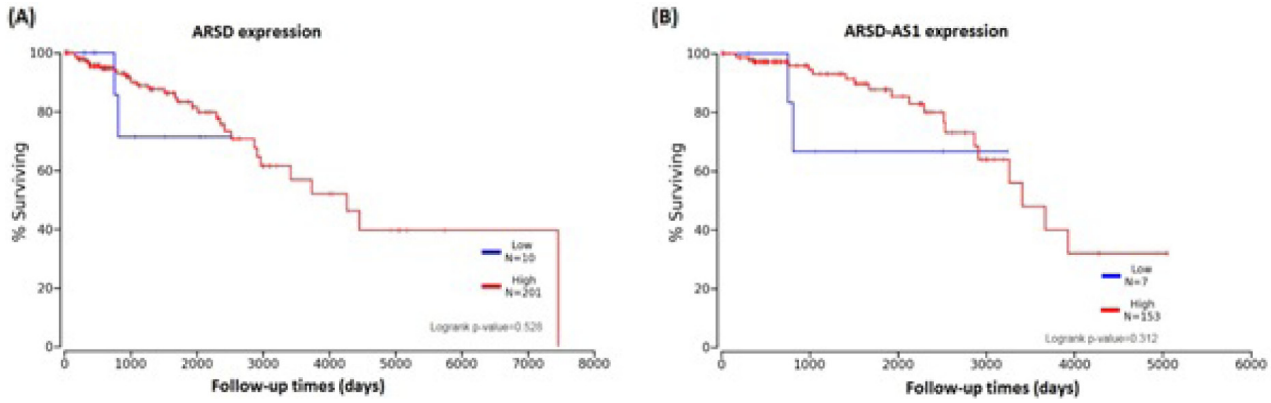

Figure 3. Kaplan-Meier plots for survival for ARSD protein and ARSD-AS1 in breast cancer. Overall survival of patients with high vs. low ARSD **(A)** and ARSD-AS1 **(B)** expression levels are illustrated. Data obtained from Oncolnc database.

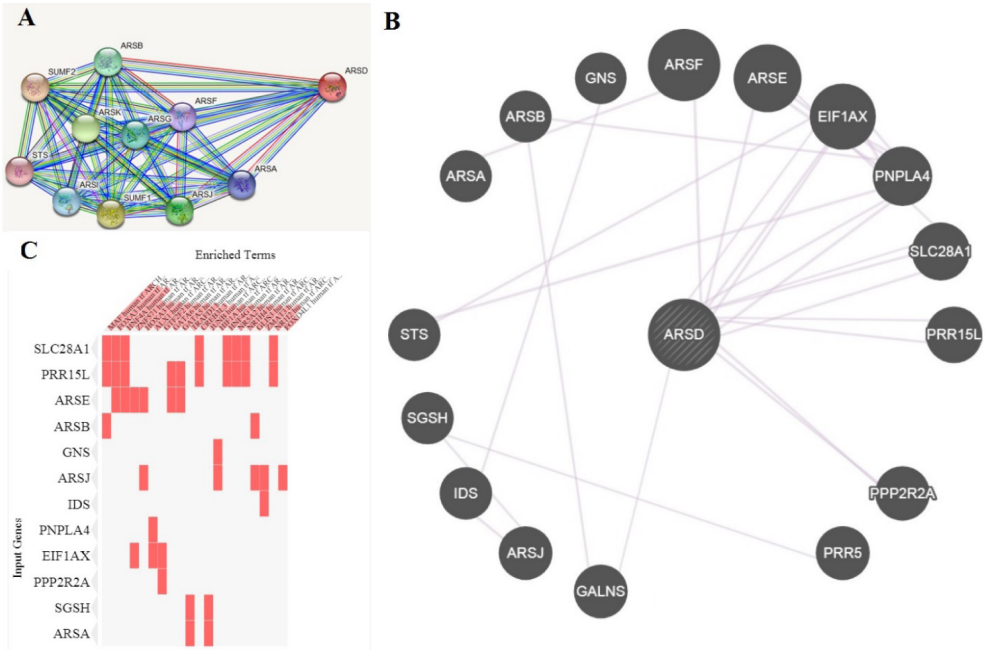

Figure 4. ARSD interaction analysis. **A:** Functional interaction network of ARSD protein (ExPASy, STRING); ARSD interacts with the members of sulfatase family and plays its role in the metabolism of estrogen by recruiting STS and MF1 molecules (protein). **B:** The co-expression analysis of ARSD gene (GeneMANIA). **C:** Transcription factors involved with the ARSD's co-expressed genes are presented in clustergram form.

ver, the steroid sulfates (STS) convert the sulfated steroid precursors to estrogens during pregnancy. According to this finding, we can infer the role of ARSD in estrogen metabolism. It is interesting to note that no functional annotations were found between ARSD and the critical cancer genes (Figure 4B).

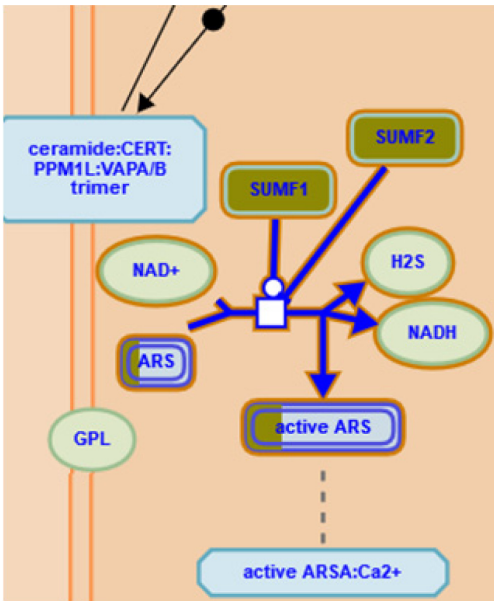

Figure 5. Pathways associated with ARSD in sphingolipid metabolism. SUMF1 mediates the activation of arylsulfatase family members. Data obtained from REACTOME database.

The GeneMANIA database was additionally applied to predict the co-expression of ARSD with other genes (Figure 4C). Subsequently, the associated transcription factors (TF) of these genes were identified by the Enrichr resource. The clustergram output of ARCHS4 TFs was painted out (Figure 4D). It is interesting to note that some of these TFs, such as MAF and GATA, had the oncogenic activity [19,20].

Furthermore, to get a better overview of downstream pathways attributed to the ARSD and its partners' function, the REACTOME database (V. 6.8) was used [21]. The findings of this database reveal that the role of ARSD protein (65 KDa) with arylsulfatase activity and metal ion binding site was involved in glycosphingolipid metabolism, as SUMF1 mediates the oxidation of cysteine to formylglycine, producing active arylsulfatases (Figure 5). The ARSD active form was accompanied by calcium ion which participates in other reactions in lysosomal lumen.

In silico expression profile of ARSD-AS1

Gene expression profiling of ASRD-AS1 was identified across different tumor and normal tissues by MiTranscriptome (http://www.mitranscriptome.org). The up-regulation of this antisense was recognized in 19 normal tissues including kidney, ovary, thyroid, prostate, adipose, and breast tis-

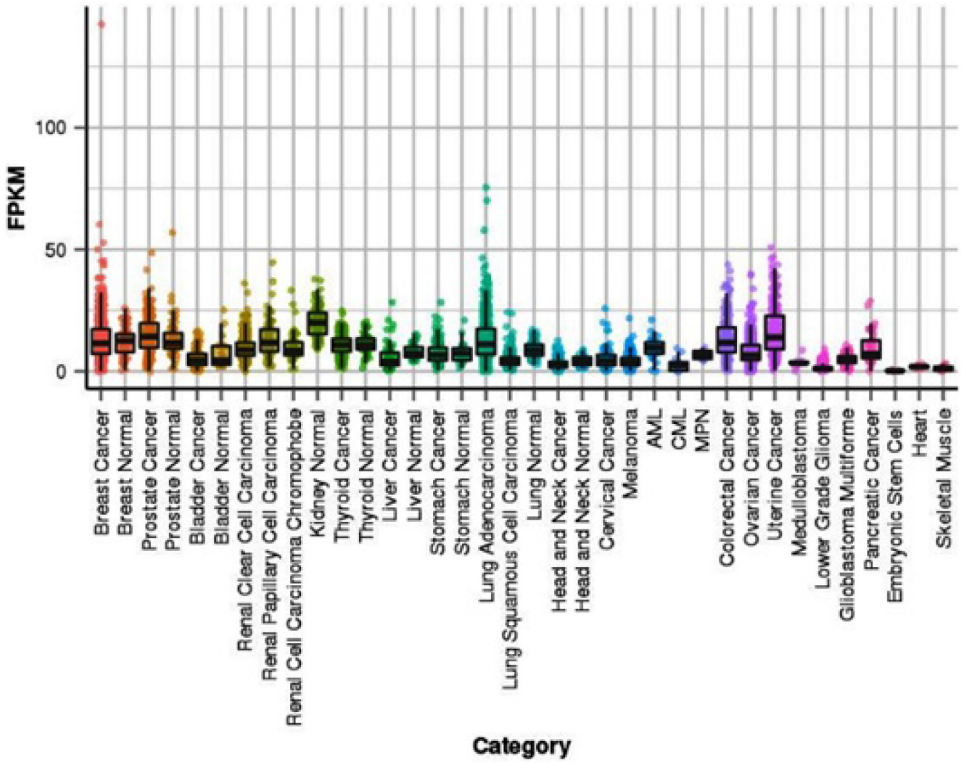

Figure 6. Gene expression profiling of ARSD-AS1 in tumor tissues vs. normal tissues. Low expression of ARSD-AS1 was demonstrated in breast cancer tissue in comparison to normal ones. Data obtained from MiTranscriptome database (http://www.mitranscriptome.org).

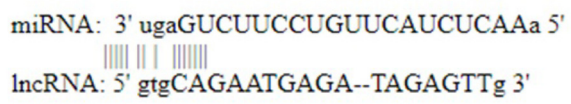

Figure 7. Interaction point of ARSD-AS1 with hsa-miR-618. Data obtained from lncRNA SNP database.

sues, respectively. However, its low expression was demonstrated in breast, bladder, and lung cancers (Figure 6).

In order to identify the binding site of transcription factors for ARSD-AS1, the ChIPBase database was applied (http://deepbase.sysu.edu.cn/chipbase). Accordingly, four different binding sites including TP53, TEAD4, KDM5B and TP63 were detected. They were located around the transcription start site of ARSD-AS1 which regulated its expression. Additionally, another binding site was observed for EZH2 and HDAC1 genes which act as chromatin remodeling factors.

Moreover, the LncRNADisease resource was used to determine the mRNA targets of ARSD-AS1 (http://cmbi.bjmu.edu.cn/lncrnadisease). This lncRNA targets the mRNA of several genes including ARSE, ARSD, GYG2 and XG, which all are located on chromosome X.

Role of polymorphism in ARSD-AS1

Recent findings demonstrated the impact of lncRNA polymorphisms in the development and progression of cancer. Therefore, to identify the ARSD-AS1 polymorphisms, the database of lncRNASNP2 was used. The data showed 11 single nucleotide polymorphisms (SNP) in ARSD-AS1 as follows: rs10218035, rs1043269, rs14004224, rs18616723, rs200994400 and rs371731641.

Subsequently, we analyzed the miRNAs binding sites according to these SNPs. We recognized that either 10 new miRNAs binding sites could be created, or there may provide 16 connectivity locations which resulted in the expression changes of a lncRNA. The obtained results of this database pointed at the interaction of ARSD-AS1 with several miRNA including miR-519a-3p, miR-143-5p, miR-541-3p, miR-525-5p, miR-331-3p, miR-149-5p, miR-516b-5p, miR-185-5p, miR-618, miR-892a, miR-34a-5p, miR-449a, miR-23b-3p, miR-128-3p and miR-221-5p.

For instance, the junction point of ARSD-AS1 with has-miR618 was displayed in Figure 7.

Discussion

Tumor markers have been considered in all aspects of cancer care, in particular for diagnosis and treatment. Identification of new tumor markers is a valuable tool for clinical practice in oncology to predict and screen the therapeutic response.

Recent researches have noted the importance of lncRNA regulatory function in the expression of protein-coding genes [22]. It was revealed that abnormal lncRNAs expressions were associated with oncogenic properties such as proliferation, angiogenesis, apoptosis and metastasis. For instance, up-regulation of HOTAIR in breast cancer is attributed to cancer progression through invasion and metastasis processes [7], while down-regulation of SPRY4-IT1 significantly reduced cell growth and induced apoptosis through SP1 transcription factor [8,23]. In addition, abnormal up-regulation of UCA1 modulates breast cancer cell proliferation and apoptosis [9]. Therefore, a demand for identification of a lncRNA as tumor marker involved in breast cancer development and progression is obligatory, since it could be considered as a potential diagnostic and prognostic marker in breast cancer treatment.

ARSD is involved in sphingolipids metabolism and other bioactive lipids by enzymatic activity [13]. It has been revealed the sphingosine-1-phosphate (S1P) regulates cancer progression processes [24,25], therefore, the ARSD can be considered as a marker in cancer prognosis and treatment. This study was set out with the aim of providing a conceptual framework based on the aberrant expression of ARSD and its antisense in breast cancer.

We have demonstrated that ARSD and ARSD-AS1 were present at lower level in breast cancer tissue samples as opposed to non-cancer tissues. The most important clinically relevant finding was the mean significant difference between ARSD and ARSD-AS1 expression. Although, these results differ from published research [13], they revealed that ARSD was significantly up-regulated in chronic lymphocytic leukemia patients with ZAP70 mutation.

We also examined the sense and antisense of ARSD expression ratio in relation to the tumor characteristics like size, cell receptors and grade. Our results showed that invasive tumors, grade III, had a lower expression level of ARSD. Some researchers reported that over-expression of lncRNAs correlates with advanced tumor stages [26,27]. Contrary to our expectations, the tumor size did not significantly correlate with the sense and antisense of ARSD expression.

According to the findings of system biology tools, we identified the interaction of ARSD with proteins that participate in glycosphingolipid and estrogen metabolisms. The presence of oncogenic

TF binding sites in ARSD gene and its antisense, such as TP53, MAF and GATA, suggested that they might participate in cancer progression.

Also, GYG2 (55 kDa, 501 aa) as a mRNA target for ARSD-AS1 was indicated by the database of LncRNADisease. This protein has a role in cancer microenvironment and was recently introduced as a target in cancer treatment [28].

The relevance of lncRNA polymorphisms in cancer progression is inarguable, therefore, we explored the ARSD-AS1 polymorphisms through lncRNA SNP database [29]. Analysis of *in silico* survey demonstrated the presence of 11 SNPs in ARSD-AS1 that results in the creation of new binding sites for miRNAs. For instance, the junction point of ARSD-AS1 with hsa-miR-618 was displayed in Figure 7, in which, low expression of miR-618 has been demonstrated in metastatic prostate cancer [30] and thyroid carcinomas [8]. Low expression of ARSD-AS1 in this study may attribute to the expression of miR-618 that plays critical role in cancer migration and invasion through FOXP2 and PI3K/Akt pathway. However, further experiments are required to evaluate the level of transcribed miR-618 in patients harboring breast cancer. This finding confirms the association between miR-618 and cancer progression which supports the idea that ARSD-AS1 may be recruited in tumor cell proliferation.

Conclusions

Overall, we demonstrated the up-regulation of ARSD in breast tumor tissue compared to ARSD-AS1. Also, down-regulation of ARSD in breast tumor tissues may be correlated to aggressive disease progression of breast cancer patients. The linkage of ARSD and ARSD-AS1 with oncogenic transcription factors, miRNAs and proteins, suggest them as great potential targets for the breast cancer prevention and treatment. Further empirical studies are needed to verify the molecular mechanisms of these genes in breast cancer.

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Conflict of interests

The authors declare no conflict of interests.

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