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Original Article

The prognostic value of microRNA-biogenesis genes *Argonaute 1* and *2* variants in breast cancer patients

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Abstract: MicroRNA machinery genes Argonaute 1 (*AGO1*) and 2 (*AGO2*) are associated with several hallmarks of cancer. They play a key role in transcriptomic silencing, regulation of the immune system, cell differentiation, and angiogenesis processes. The present pilot study aims to explore the impact of genetic variants rs636832 and rs2977490 of *AGO1* and *AGO2*, respectively, on breast cancer (BC) risk in a sample of Mediterranean population. TaqMan genotyping assay of 93 consecutive breast cancer female patients and age- as well as ethnicity-matched controls, was done by Real-Time allele discrimination polymerase chain reaction. Association with the available clinical, histopathological and immunohistochemistry assessments was applied. *In silico* data analysis was also executed. Although allele and genotype frequencies distribution of both study variants were comparable in BC and healthy control cohorts, *AGO1*G* variant conferred a significant BC risk under recessive model [adjusted odds ratio (95% confidence interval); 4.90 (1.03-23.39), $P = 0.024$], and was significantly associated with lymph node infiltration ($P = 0.037$), distant metastasis ($P = 0.019$), advanced clinical stage ($P < 0.001$), recurrence ($P = 0.032$), and shorter overall survival ($P = 0.001$). Furthermore, *AGO2*G/G* genotype showed an association with poor pathological grade ($P = 0.029$). Our results suggested for the first time that rs636832 and rs2977490 variants of the miRNA-machinery genes *AGO1* and *2*, respectively, may impact susceptibility and/or clinical outcomes of BC patients in the study population.

Keywords: Breast cancer, single nucleotide polymorphism, AGO1, AGO2, real-time PCR

Introduction

Breast cancer (BC) is the most common carcinomas associated with high incidence of morbidity and mortality rate among females worldwide [1]. Although recent decade has evident progress in diagnosis and targeted therapy of BC, it still represents an overload on any country resources [2]. Therefore, a better understanding of the cellular and molecular mechanisms participating to BC is required.

In the last several years, a growing body of evidence indicates that microRNA (miRNA) machinery proteins deregulation could play a vital role in tumorigenesis and cancer progression [3-5]. Moreover, they could enrich the world of RNA interference (RNAi)-based therapy [6].

Although different clusters of small RNAs are generated by distinct biogenesis processes, all mature miRNAs associate with Argonaute proteins (i.e. one sub-family of miRNA biogenesis proteins) to form the RNA-induced silencing complex (RISC) [3, 6, 7]. The miRNA biogenesis includes sequential steps that aim to process the primary (pri-miRNA) and the precursor (pre-miRNA) forms of miRNA transcript to the biologically active mature form [8].

In silico analysis and published literature data mining revealed that Argonaute RISC Component 1 (AGO1) and 2 (AGO2), also known as "Eukaryotic Translation Initiation Factors 2C 1 and 2 (EIF2C1/2)", are two members of the Argonaute protein family, which are coded by *AGO1* and *AGO2* genes, respectively. The human *AGO1* (*EIF2C1*) lies along chromosome 1p34.3, contains 20 exons which encodes for a protein of 857 amino acids, while *AGO2* (*EIF2C2*), is located on chromosome 8q24.3, contains 22 exons and encodes for a protein of 859 amino acids in length. Both proteins share 85% amino acid identity and several splice variant transcripts could be formed from each gene.

Accumulating evidence revealed that "single nucleotide polymorphisms (SNPs)" in miRNA biogenesis genes could be implicated in the risk of tumorigenesis, prognosis (including patients' survival) and treatment response [9-14]. More specifically, SNPs in the main components of the silencing machinery, including *AGO1* and *2*, may affect the overall silencing efficiency with subsequent target upregulation of oncogenes or down-regulation of tumor suppressor genes in case of risky variant, and vice versa in protective one [15]. Furthermore, as different targets may be more or less sensitive to the silencing efficiency, such SNPs may affect several pathways with variable sensitivity, contributing to the genetic variation observed in specific phenotypes [9].

Although several *AGO1* and *AGO2* SNPs were investigated for their association with susceptibility and/or prognosis of different solid cancers in several populations, to the best of authors' knowledge, no previous study was conducted to uncover the association and impact of the intronic rs636832 and rs297-7490 variants on the susceptibility and/or outcomes of breast cancer in a sample of Middle East population. This may provide novel genetic biomarkers of breast cancer susceptibility and good basis for "miRNA-based therapeutic approaches" in the future.

Materials and methods

Study population

The study included a total of 186 women (93 consecutive primary breast cancer and 93 unrelated matched controls). Patients were obtained from the General Surgery Department, Suez Canal University Hospital, Ismailia, Egypt. They were diagnosed clinically, radiologically and confirmed by biopsy [16]. They did not have a history of receiving any line of treatment as radiotherapy, immunotherapy, chemotherapy or hormonal therapy prior to blood sampling. Patients with chronic diseases, or other malignancies were excluded. Healthy attendees of the outpatient clinics for routine check-up were enrolled as controls. They had no evidence of chronic disease, recent pregnancy or lactation within the last two years, and/or any concomitant malignancy. The study was approved by the institutional research ethics committee of Faculty of Medicine, Suez Canal University and conducted according to the principles of the Declaration of Helsinki. Written informed consents were obtained from all participants prior to the start of the study.

Histopathological assessment

Histopathological analysis of breast cancer tissue specimens was performed post-operatively. Evaluation of pathological grade and clinical stage were carried out using Elston and Ellis modification of Scarff-Bloom-Richardson system and the American Joint Committee on Cancer (AJCC) tumor-lymph node-metastasis (TNM) staging system [17] (**Figure 1A** and **1B**). Assessment for hormonal receptors for molecular subtyping was done by an independent pathologist [18] (**Figure 1C** and **1D**). Patients were clustered into four groups: Luminal A, Luminal B, $HER2^+$, and triple negative BC as detailed previously [19].

Clinical assessment and prognostic evaluation

Clinical features, risk factor assessment, and investigations were reported. Nottingham Prognostic Index (NPI) and Immunohistochemical Prognostic Index (IHPI) were applied as previously described [19]. Patients were then classified to have good, moderate, and poor prognosis. Follow-up period lasted for up to 3 years for overall survival (OS) and disease-free survival (DFS) assessment.

Sample collection and DNA extraction

Whole blood samples (5 mL) were drawn into EDTA Vacutainers. DNA was isolated using

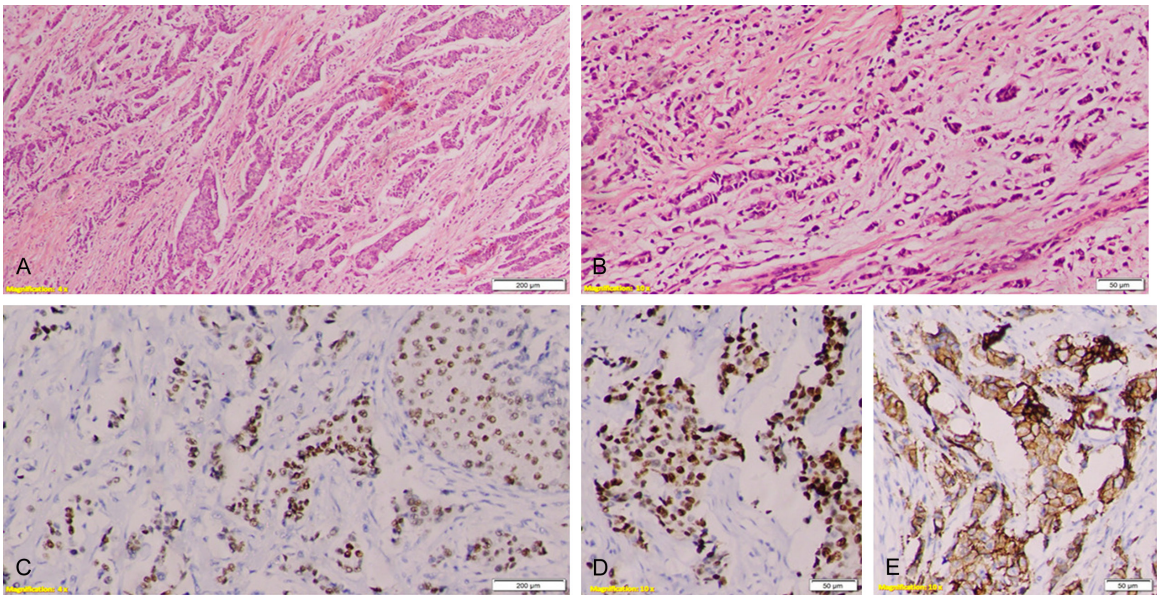

Figure 1. Histopathological analysis of breast cancer samples. Photo (A) Mixed invasive duct & lobular carcinoma showing sheets of infiltrating large sized malignant ductal cells within moderate desmoplastic reaction & other groups of small sized monotonous malignant epithelial cells with little vacuolated cytoplasm & arranged in thin cords & trabeculae of cells showing Indian File pattern at low power 4× magnification. Photo (B) Case of infiltrating lobular carcinoma pleomorphic variant high grade showing markedly atypical hyperchromatic & pleomorphic nuclei with little vacuolated cytoplasm & surrounded by marked desmoplastic reaction. Tumor cells arranged in thin cords with Indian File pattern at 10× power. Photo (C) Slide of infiltrating duct carcinoma stained by oestrogen immuno-histochemical stain showing moderate positivity for hormonal nuclear receptors at about (65%) of tumor cells score (6/8). At 4× power. Photo (D) Slide of infiltrating duct carcinoma stained by progesterone immuno-histochemical stain showing marked positivity for hormonal nuclear receptors at about (65%) of tumor cells score (7/8). At 10× power. Photo (E) Slide of infiltrating duct carcinoma high grade showing complete intense membranous staining for Her2 protein over-expression score (+3). At 10× power.

ABIOpure TM TOTAL DNA extraction kit (Catalog no. M501DP100, Alliance Bio, USA) following manufacturer's instructions. NanoDrop ND-1000 spectrophotometer (NanoDrop Tech., Inc. Wilmington, DE, USA) was used to assess the purity and concentration of the extracted DNA.

Allelic discrimination analysis

Real-time Polymerase Chain Reaction was performed in the StepOne™ Real-Time PCR System (Applied Biosystems, USA) using TaqMan Genotyping PCR Master Mix, No UNG (4440043), and TaqMan SNP Genotyping Assay Mix (assay ID C___1079151_10 for rs636832 and C___9176197_10 for rs2977490, Thermo Fisher Scientific). In brief, 20 ng genomic DNA diluted to 11.25 µL with nuclease-free water was added to the reaction mix contained 12.5 µl Taqman Master Mix, and 1.25 µl TaqMan SNP Genotyping Assay (20×) Mix. The PCR program was detailed previously [20]. Genotyping was performed blinded to the case/control status and negative controls were used in each run. Twenty percent of samples were analyzed in duplicate with 100% concordance rate for genotype calls. Genotype calling was performed using the SDS software version 1.3.1 (Applied Biosystems).

Bioinformatics analysis

Genomic and protein structure were retrieved from the Ensembl genome browser (www.ensembl.org). Subcellular localization was identified using Compartment database (http://compartments.jensenlab.org/). Functional enrichment in cancer hallmarks was determined via Cancer Hallmark Analytics Tool (http://chat.lionproject.net/). The gene-gene network was built in GeneMania (https://genemania.org/). Protein-protein interaction was identified using STRING version 11.0 (https://string-db.org). Genetic alterations in breast cancer studies were screened in cBioPortal for cancer genom-

Table 1. Demographic data of the study groups

Demographic data		Controls	Patients	*P* value
Age				
Sex	Female	93 (100)	93 (100)	1.0
Residence	Rural	47 (50.5)	50 (53.8)	0.769
	Urban	46 (49.5)	43 (46.2)	
Marital status	Divorced	20 (21.5)	17 (18.3)	0.238
	Married	60 (64.5)	54 (58.1)	
	Single	13 (14.0)	22 (23.7)	
Occupation	Housewife	56 (60.2)	66 (71.0)	0.099
	Retired	3 (3.2)	0 (0.0)	
	Worker	34 (36.6)	27 (29.0)	

Data are presented as numbers and percentages. Chi-square test was used.

ics (www.cbioportal.org). Association of AGO1 and AGO2 with survival in breast cancer patients was plotted in Kaplan-Meier curves using Kaplan-Meier Plotter (http://kmplot.com/analysis/index.php?p = service&default = true) [21]. Role of AGO1 and AGO2 in prior cancer studies were systematically collected. Next, variant analysis was performed in the Ensmebl genome browser, ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/), dbVar (https://www.ncbi.nlm.nih.gov/dbvar), MedGen (https://www.ncbi.nlm.nih.gov/medgen/), ClinGen (https://www.ncbi.nlm.nih.gov/clinvar/docs/clingen/), and 1000 Genomes Browser (https://www.ncbi.nlm.nih.gov/variation/tools/1000genomes/). The studied variants were selected according to set conditions: (1) Biallelic single nucleotide polymorphism, (2) Minor allele frequency (MAF) > 0.35, and (3) MAF in African > 0.45.

Statistical analysis

Data distribution was checked using the "Kolmogorov-Smirnov test". Chi-square and Fisher's exact tests were applied for categorical variables, while student's t and one-way analysis of variance (ANOVA) tests were used for quantitative variables. Genotype frequencies were evaluated for Hardy-Weinberg equilibrium (HWE) using the Online Encyclopedia for Genetic Epidemiology studies; OEGE (www.genes.org.uk) and tested using Chi-square test to compare the expected genotype frequencies among patient and control groups. Due to the differences in the cliniopathological characteristics between patients and controls, binary logistic regression analysis was performed to adjust confounding variables. Data were analyzed using R version 3.6 and R studio version 1.1.383. Single and multiple SNP analyses were done using SNPStats (www.snpstats.net). Adjusted odds ratios (OR) with a 95% confidence intervals (CI) were calculated for different genetic association models [22]. Principal component analysis (PCA) was plotted using the 'factoextra' and 'FactoMineR' packages. A two-sided *P-value* < 0.05 was considered significant.

Results

Single and multiple SNP analyses

Tables 1 and **2** demonstrated the demographic and the clinicopathological characteristics of the study population, respectively. The genotype frequencies of the intron variants *AGO1* (rs636832; A > G) and *AGO2* (rs2977490; A > G) showed no deviation from the Hardy-Weinberg equilibrium (all *P* > 0.05). The MAF of rs636832*G and rs2977490*G alleles were 0.23 and 0.48, respectively (**Table 3**). Patients carrying the G/G genotype for *AGO1* showed five times more risk to develop breast cancer under recessive model (OR = 4.90, 95% CI = 1.03-23.3, P = 0.024) (**Table 4**). The gene-gene interaction did not reveal a significant association with breast cancer disease (**Table 5**).

In silico data analysis

AGO1 and AGO2 were highly enriched within the nucleus and cytoplasmic compartment (**Figure 2A** and **2B**). AGO2 was also abundant in the extracellular space (**Figure 2B**). Both genes played key role in various hallmarks of cancer, in particular genome instability and mutation, evading growth suppressors, and sustaining proliferative signaling. AGO1 was involved in deregulating cellular energetics, while AGO2 can lead to the acquisition of invasion and metastasis (**Figure 3A**). Gene-gene network analysis demonstrated their role in transcriptomic silencing, regulation of immune system process, regulation of cell differentiation, and regulation of angiogenesis (**Figure 3B** and **3C**).

Screening of 8874 breast cancer patients across 15 studies showed *AGO1* mutations in 0.8% (66 out of 8508) of cases, while genetic alterations in *AGO2* gene accounted for 10% of

Table 2. Clinicopathological characteristics of breast cancer patients (N = 93)

Variable		Number	Percentage	Variable		Number	Percentage
Clinical presentation				Site of metastasis	Bone	16	17.2
Mastalgia	Positive	28	30.1		Liver	6	6.5
Breast mass	Positive	75	80.6		Lung	18	19.4
Skin lesions	Positive	15	16.1	LVI	Negative	48	51.6
Nipple changes	Positive	18	19.4		Positive	45	48.4
Axillary pain	Positive	7	7.5	Skin infiltration	Negative	74	79.6
Axillary mass	Positive	7	7.5		Positive	19	20.4
Side	Left	35	37.6	Receptor status			
	Right	57	61.3	ER/PR	Negative	38	40.9
Site	LOQ	8	8.6		Positive	55	59.1
	UIQ	18	19.4	$HER2^+$	Negative	78	83.9
	UOQ	44	47.3		Positive	15	16.1
	Retro	23	24.7	Molecular subtype	Luminal A	45	48.4
No. of masses	Single	70	75.3		Luminal B	10	10.8
	Multiple	23	24.7		$HER2^+$	5	5.4
Pathological analysis					TNBC	33	35.5
HPD	Ductal	33	35.5	IHPI score	Good	55	59.1
	Lobular	25	26.9		Moderate	33	35.5
	Medullary	14	15.1		Poor	5	5.4
	Mucinous	9	9.7	Follow-up			
	Tubular	6	6.5	Clinical stage	IIA	17	18.3
	Metaplastic	6	6.5		IIB	13	14.0
Grade	G2	76	81.7		IIIA	12	12.9
	G3	17	18.3		IIIB	11	11.8
T stage	T2	47	50.5		IV	40	43.0
	T3	21	22.6	NPI score	Good	45	48.4
	T4B	19	20.4		Poor	48	51.6
	T4D	6	6.5	ESMO score	Low risk	36	38.7
N stage	N0	27	29.0		High risk	57	61.3
	N1	31	33.3	Recurrence	Negative	43	46.2
	N2	29	31.2		Positive	50	53.8
	N3	6	6.5	DFS	Prolonged	45	48.4
M stage	M0	40	43.0		Short	48	51.6
	M1	40	43.0	OS	Prolonged	38	40.9
	Mx	13	14.0		Short	55	59.1

Data are presented as numbers and percentages. LOQ: lower outer quadrant, UIQ: upper inner quadrant, UOQ: upper outer quadrant, HPD: Histopathological diagnosis, LVI: lymphovascular invasion, ER: estrogen receptor, PR: progesterone receptor, HER2: human epidermal growth factor receptor 2, TNBC: triple negative breast cancer, IHPI: immunohistochemical prognostic index estimated based on the three receptor status (HER2, ER, and PR), NPI: Nottingham prognostic index, calculated as [0.2× tumor size in cm] + tumor grade [1-3] + lymph node stage [1-3, according to stages A-C], ESMO: European Society of Medical Oncology for risk estimation of recurrence, DFS: Disease free survival, OS: overall survival.

patients mainly copy number variations (**Figure 2C**). Mutual exclusivity analysis showed that co-occurrence of *AGO1/AGO2* mutations was significant (Adjusted *P* value < 0.001). Survival analysis of 3591 TCGA patients showed an association between the gene expression of *AGO1* and *AGO2* and overall survival (**Figure 2D** and **2E**).

AGO1 can form three splice variant transcripts. The intron variant rs636832 of *AGO1* gene is caused by G to A substitution and is located within intron 8 out of 18 (2605 of 6204) at 1:36363475 [ENST00000373204.6: c.1020+2605G > A, ENST00000373206.5: c.795+2605G > A, and ENST00000635259.1: c.469+2605G > A]. MAF accounted for 0.37, with

Table 3. Genotype and allele frequencies of *AGO1* and *AGO2* genes

Gene	SNP ID	Variant	All		Controls		Patients		*P*-value
			N	Proportion	N	Proportion	N	Proportion	
AGO1	rs636832	A/A	112	0.6	58	0.62	54	0.58	0.093
		A/G	63	0.34	33	0.35	30	0.32	
		G/G	11	0.06	2	0.02	9	0.1	
	P_{HWE}		0.54		0.51		0.17		
		A	287	0.77	149	0.8	138	0.74	0.177
		G	85	0.23	37	0.2	48	0.26	
AGO2	rs2977490	A/A	51	0.27	24	0.26	27	0.29	0.669
		A/G	92	0.49	45	0.48	47	0.51	
		G/G	43	0.23	24	0.26	19	0.2	
	P_{HWE}		0.88		0.84		1.00		
		A	194	0.52	93	0.5	101	0.54	0.408
		G	178	0.48	93	0.5	85	0.46	

Data are presented as numbers and percentages. SNP: single nucleotide polymorphism, P_{HWE}: *P* value of Hardy-Weinberg equilibrium. Chi-square test was applied.

Table 4. Genetic association models for disease risk assessment

Gene	Model	Genotypes	Controls	Patients	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
AGO1	Codominwant	A/A	58 (62.4%)	54 (58.1%)	1.00	0.078	1.00	0.077
		G/A	33 (35.5%)	30 (32.3%)	0.98 (0.53-1.81)		0.98 (0.53-1.83)	
		G/G	2 (2.1%)	9 (9.7%)	4.83 (1.00-23.37)		4.87 (1.00-23.63)	
	Dominant	A/A	58 (62.4%)	54 (58.1%)	1.00	0.55	1.00	0.55
		G/A-G/G	35 (37.6%)	39 (41.9%)	1.20 (0.66-2.15)		1.20 (0.66-2.17)	
	Recessive	A/A-G/A	91 (97.8%)	84 (90.3%)	1.00	0.024	1.00	**0.024**
		G/G	2 (2.1%)	9 (9.7%)	**4.87 (1.02-23.21)**		**4.90 (1.03-23.39)**	
	Overdominant	A/A-G/G	60 (64.5%)	63 (67.7%)	1.00	0.64	1.00	0.64
		G/A	33 (35.5%)	30 (32.3%)	0.87 (0.47-1.59)		0.86 (0.47-1.59)	
	Log-additive	---	---	---	1.39 (0.86-2.24)	0.18	1.39 (0.86-2.27)	0.18
AGO2	Codominant	A/A	24 (25.8%)	27 (29%)	1.00	0.67	1.00	0.67
		G/A	45 (48.4%)	47 (50.5%)	0.93 (0.47-1.84)		0.92 (0.46-1.85)	
		G/G	24 (25.8%)	19 (20.4%)	0.70 (0.31-1.59)		0.70 (0.31-1.60)	
	Dominant	A/A	24 (25.8%)	27 (29%)	1.00	0.62	1.00	0.62
		G/A-G/G	69 (74.2%)	66 (71%)	0.85 (0.45-1.62)		0.85 (0.44-1.63)	
	Recessive	A/A-G/A	69 (74.2%)	74 (79.6%)	1.00	0.38	1.00	0.38
		G/G	24 (25.8%)	19 (20.4%)	0.74 (0.37-1.47)		0.74 (0.37-1.47)	
	Overdominant	A/A-G/G	48 (51.6%)	46 (49.5%)	1.00	0.77	1.00	0.77
		G/A	45 (48.4%)	47 (50.5%)	1.09 (0.61-1.94)		1.09 (0.61-1.94)	
	Log-additive	---	---	---	0.84 (0.56-1.27)	0.41	0.84 (0.56-1.27)	0.4

Data are presented as number (percentage). Adjusted odds ratio (OR) by age. CI: confidence interval. The values highlighted in bold are statistically significant.

Africans were the highest population representing 0.49. On the other hand, *AGO2* has 10 splicing variants. The intronic variant rs2977490, at 8:140563259 covers only three transcripts [ENST00000220592.10: c.337-625G > A, ENST00000519980.5: c.337-625G > A, and ENST00000523609.5: c.144-625G > A] with MAF at 0.49.

Multivariate analysis

Data exploration by principal component analysis classified patients into three distinct groups, one influenced by prolonged survival, another with high grade and LN invasion, and a third group with recurrence, lower survival, and advanced clinical stage (**Figure 4**). Gene-

Table 5. Combined genotype association with disease risk

	AGO1	*AGO2*	Total	Controls	Patients	Crude OR (95% CI)	*P*-value	Adjusted OR (95% CI)	*P*-value
1	A	G	0.3916	0.4452	0.3431	1.00	---	1.00	---
2	A	A	0.3799	0.3559	0.3988	1.41 (0.84-2.35)	0.19	1.42 (0.85-2.39)	0.18
3	G	A	0.1416	0.1441	0.1442	1.33 (0.69-2.55)	0.39	1.36 (0.70-2.63)	0.37
4	G	G	0.0869	0.0548	0.1139	2.22 (0.87-5.71)	0.099	2.26 (0.87-5.83)	0.095

Adjusted odds ratio (OR) by age. CI: confidence interval. Global haplotype association *P*-value: 0.27.

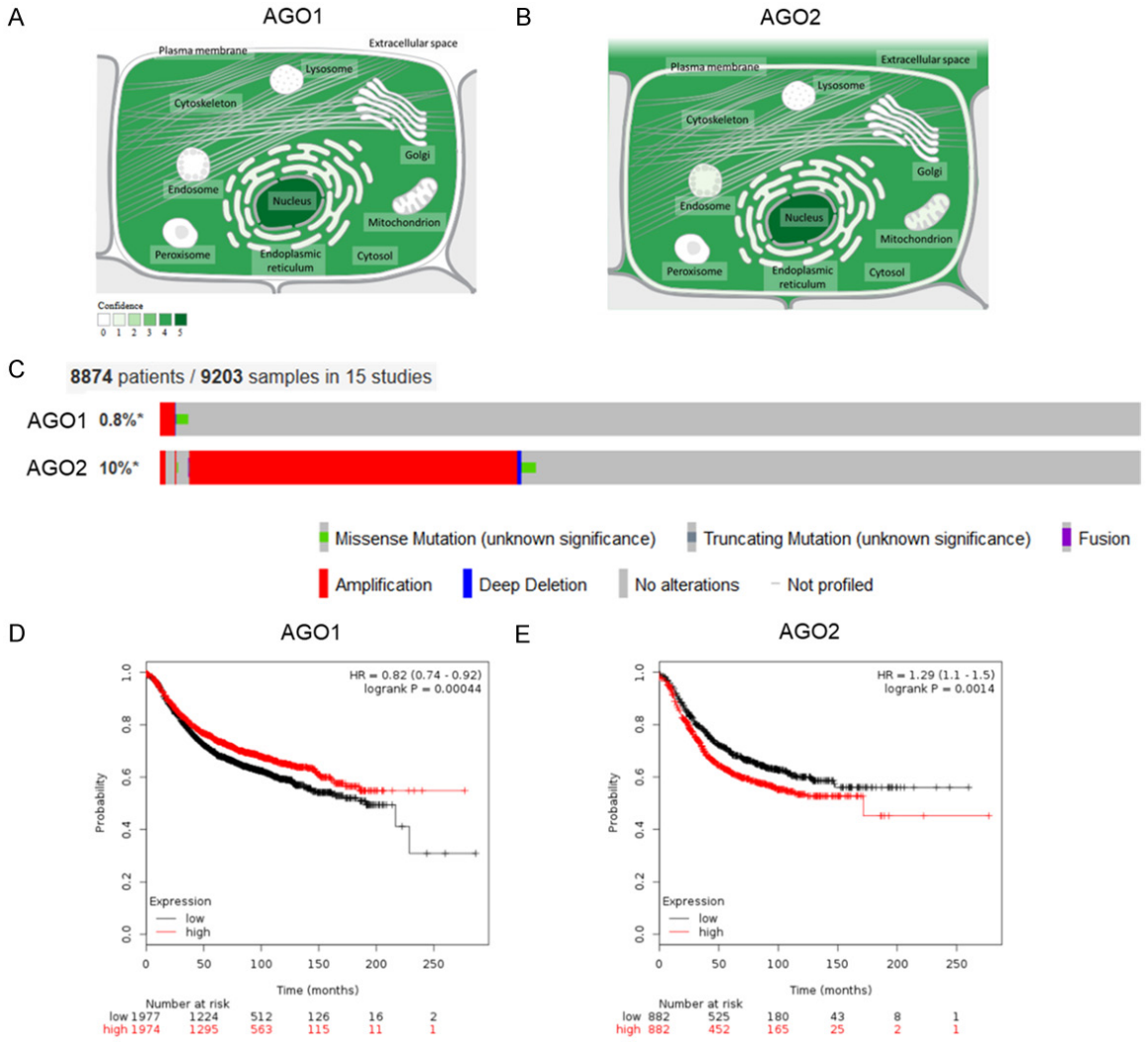

Figure 2. *In silico* data analysis on AGO1 and AGO2. (A, B) Cellular localization of AGO1 and AGO2 proteins showing widely spread of the proteins within the cytosol and the nucleus. Data source: Compartment (https://compartments.jensenlab.org/) which integrates evidence from manually curated literature, high-throughput screens, automatic text mining, and sequence-based prediction methods. (C) Genetic alterations of AGO1 and AGO2 of 8874 breast cancer patients in 15 studies. Studies were retrieved from cBioportal for Cancer Genomics (www.cbioportal.org). After combination, the frequency and type of *AGO1* and *AGO2* genetic alterations were calculated. Both somatic mutations and copy number variations were screened. (D, E) Kaplan-Meier curve of 3591 breast cancer patients showing poor survival of breast cancer patients with low *AGO1* gene expression and high *AGO1* expression profile. Expression level is split by the median. Number of included patients is displayed below the figure. Data source: Kaplan-Meier Plotter (https://kmplot.com/analysis/index.php?p=service).

environment interaction was performed. Multiple regression analysis demonstrated AG/GG genotypes of *AGO1* had a higher risk of nodal infiltration (OR = 2.90, 95% CI = 1.03-8.17, P =

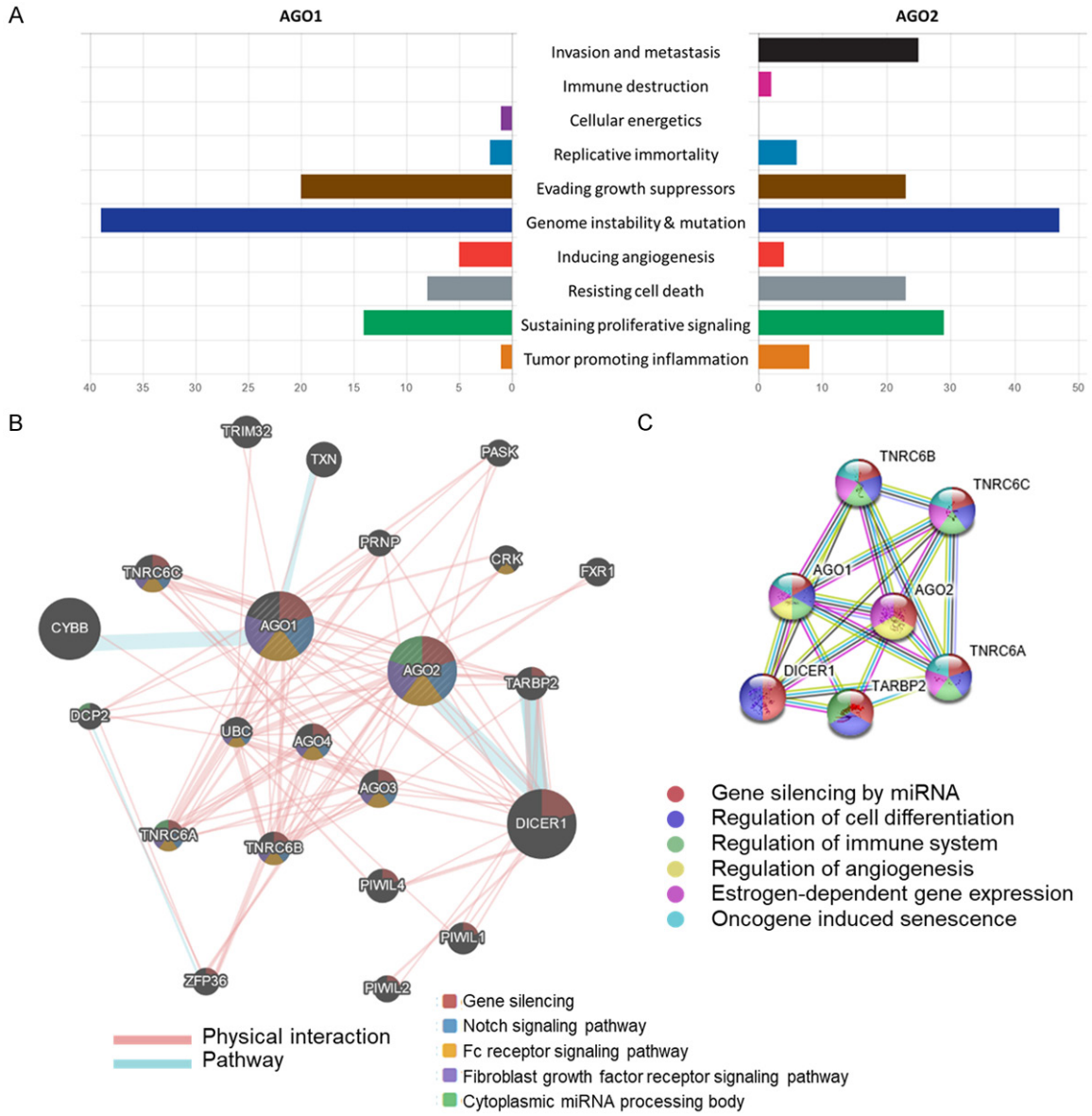

Figure 3. Functional enrichment analysis of AGO1 and AGO2. (A) AGO1 and AGO2 are involved in various cancer hallmarks. Comparison between the functional role of AGO1 and AGO2 in cancer. Cancer Hallmarks Analytics Tool (CHAT) was used (http://chat.lionproject.net). It is a text mining approach that organizes and evaluates scientific literature on cancer. They showed various alterations in cell behavior in cancer state. (B) Gene-gene network analysis revealing interaction of AGO1 and AGO2 with various cancer-related genes and microRNA machinery component pathway genes. Data source: GeneMania (GeneMania.org). (C) Protein-protein network using STRING version 11.0 (https://string-db.org/) showing significant biological processes enriched in tumorigenesis as senescence, angiogenesis, and cell differentiation.

0.037), distant metastasis (OR = 4.46, 95% CI = 1.18-16.87, P = 0.019), advanced clinical staging (OR = 6.54, 95% CI = 2.06-20.75, P < 0.001), overall survival (OR = 2.54, 95% CI = 1.08-5.99, P = 0.032), and recurrence (OR = 5.22, 95% CI = 1.73-15.74, P = 0.001). While the homozygosity GG of *AGO2* variant was associated with increased susceptibility to poor differentiation (OR = 4.01, 95% CI = 1.15-14.02, P = 0.029) (**Table 6**).

Discussion

Given the essential regulatory roles of miRNAs on approximately two thirds of human genes [23] from which most of them are related to

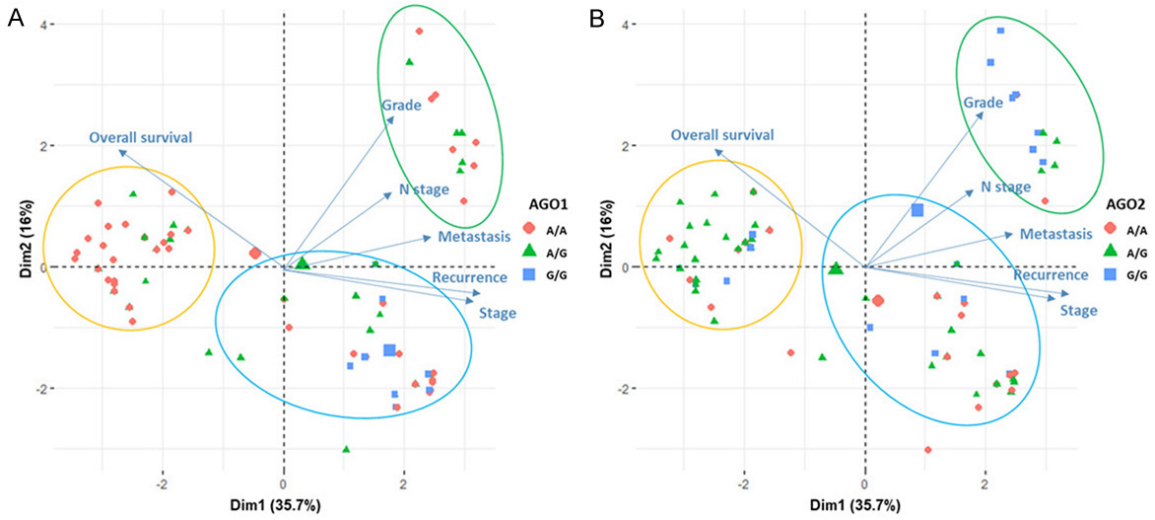

Figure 4. Principal component analysis. Multivariate analysis revealed clustering of BC patients into three groups. Orange circled patients had prolonged survival; the group with green circle had high pathological grade and LN infiltration, while the blue circle group experienced recurrence and progression.

Table 6. Multiple regression analysis for the role of *AGO1* and *AGO2* variants in disease outcomes

Characteristics		*AGO1*		Adjusted OR (95% CI)	*P* value	*AGO2*		Adjusted OR (95% CI)	*P* value
		AA	AG/GG			AA/AG	G/G		
Grade	≤ 2	44 (57.9)	32 (42.1)	1	0.57	65 (85.5)	11 (14.5)	1	**0.029**
	> 2	10 (58.8)	7 (41.2)	0.71 (0.22-2.27)		9 (52.9)	8 (47.1)	**4.01 (1.15-14.02)**	
T stage	≤ 2	20 (74.1)	7 (25.9)	1	0.23	24 (88.9)	3 (11.1)	1	0.7
	> 2	34 (51.5)	32 (48.5)	1.82 (0.68-4.86)		50 (75.8)	16 (24.2)	1.30 (0.35-4.79)	
N stage	N0	29 (63)	17 (37)	1	**0.037**	40 (87)	6 (13)	1	0.53
	N1-3	25 (53.2)	22 (46.8)	**2.90 (1.03-8.17)**		34 (72.3)	13 (27.7)	1.56 (0.38-6.46)	
M stage	M0	24 (60)	16 (40)	1	**0.019**	36 (90)	4 (10)	1	0.54
	M1	19 (47.5)	21 (52.5)	**4.46 (1.18-16.87)**		29 (72.5)	11 (27.5)	1.74 (0.30-10.15)	
Clinical stage	≤ 2	31 (73.8)	11 (26.2)	1	**< 0.001**	37 (88.1)	5 (11.9)	1	0.55
	> 2	23 (45.1)	28 (54.9)	**6.54 (2.06-20.75)**		37 (72.5)	14 (27.4)	1.52 (0.39-6.00)	
NPI	Good	29 (64.4)	16 (35.6)	1	0.18	38 (84.4)	7 (15.6)	1	0.75
	Poor	25 (52.1)	23 (47.9)	1.89 (0.74-4.84)		36 (75)	12 (25)	0.80 (0.21-3.03)	
ER/PR	Negative	17 (50)	17 (50)	1	0.87	29 (85.3)	5 (14.7)	1	0.51
	Positive	26 (56.5)	20 (43.5)	0.92 (0.34-2.50)		36 (78.3)	10 (21.7)	1.57 (0.41-6.00)	
HER2$^+$	Negative	40 (61.5)	25 (38.5)	1	0.1	52 (80)	13 (20)	1	0.12
	Positive	3 (20)	12 (80)	4.33 (0.67-28.01)		13 (86.7)	2 (13.3)	0.17 (0.02-1.97)	
IHPI	Good	33 (60)	22 (40)	1	0.66	42 (76.4)	13 (23.6)	1	0.42
	Poor	21 (55.3)	17 (44.7)	1.21 (0.52-2.80)		32 (84.2)	6 (15.8)	0.63 (0.21-1.95)	
OS	Prolonged	37 (67.3)	18 (32.7)	1	**0.032**	44 (80)	11 (20)	1	0.97
	Short	17 (44.7)	21 (55.3)	**2.54 (1.08-5.99)**		30 (79)	8 (21.1)	0.98 (0.33-2.88)	
Recurrence	Negative	31 (72.1)	12 (27.9)	1	**0.001**	37 (86)	6 (13.9)	1	0.98
	Positive	23 (46)	27 (54)	**5.22 (1.73-15.74)**		37 (74)	13 (26)	1.02 (0.26-3.95)	

Data are presented as number (percentage). Binary logistic regression analysis was applied. NPI: Nottingham Prognostic Index, calculated as [0.2× tumor size in cm] + tumor grade [1-3] + lymph node stage [1-3, according to stages A-C], ER/PR: estrogen and progesterone receptor, HER2$^+$: HER2/neu receptor, IHPI: Immunohistochemical Prognostic Index estimated based on the three receptor status (HER2, ER, and PR). OR: adjusted odds ratio by clinicopathological parameters, CI: confidence interval. The values highlighted in bold are statistically significant.

tumor development and/or progression, miRNAs-related SNPs, including polymorphisms in "miRNA genes, miRNA binding site and miRNA processing machinery" [24] could have modu-

latory effects on miRNA and their target genes expression, contributing to tumorigenesis and patients' prognosis [9, 25].

In this work, we focused on AGO1 and AGO2 among human AGO subfamily as they have considerable protein and mRNA levels in many cell types compared to lower levels of other members (i.e. AGO3 and 4) [26]. The current study revealed that *AGO1*G* of rs636832 variant could confer a significant BC risk under a recessive model in our population and could be associated with poor prognostic indices, including lymph node infiltration, distant metastasis, advanced clinical stage, recurrence, and shorter overall survival. The miRNA machinery genes variants might impact miRNA maturation and regulatory function by influencing the transcription's ability of genes or protein function, thus manifesting as a change in cancer susceptibility and malignant behavior [27].

AGO1 has been found to regulate many actively transcribed genes implicated in growth, cell cycle progression and survival [28], mediating its suppressive role in cell proliferation and motility while inducing apoptosis [29]. In this sense it is not surprising that AGO1 was reported to be lost in several cancers, including "Wilms tumors, neuroblastoma, and carcinomas of the breast, liver, and colon" [30].

The rs636832 polymorphism has been suggested to affect the precursor mRNA splicing and proteins conformation and function [31] without any significant influence on *AGO1* expression [32]. Previous studies supported the association of this variant with many disorders and cancers, including depression and suicidal tendency [33], autoimmune thyroid diseases [32], Renal cell carcinoma in the context of a haplotype with another *AGO1* variant [34], lung cancer [35], and lymphatic metastasis of gastric cancer [36].

According to previous *in vitro* work done by Iio et al., "DEAD-box RNA helicase 6 (DDX6), which directly interacts with AGO1 in RISC", was reported to down-regulate miR-143 and -145 cluster expressions by prompting their host genes product degradation [37]. Interestingly, later investigations revealed that this microRNA cluster could act synergistically to regulate ERBB3, "one of the ErbB family of receptor tyrosine kinases that plays an important role in breast cancer etiology and progression" to suppress cell proliferation and invasion in BC [38]. Additionally, this cluster has showed functional properties and expression patterns typical for tumor suppressors in malignant epithelial cells and stromal fibroblasts in BC [39]. We speculated that the aforementioned molecular players might explain in part the association of the study *AGO1* variant with BC susceptibility and poor prognosis at the cellular level. However, this will need further functional validation studies in the near future.

Although in our study, *AGO2* rs2977490 variant frequencies did not show a difference between BC cases and controls, the *AGO2*G/G* genotype showed significant association with poor pathological grade in our cases. Given that this variant is located in the intron of *AGO2* gene, this polymorphism may not affect the AGO2 quantitation as previously evidenced [32]. However, by creating distinct sequences caused by the study polymorphism, conformational changes of AGO2 might be associated with binding to different miRNAs which are associated with cancer progress and prognosis [32]. In this regard Mullany et al., also did not observe a significant association between this polymorphism and colon cancer risk or the corresponding mRNA expression in their genome-wide association study [40].

By repressing mRNA translation or inducing deadenylation-dependent mRNA decay, AGO2-associated RISC contributes to silencing of gene expression and "non-redundant slicer-independent function" [41-44]. Of note, AGO2 is the only member among human AGO protein family (AGO1-4) implied in the endonuclease activity that is independent on the nature of the guide RNAs [45-47]. Interestingly, apart from miRNA biogenesis regulation, recent findings highlighted the non-canonical functions of Argonaute proteins [48]. Independent of the catalytic activity, AGO2 can bind directly to transfer RNA genes and promotes their repression [49]. Additionally, it can recruit the chromatin-modifying enzymes as methyltransferase and acetyltransferase (alongside DNA damage-induced RNAs) to reconfigure damaged DNA upon double-strand break, facilitating the repair process and maintaining the genome integrity [50]. The diversity of these canonical and non-canonical functions could reflect the importance and implication of Argonaute proteins in several oncogenic pathways and cancer prognosis.

AGO2 deregulation has been associated previously with cancer progression [47, 51], and a growing body of evidence has reported AGO2 deregulation also in BC, but with conflicting results [51, 52]. Interestingly, recent findings of Bellissimo et al. supported the essential contribution of AGO2 to miR-145-5p tumor-suppressor activity in BC [53]. Functionally, the later microRNA could not exert its inhibitory effect on cell migration without the presence of AGO2.

Although a number of studies suggested an association between other variants of *AGO2* and the risk of BC [10, 13], currently to our knowledge, there are no literature we identified investigated *AGO2* rs2977490 polymorphism with BC.

Taken together, we can conclude that *AGO1* rs636832 and *AGO2* rs2977490 variants could be implicated in BC risk and prognosis in the study population. These variants might be included within the BC susceptibility/poor prognosis-associated variant list that could be helpful in risk stratification and targeted therapy of BC patients in near future. However, the present results will require validation in larger multicentre BC cohorts, and further laboratory-based functional studies will be needed to uncover the molecular basis by which these variants were implicated in BC.

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Disclosure of conflict of interest

None.

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