miRNA expression pattern associated with prognosis in elderly patients with advanced OPSC and OCC

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Abstract. The long-term survival for elderly patients with advanced ovarian papillary serous carcinoma (OPSC) does not exceed 30%, and the incidence and prognosis rise continuously after menopause. The aim of this study was to identify the differences in key miRNAs and their potential regulators through miRNA microarray analysis, functional target prediction, and clinical outcome between the elderly patients with advanced OPSC and ovarian clear cell carcinoma (OCC) who all suffered poor prognosis, to identify the pathogenetic basis, and to improve the understanding of the molecular basis of advanced OPCS in elderly patients. Through microarray analysis, we found 52 unique miRNAs with significant fold-change in expression levels, of which 9 were upregulated, whereas 43 were downregulated in OCC patients compared to elderly OPSC patients with advanced stage. Among these prediction miRNAs, miR-30a*, miR-30e* and miR-505* were found to have some common cancer-related targets. Lower expression of these three miRNAs of advanced OPSC in elderly patients, all associated with significantly poorer survival rate, strongly suggesting that they could be critical oncogenes and take important roles in OPSC etiology in elderly patients with advantaged stage. Functional analyses support the above hypothesis. Their targets, ATF3, STMN1 and MYC suggest that OPSC also has aggressive biological behavior when presented with advanced stage, and support the epidemiology results that incidence and mortality of advanced OPSC increases continuously. miR-30a*, miR-30e* and miR-505* may be important pathogenetic factors for OPSC in elderly patients with advanced stage. Age could be regarded as a continuous covariate in this process. This may improve the understanding of molecular underpinnings of advanced OPSC in elderly patients, and provide improved diagnostic, prognostic and therapeutic approaches.

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Introduction

The increasing age of the world's population has been the most dramatic demographic change since the 20th century (1). As the fifth leading cause of death in women and the first cause of gynecologic cancer death, ovarian cancer incidence and mortality rate increase continuously with advancing age and peaks in the 7th to 8th decades of life (2-6). Due to lack of symptoms in early-stage of this disease, in China, indeed the long-term survival for elderly patients with advanced disease does not exceed 20%. Therefore, the poor prognosis of ovarian cancer in the elderly has been recently recognized (4). A prior report showed that, 28,082 women were diagnosed with epithelial ovarian cancer (EOC) from 1988 to 2001. The largest histology subgroup, 49.3% (13,835) of patients had ovarian papillary serous carcinoma (OPSC) (7). OPSC increases steadily after menopause, and the major patients of OPSC with advanced-stage (FIGO stages III or IV) diagnoses have a <30% 5-year survival rate (7,8). Thus, age should be considered as an important prognostic variable. In addition, ovarian clear cell carcinoma (OCC) was frequently present at early stage (9), and has a distinct, aggressive biologic behavior with poor survival rates compared to other epithelial counterparts (7,10-12). Given that elderly advanced OPSC and OCC all carry poor prognosis, it is advantageous to study them together, find the essential differences of cancer epidemiology and biology, and then improve the understanding of the molecular basis which plays an important part in pathogenesis of elderly advanced OPCS or OCC cases.

MicroRNAs (miRNAs) are small non-coding RNA molecules of ~22 nucleotides that act post-transcriptionally to regulate gene expression (8). They show great potential in discovering new biological pathways; could be used as a diagnostic and prognostic tool for cancer patients and even serve as molecular targets for therapy.

Considering the above evidence, the OPSC patients aged ≥50 years with advanced-stage and OCC patients, who all carry poor prognosis, were studied. The goal was to identify the differences of key miRNA and possible regulators through miRNA microarray chip, functional target prediction, and clinical outcome between the elderly advanced OPSC and OCC

patients to find the pathogenic basis, and to provide insight into clinical diagnosis and therapy for advanced OPSC, especially in elderly patients.

Materials and methods

Patients and samples. Patients, who were surgically treated for ovarian cancer at the Obstetrics and Gynecology Hospital, Dalian, China between July 2004 and November 2010 were identified. The study was approved by a central and by institutional ethics committees. Corresponding 58 tumor specimens; OPSC (n=30); OCC (n=28) were dissected during the operations, and formalin fixed paraffin embedded (FFPE) blocks were further obtained after surgery. All pathological specimens from primary surgery were reviewed by two independent pathologists with no knowledge of patients' clinical data. Cases were classified according to the FIGO staging system. All cases were newly diagnosed, and only serous papillary (≥50 years and FIGO stages III or IV tumors) and OCC histology were included.

MicroRNA array and data analysis. A microarray platform optimized for the analysis of a panel of 768 human miRNAs was used to analyze and compare the pattern of miRNA expression between OCC (n=9) and elderly advanced OPSC (n=8). Total RNA that enriched miRNAs was extracted from the FFPE tissue by using the Ambion mirVana microRNA isolation kit (Ambion, Austin, TX). The quality of total RNA was assessed using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA). Individual quantitative real-time polymerase chain reaction assays were formatted into a TaqMan low-density array (TLDA; Applied Biosystems), which was performed at the Shannon McCormack Advanced Molecular Diagnostics Laboratory Research Services, Dana Farber Cancer Institute, Harvard Clinic and Translational Science Center. The normalized microarray data were managed and analyzed by Statminer version 3.0 (Integromics™).

MiRNA targets prediction and pathway analysis. The analysis of miRNA predicted targets was determined using several computational approaches, the MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and miRBase (http://www.mirbase.org/). The target prediction algorithm used here is estimated to have a 20-30% false positive rate. This level of false discovery is unlikely to affect the overall network findings obviously, although the number of top predicted gene targets is large. Functional analysis of these predicted gene targets identified biologic pathways with significant involvement for gene expression. In order to retrieve only the most relevant targets, we listed only genes targeted by the miRNAs that were differentially expressed in the patient with OPSC and OCC. To further understand and interpret literature information of our unique miRNAs, an analysis of biologic pathway relationships was performed using commercially available software (Ingenuity Systems, Redwood City, CA).

Quantitative real-time RT-PCR. To validate key microarray results, quantitative reverse transcription was performed using the TaqMan MicroRNA Reverse Transcription Kit [Applied Biosystems (ABI), Foster City, CA] with ABI miRNA specific primers and primer kits on an Agilent Technologies Stratagent Mx3000P. Specific kits used were as follows: hsa-miR-505*: ABI#4395198; hsa-miR-30a*: ABI#4373062; hsa-miR-30e*: ABI#4427975. Relative expression levels were calculated using the comparative Ct ($2^{-\Delta\Delta Ct}$) method with U6 small nuclear RNA as the endogenous control. Samples were analyzed in triplicate. Technical validations were carried out using a subset of the original samples that were used in the discovery phase of the study with miRNA array. Biological validation was performed using additional FFPE cases of OPSC and OCC (n=21 in total) (13).

qRT-PCR was performed to validate target prediction results. Total RNA (0.5 μg) in 1 μl of RNase-free water was used in 20 μl of RT mix. Primer pairs were as follows: activating transcription factor 3 (ATF3) forward 5'-CTGCAGAAAGAGTCGGAG-3' and reverse 5'-TGAGCCCGGACAATACAC-3'; β-actin forward 5'-GACTACCTCATGAAGATC-3' and reverse 5'-GATCCACA TCTGCTGGAA-3' (Invitrogen). Real-time PCR was done using the LightCycler system and Roche fast-Start Light Cycler-Master Hybridization Probes master mix (Roche Diagnostics), and the product was detected with SYBR-Green I. Samples from at least three independent experiments, each measured in duplicate, were analyzed and the data expressed as the averages ± SE.

Immunohistochemistry (IHC). The IHC staining procedure was described previously (14) with modification. Briefly, after dewaxing in xylene and descending concentrations of ethanol, the sections were incubated in 3% H_2O_2 for 30 min to suppress endogenous peroxidase activity. The sections were further blocked with a mixed solution [10% goat serum and 3% albumin bovine (BSA) in PBS] for 1 h and incubated with the primary antibodies of ATF3 (1:100 dilution; Bioworld, cat#BS1245); Stmn1 (1:100 dilution; Bioworld, cat#BS3615); or MYC (1:100 dilution; Bioworld, cat#BS2261) overnight at 4˚C. On the second day, after rinsing for 10 min x 3, the sections were incubated with streptavidin-peroxidase staining system kit according to the manufacturer's instructions (SP-9001, Zhongshan Golden Bridge Biotechnology Company, Beijing, China). Finally, 3,3'-diaminobenzidine (DAB) liquid chromogen substrate kit (ZLI-9032, Zhongshan Golden Bridge Biotechnology Company) offered a clean detection performance and subsequent hematoxylin staining provided a light blue counter stain contrasting with DAB. For negative controls, all the conditions were performed at the same procedures except that primary antibody was eliminated.

Statistical analysis. The results were analyzed by SPSS 15.0 (Chicago, IL). Data are expressed as arithmetic means ± SEM of the number (n) of experiments. Samples were analyzed with repeated measures analysis of variance; differences in the incidence were analyzed using ANOVA. The differences of the positive area and integrate optical density (IOD) per vision-field of x400 immunohistochemistry photographs were taken with Image-Pro plus vision 6.0. Overall survival was defined as the time from initial cytoreductive surgery to date of last follow-up or death. Survival time course was studied using the Kaplan-Meier method, and groups were compared using the log-rank test. $P<0.05$ was considered statistically significant.

Table I. Clinicopathological characteristics of the patients.

Characteristics	Total (n=58) No. (%)	Advance OPSC ≥50 (n=30) No. (%)	OCC (n=28) <50 (n=16) No. (%)	OCC (n=28) ≥50 (n=12) No. (%)	OCC (n=28) Total OCC (n=28) No. (%)	P-value[a]
Age at diagnosis						
Median	54.1	59.9	38.6	54.7	45.5	0.035
Range	(29-74)	(50-74)	(29-48)	(50-62)	(29-62)	
FIGO stage at diagnosis						<0.001[c]
I	23 (39.7)	0 (0.0)	12 (75.0)	11 (91.7)	23 (82.1)	
IA	13 (22.4)	0 (0.0)	6 (37.5)	7 (58.3)	13 (46.4)	
IC	10 (17.2)	0 (0.0)	6 (37.5)	4 (33.3)	10 (35.7)	
II	2 (3.4)	0 (0.0)	2 (12.5)	0 (0.0)	2 (7.1)	
IIC	2 (3.4)	0 (0.0)	2 (12.5)	0 (0.0)	2 (7.1)	
III[b]	31 (53.4)	28 (93.3)	2 (12.5)	1 (8.3)	3 (10.7)	
IIIB	3 (5.2)	1 (3.3)	1 (6.3)	1 (8.3)	2 (7.1)	
IIIC	27 (46.6)	26 (86.7)	1 (6.3)	0 (0.0)	1 (3.6)	
IV	2 (3.4)	2 (6.7)	0 (0.0)	0 (0.0)	0 (0.0)	
Grade[d]						
1	2 (3.4)	1 (3.3)	0 (0.0)	1 (8.3)	1 (3.6)	
2	9 (15.5)	6 (20.0)	1 (6.3)	2 (16.7)	3 (10.7)	
3	29 (50.0)	22 (73.3)	4 (25)	3 (25.0)	7 (25.0)	
Unknown	18 (31.0)	1 (3.3)	11 (68.8)	6 (50.0)	17 (63.0)	
Lymphadenectomy						
Yes	44 (75.9)	20 (66.7)	15 (93.8)	9 (75)	24 (85.7)	
No	12 (20.7)	9 (30.0)	1 (6.2)	2 (16.7)	3 (10.7)	
Unknown	2 (3.5)	1 (3.3)	0 (0.0)	1 (8.3)	1 (3.6)	
Median no. nodes resected	20	19	18	23	20	
Presence of positive nodes						0.002
Yes	14 (24.1)	12 (40.0)	1 (6.3)	1 (8.3)	2 (7.1)	
No	30 (51.7)	8 (26.7)	14 (87.5)	8 (66.7)	22 (78.6)	
Unknown	14 (24.1)	10 (33.3)	1 (6.3)	3 (25.0)	4 (14.3)	

FIGO, International Federation of Obstetrics and Gynecology. [a]Difference between groups calculated by AVON test for age and χ^2 test for all others. [b]Numbers do not add up to 100% due to small numbers of patients with unknown status. [c]FIGO stage according to early stage (stages I and II) and advanced stage (stages III and IV) in elder advanced OPSC and total OCC patients. [d]Grade according to the American Joint Committee on Cancer (AJCC) of ovarian cancer.

Results

Patient characteristics. Patient characteristics are shown in Table I. Fifty-eight patients were identified to fit the study criteria, including 30 OPSC patients (age ≥50 years with advanced-stage) and 28 OCC patients. The percentage of patients with stage III disease and the presence of positive lymph nodes were all significantly higher in those patients with elderly advanced OPSC compared with those patients with elderly OCC (8.3 vs. 93.3%).

miRNA expression pattern of OPSC and OCC. miRNAs have been reported in different types of tumors derived from different organs, including ovarian cancer. However, the pathobiological significances of aberrant miRNA expression in human ovarian cancer have not been well documented. To further characterize the unique miRNAs in EOC development, we initially analyzed miRNA expression in tumors of 8 elderly OPSC patients with advanced stage and 9 OCC patients for microarray analysis. According to the background-subtracted and normalized fluorescent intensities of array sample results, 52 unique miRNAs were detected with significant ($p<0.00001$ for all), fold-change (FC) in expression level, of which 9 were upregulated, whereas 43 miRNAs were downregulated in OCC patients compared to elderly OPSC patients with advanced stage (Table II and Fig. 1A). Moreover, among the top significant unique miRNAs (FC >4), 9 were preferentially expressed in the OCC patients, whereas only 1 miRNA was most highly expressed in the elderly OPSC with advanced stage (Table II). The prediction targets of these 9 miRNAs are shown in Fig. 1B and C.

Table II. The miRNA expression in elderly OPSC and OCC patients with advanced stage.

miR name	P-value	FDR (%)[a]	Fold-change (FC)	Fold-change grade	Higher expression associated with	Cancer involvement[b]
hsa-miR-130a*	1.40E-08	<0.01	0.003552833	IV	OPSC	Brain, liver
hsa-miR-188-3p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-29b-2*	3.33E-07	<0.01	10.83658116	III	OCC	Thyroid, uterus, AML
hsa-miR-208a	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-208b	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-219-2-3p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-220b	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-220c	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-30a	1.47E-06	<0.01	4.376533535	I	OPSC	Lung, colon, myeloid leukemia, renal
hsa-miR-30a*	1.30E-08	<0.01	11.97926536	II	OCC	Colon, breast, esophagus, bladder
hsa-miR-30c-2*	2.72E-07	<0.01	226.4780393	IV	OCC	Endometrial, stomach, ovarian, breast, colon
hsa-miR-30e*	5.97E-09	<0.01	8.945290166	II	OCC	Ovarian, brain, epithelium, esophagus, breast, lung, colon
hsa-miR-302a	8.87E-06	<0.01	0.62304526	I	OPSC	Skin, germ cell
hsa-miR-302c	8.87E-06	<0.01	0.62304526	I	OPSC	Breast
hsa-miR-325	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-326	8.87E-06	<0.01	0.62304526	I	OPSC	Brain
hsa-miR-346	8.87E-06	<0.01	0.62304526	I	OPSC	Follicular thyroid
hsa-miR-367	8.87E-06	<0.01	0.62304526	I	OPSC	Ependymomas
hsa-miR-376b	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-377	8.87E-06	<0.01	0.62304526	I	OPSC	Breast
hsa-miR-380	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-384	8.87E-06	<0.01	0.62304526	I	OPSC	Laryngeal, breast
hsa-miR-448	8.87E-06	<0.01	0.62304526	I	OPSC	Prostate, breast
hsa-miR-485-5p	8.87E-06	<0.01	0.62304526	I	OPSC	Ovarian
hsa-miR-490-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Colon
hsa-miR-496	8.87E-06	<0.01	0.62304526	I	OPSC	Ovary, AML
hsa-miR-505*	4.98E-06	<0.01	6.122042066	II	OCC	Bladder
hsa-miR-516b	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-518a	8.87E-06	<0.01	0.62304526	I	OPSC	Follicular lymphoma
hsa-miR-518c	8.87E-06	<0.01	0.62304526	I	OPSC	Colon, bladder
hsa-miR-518d-5p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-519c-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Lung
hsa-miR-520e	8.87E-06	<0.01	0.62304526	I	OPSC	Cholangio, liver
hsa-miR-524-5p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-525-5p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-544	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-548a-5p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-548b-3p	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-556-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Prostate
hsa-miR-556-5p	8.87E-06	<0.01	0.62304526	I	OPSC	Prostate
hsa-miR-576-5p	8.87E-06	<0.01	0.62304526	I	OPSC	Ovary
hsa-miR-615-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Breast, colon, prostate, blood, ovary

Table II. Continued.

miR name	P-value	FDR (%)[a]	Fold-change (FC)	Fold-change grade	Higher expression associated with	Cancer involvement[b]
hsa-miR-615-5p	8.87E-06	<0.01	0.62304526	I	OPSC	Breast, colon, prostate, blood, ovary
hsa-miR-628-3p	2.43E-07	<0.01	6.140440621	II	OCC	Brain, breast, thyroid
hsa-miR-624	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-875-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Pancreas
hsa-miR-885-3p	8.87E-06	<0.01	0.62304526	I	OPSC	Lung, brain
hsa-miR-885-5p	5.11E-06	<0.01	8.409273981	II	OCC	Renal, brain, ovary
hsa-miR-890	8.87E-06	<0.01	0.62304526	I	OPSC	Nasopharyngeal
hsa-miR-891b	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-miR-892a	8.87E-06	<0.01	0.62304526	I	OPSC	
hsa-let-7e*	5.99E-06	<0.01	4.703680442	I	OPSC	Head and neck, retinoblastoma, pleural

OCC patients vs. stage III, IV of elderly OPSC with advanced stage, p<0.00001 for all. FDR, false discovery rate; FC, fold-change; AML, acute myeloid leukemia; FC grade I, 1<FC ≤5; grade II, 5<FC ≤10; grade III, 10<FC ≤15; grade IV, FC >200. [a]1% FDR predicts that this list is 99% accurate. [b]Information obtained from PubMed (http://www.ncbi.nlm.nih.gov/pubmed/), Elsevier ScienceDirect (http://www.sciencedirect.com/) and previous reports.

Unique miRNAs and their co-target prediction. The Micro Cosm Targets version 5 and miRBase were used to analyze predicted targets for the 10 top significantly unique miRNAs highly expressed in OPSC vs. OCC with p-values <0.00001, and FC >4-fold. The target prediction algorithm used here is estimated to have a 20-30% false positive rate. This level of false discovery is unlikely to affect the overall network findings obviously, although the number of top predicted gene targets is large. Functional analysis of these predicted gene targets identified biologic pathways with significant involvement for gene expression. In order to retrieve only the most relevant targets, we listed only genes targeted by miR-30a*, miR-30e* and miR-505* that were found having some targets in common suggesting they might play important roles in pathogenesis between OCC and elderly advanced OPSC together or partly (Figs. 1D and 2). Fig. 3 show the function analysis of miR-30a*, miR-30e* and miR-505*.

qRT-PCR validation for microarray results. In order to confirm microarray results, RNA was isolated from a new set of FFPE tissues as described above to increase the likelihood that the observed differences in miRNA expression profiles represent biologically significant changes. MiR-30a*, miR-30e* and miR-505* were the upregulated miRNAs in OCC with different fold-changes (from 6-12) when compared with OPSC by using microarray analysis. Validation of miRNA expression analysis was repeated with qRT-PCR (miR-30a*, miR-30e* and miR-505*) and representative analyses are shown in Fig. 4A (unsupervised hierarchical clustering of validation) and Fig. 4B. Through this additional analysis, the expression patterns found in the arrays could be confirmed. In keeping with microarray results, miR-30a*, miR-30e* and miR-505* were all highly expressed in OCC with statistical significance.

Validations of miRNA target prediction and their top co-targets. In target prediction experiment using multiple software, ATF3 was predicted as a potential co-target of miR-30a*, miR-30e* and miR-505*, and presented as a regulator in the different pathways, which include cancer and cell death. At the same time, we found that MYC is the co-target of miR-30a* and miR-30e*; stathmin1 (STMN1) is co-target of miR-30a* and miR-505*; HLA-DPB1 is co-target of miR-30e* and miR-505*. To examine the biological significance of these miRNAs in ovarian cancer, we focus on the ATF3, STMN1 and MYC, which were already proven to be cancer markers.

Immunohistochemical assay for the ATF3, STMN1 and MYC proteins showed a relevant upregulation in OPSC cells compared to OCC cells (Fig. 5A). It is clear that these co-targets were all extensively distributed in the cytoplasm of cancer cells in the tissue of OPSC samples (Fig. 5A1-3) comparing with the OCC sections (Fig. 5A4-6). Through analysis with Image-Pro plus vision 6.0, positive area and IOD per vision-field of x400 immunohistochemistry photographs were detected. These results also support the conclusions of the immunohistochemical assay (Fig. 5B and C).

Overall survival analysis of elderly advanced OPSC and OCC patients. We next compared the prognosis of elderly advanced OPSC patients in groups stratified according the expression levels of individual miRNAs. For each miRNA, we divided the samples into two sub-sections according to high and low expression level of the miR-30a*, miR-30e* and miR-505* (Fig. 6A-C). The association of these three miRNAs with survival indicated that lower expression of miR-30a*, miR-30e* and miR-505*, all associated with poorer prognosis. We also studied the overall survival of ovarian cancer patients with OPSC and OCC (Fig. 6D). They were

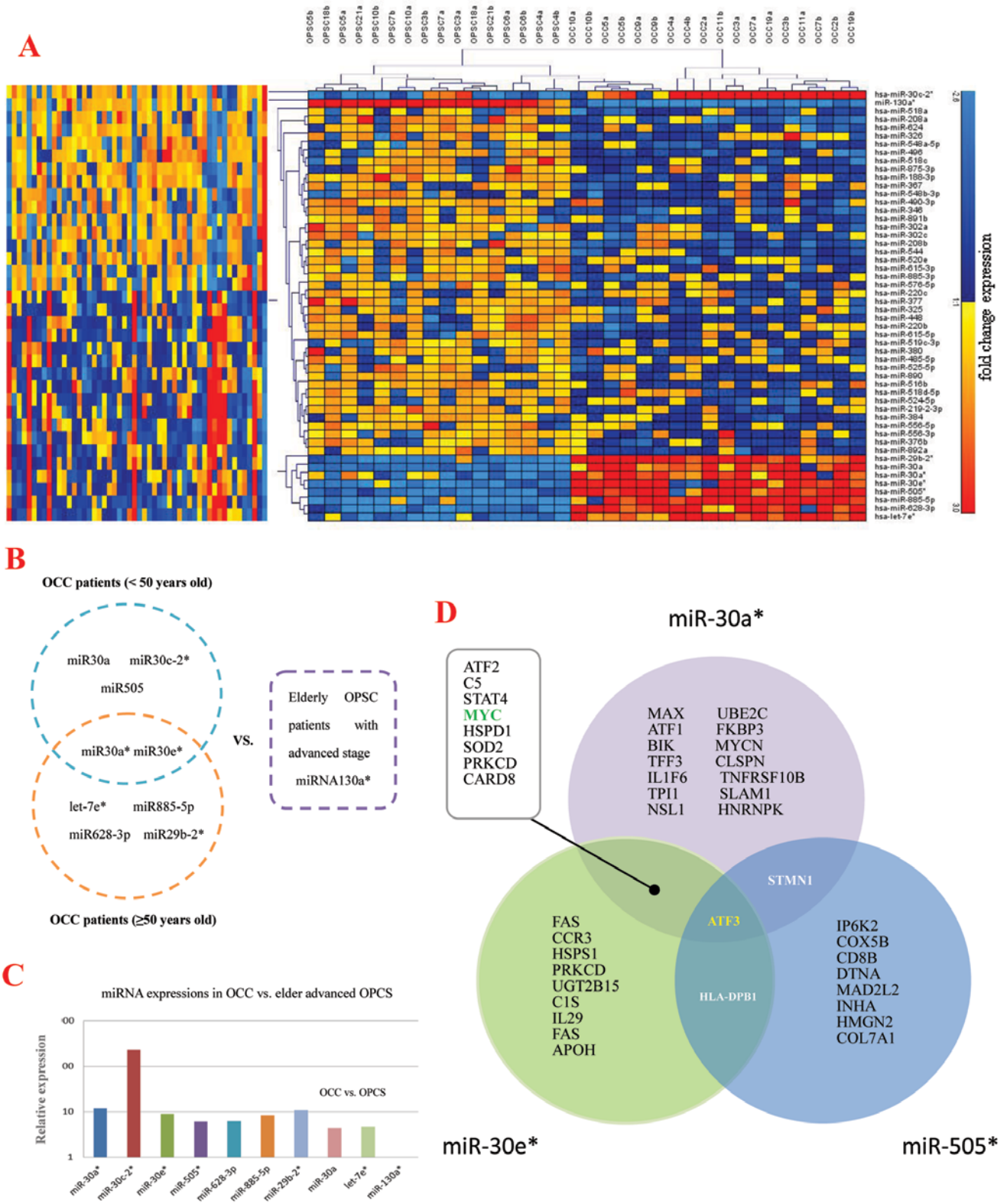

Figure 1. Target predictions of miRNAs through microarray analysis. (A) Results of the unsupervised hierarchical clustering of microarray assay by qRT-PCR. Among the top significantly unique miRNAs (fold-change >4, FDR <0.01, B and C) using microarray platform to predict target miRNAs, 9 miRNAs are preferentially expressed in the OCC patients when compared to the elderly OPSC with advanced stage, and their relative expressions are shown in the column map. Only miR-130a*, is highly expressed in OPSC (its FC value is 0.0035528). These 9 miRNAs are distributed in different ages of OCC patients, and miR-30a* and miR-30e* are the co-prediction targets of younger (<50 years) and elderly (≥50 years) OCC patients. (D) Venn diagram of selected miRNAs and their putative gene targets. The MicroCosm Targets version 5 and miRBase were used to analyze predicted targets for the 10 top significantly unique miRNAs which were highly expressed in OPSC vs. OCC with p<0.00001, and change >4-fold. Three miRNAs were found with some common targets, including miR-30a*, miR-30e* and miR-505*. These events suggest they might have an important role in ovarian cancer differences between OCC and elder advanced OPSC together or partly, and ATF3 is the co-target of these relevant miRNAs.

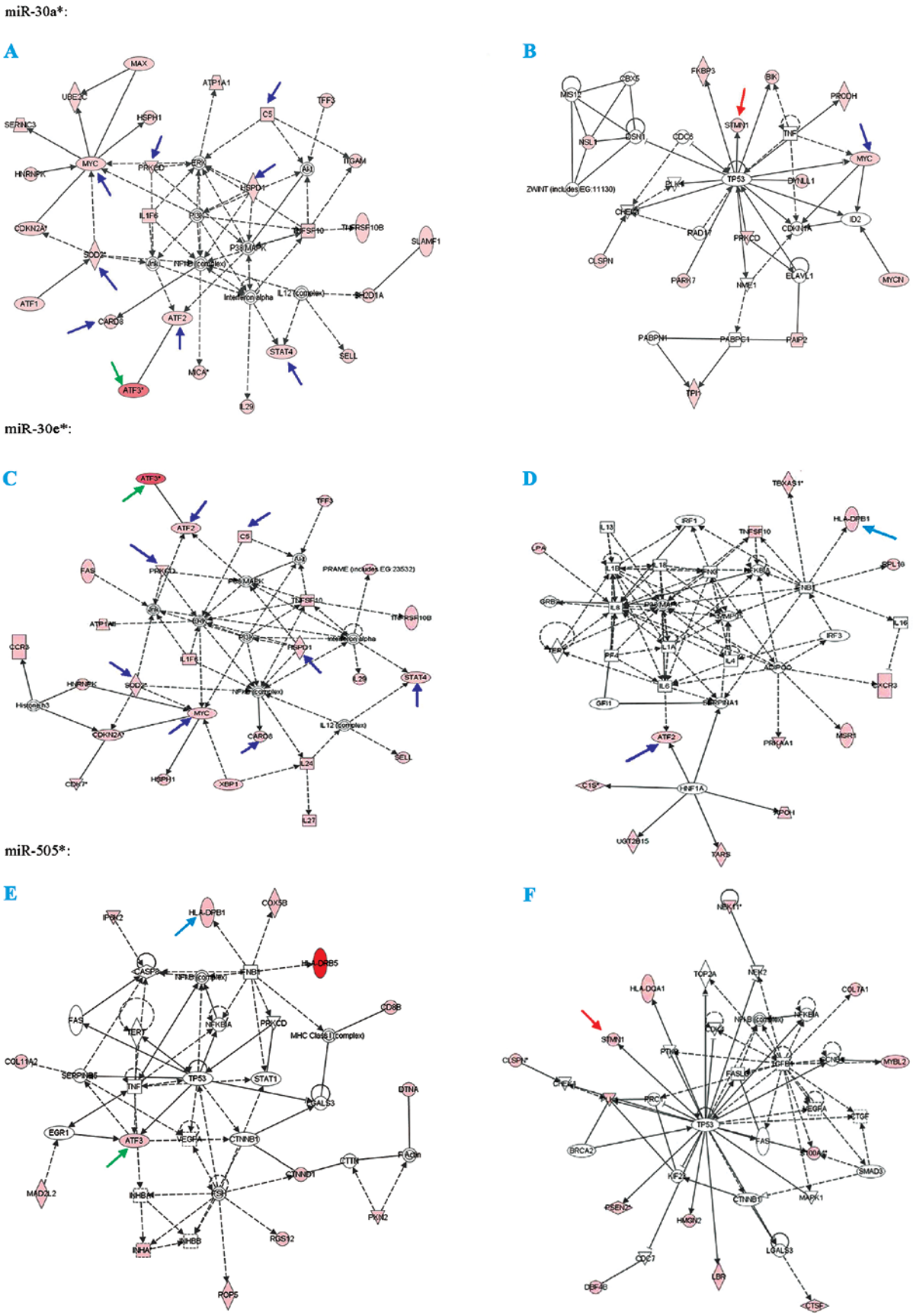

Figure 2. The analysis of prediction protein targets for miR-30a*, miR-30e* and miR-505* were determined through using the MicroCosm Targets version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5/) and miRBase (http://www.mirbase.org/). Green arrows, co-targets of miR-30a*, miR-30e* and miR-505*; light blue arrows, co-targets of miR-30e* and miR-505*; blue arrows, co-targets of miR-30a* and miR-30e*; and red arrows, co-targets of miR-30a* and miR-505*. Associated network functions are: (A) DNA replication, recombination and repair, cancer, cell death of miR-30a*; (B) cell cycle, cancer, cell death of miR-30a*; (C) cell morphology, cancer, cell death of miR-30e*; (D) dermatological diseases and conditions, cellular movement, hematological system development and function of miR-30e*; (E) cancer, cellular growth and proliferation, cell death of miR-505*; and (F) cell cycle, cancer, renal and urological disease of miR-505*.

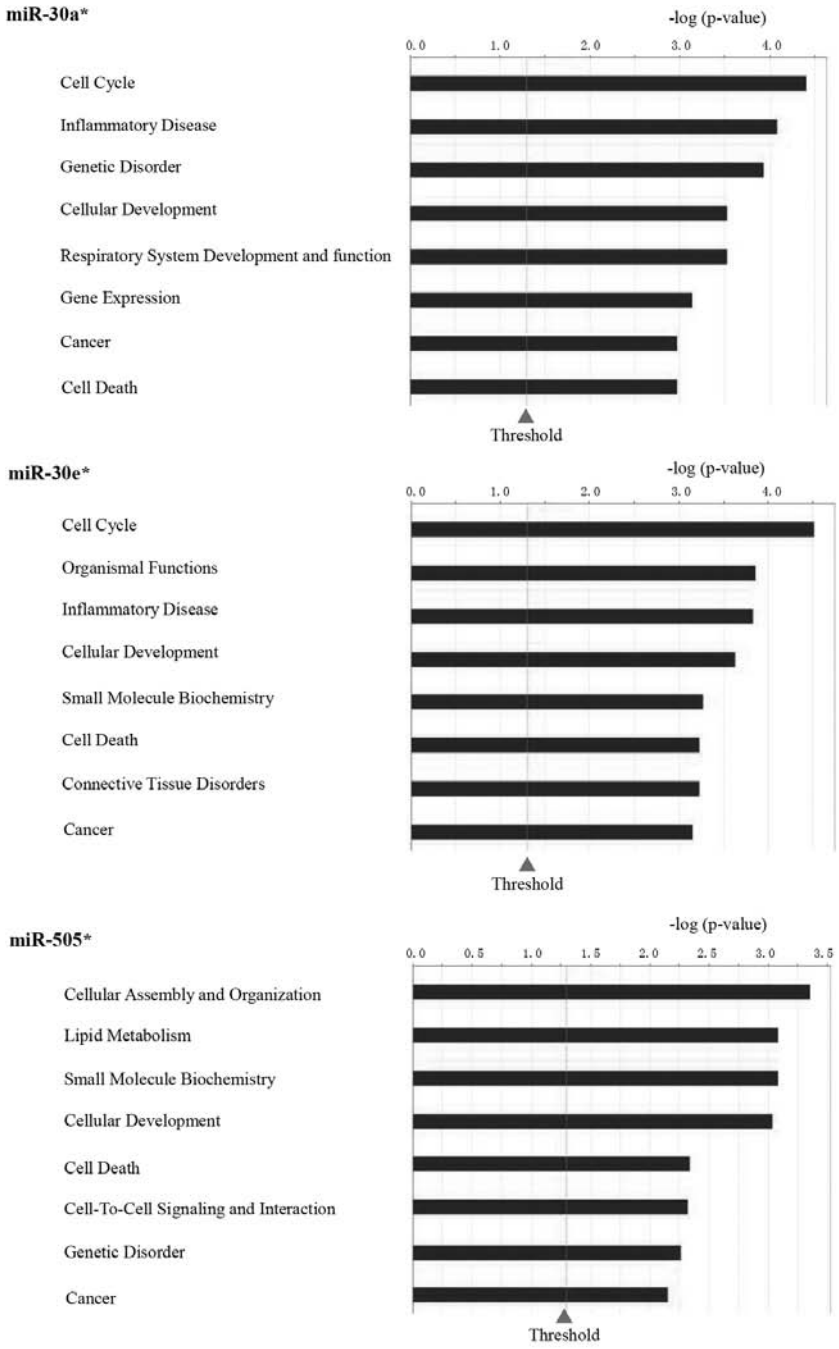

Figure 3. Analysis of miR-30a*, miR-30e* and miR-505*.

associated with significant differences (OPSC was lower, log-rank p<0.01) in overall survival.

Discussion

EOC is the most important cause of gynecologic malignancy-related mortality in women (15) rising continuously with advancing age. Some recent reports show that cancer incidence is 11-fold greater for the older population (4,16). Therefore, it is necessary to study and understand cancer epidemiology, biology and therapy to the elderly patient. The incidence of OPSC increases steadily after menopause, and this histologic subgroup makes up the largest part of EOC patients.

Considering that for most women menopause is after the age of 50 years, OPSC patients aged ≥50 years with advanced-stage as well as OCC patients were studied. The purpose was to apply microarray analysis to investigate the difference, especially molecular mechanism between elderly OPSC with advanced stage and OCC, which both carry poor prognosis (8,9). Through miRNA microarray and target prediction analysis, 10 miRNAs were found to be differentially expressed in tumor from OPSC vs. OCC (p<0.00001 and FC >4). Moreover, in order to retrieve only the most relevant targets, we only investiged three miRNAs (miR-30a*, miR-30e* and miR-505*) which were found with some common cancer associated pathways. We confirmed these predictions and relations. Biological pathways were predicted

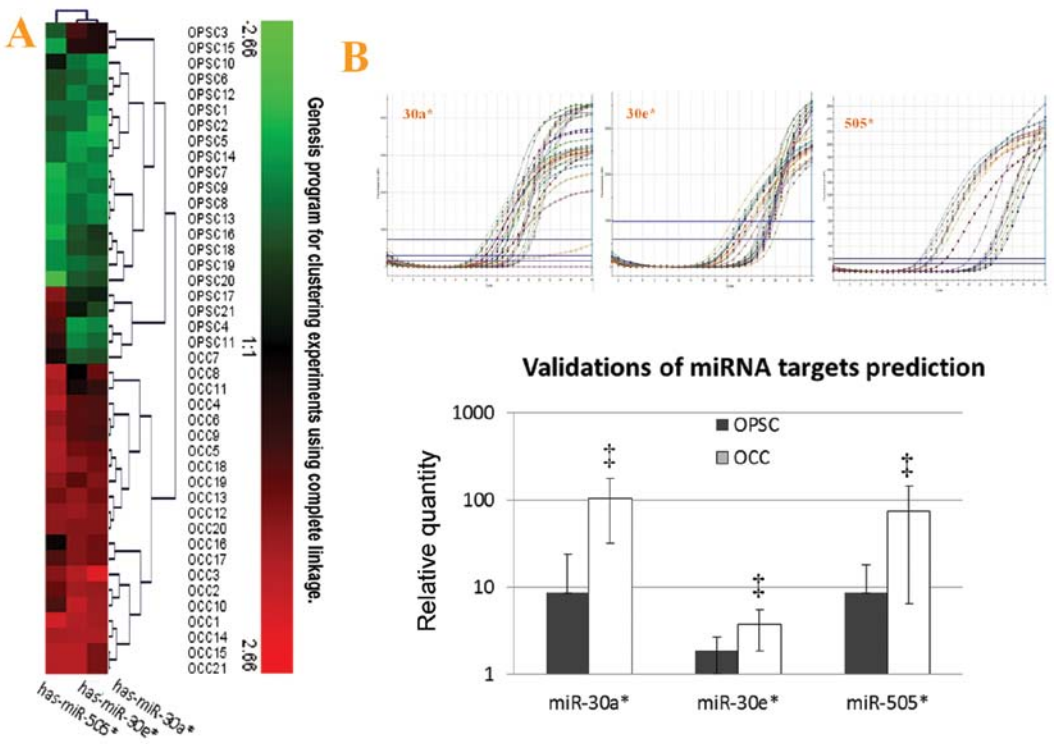

Figure 4. MicroRNA validation with real-time PCR for microarray analysis. (A) Unsupervised clustering of validation set samples, according to miR-30a*, miR-30e* and miR-505* expressions by qRT-PCR. (B) Column map of target validation with qRT-PCR for miR-30a*, miR-30e* and miR-505*, which were found with some common targets. In keeping with microarray results, miR-30a*, miR-30e* and miR-505* were highly expressed in OCC with significance. Compared with the OCC, ‡p<0.01.

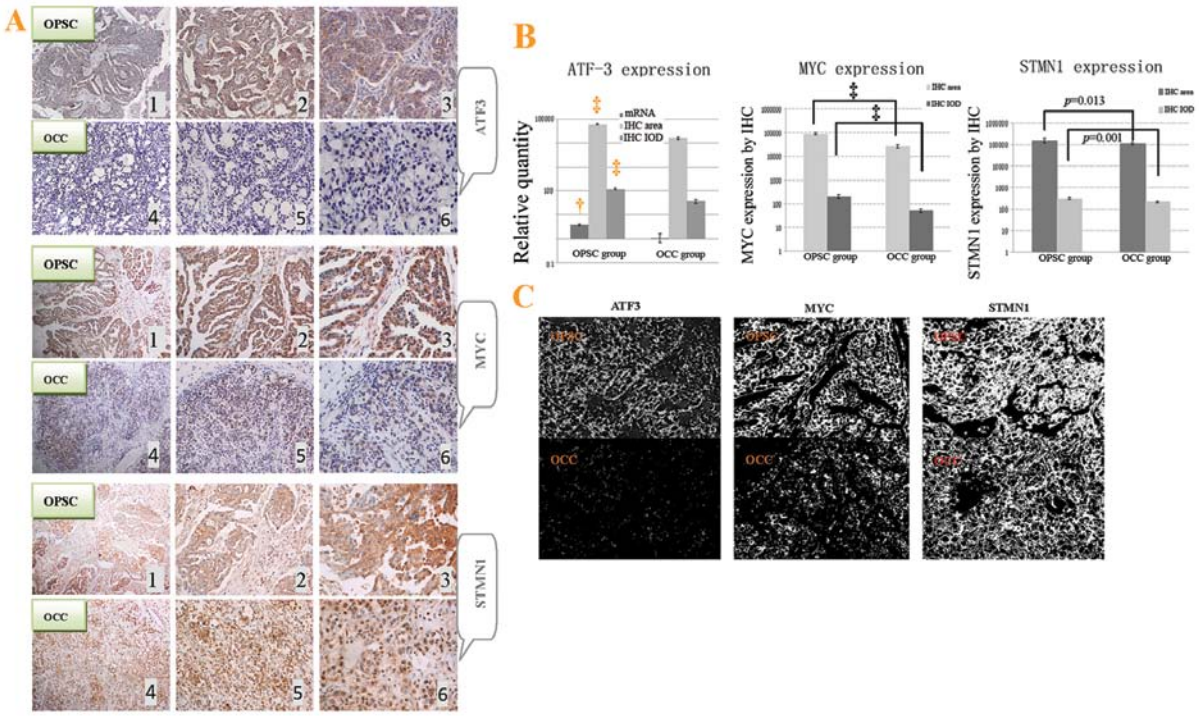

Figure 5. ATF3, STMN1 and MYC protein expressions in paraffin-embedded tissues in the immunohistochemical assay, and overall survival of ovarian cancer patients with elderly advanced OPSC and OCC. (A) Different expression of ATF3, STMN1 and MYC protein between OPSC and OCC cells by immunohistochemistry (IHC) with hematoxylin counter staining. ATF3, STMN1 and MYC, are extensively expressed in the cytoplasm of OPSC tumor cells (A1, 2 and 3) and poorly expressed in OCC tissues (A4, 5 and 6). Magnifications from left to right are x100, x200 and x400, respectively. (B) As the only co-target of these miRNAs, ATF3 mRNA expression was also detected through real-time PCR. Different expressions for positive area and integrate optical density (IOD) of ATF3, STMN1 and MYC (per vision-field of x400 photograph taken with Image-Pro plus vision 6.0) between OPSC and OCC tumor through immunohistochemistry staining, respectively, are shown. (C) Image (x400) showing positive staining area of above proteins by Image-Pro plus vision 6.0, respectively. Compared with the OCC, †p<0.05, and ‡p<0.01.

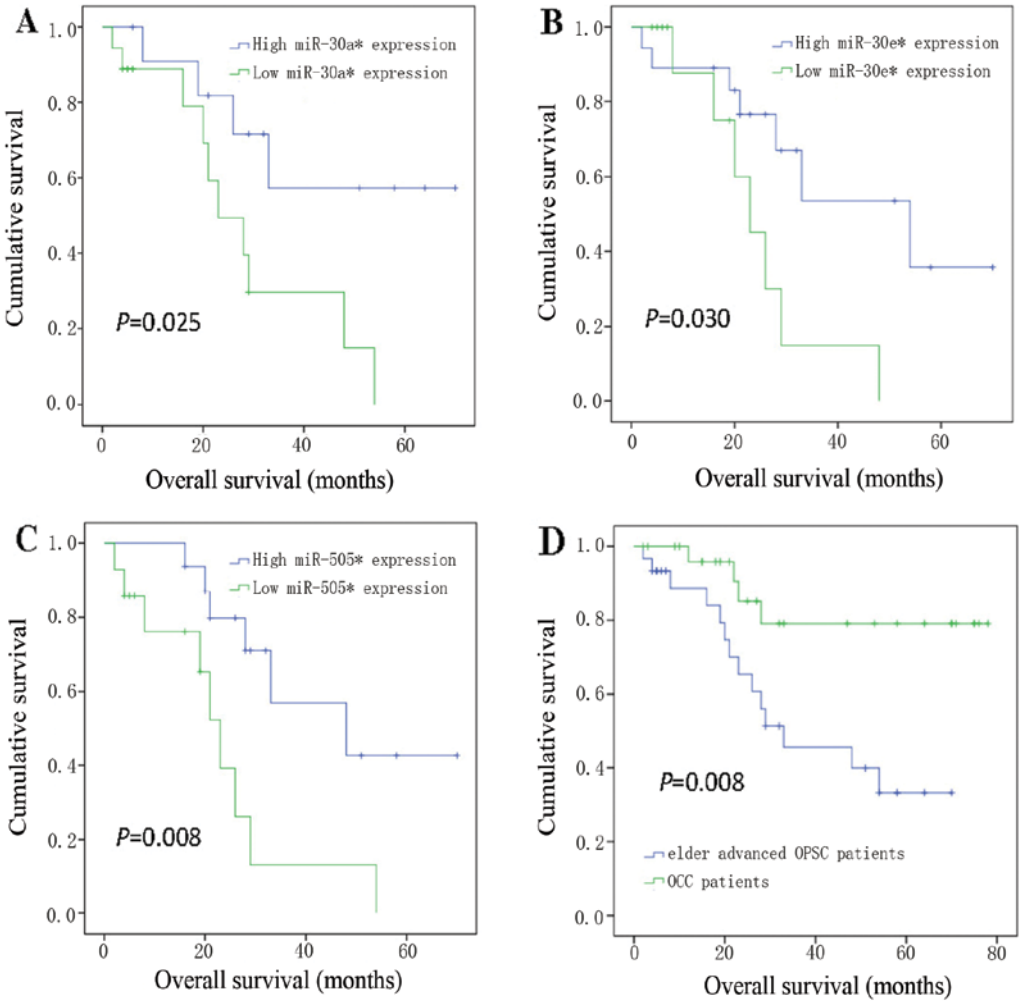

Figure 6. Kaplan-Meier curves showing overall survival for groups of elderly OPSC patients with advanced disease (A, B and C), stratified by expression levels of (A) hsa-miR-30a*, (B) hsa-miR-30e* and (C) hsa-miR-505*; (D) overall survival of ovarian cancer patients with OPSC and OCC. The samples of elder advanced OPSC patient were divided into two groups with high expression levels (blue line) and low expression levels (green line) of (A) hsa-miR-30a* (p=0.0025), (B) hsa-miR-30e* (p=0.030) and (C) hsa-miR-505* (p=0.008), n=30 for all. Lower expressions of miR-30a*, miR-30e* and miR-505*, all associated with poorer prognosis. (D) Overall survival associated with the histological type, elder advanced OPSC and OCC, was also tested (n=58, p=0.008). The overall survival of elder advanced OPSC is obviously lower than OCC patients. P-values are calculated by log-rank test comparing the low and high expression groups. Censoring events are marked by vertical lines.

using multiple software, and ATF3 was indicated as an only potential co-target of these three miRNAs.

Advanced OPSC has its own specific pathogenic factors, especially in elderly patients. Lower expressions of miR-30a*, miR-30e* and miR-505* were validated in elderly advanced OPSC patients consistent with microarray analysis. The survival analysis was investigated for elderly advanced OPSC patients in groups stratified according the expression levels of individual miRNAs, and the results revealed that lower expressions of miR-30a*, miR-30e* and miR-505*, all associated with significantly poorer prognosis. The results strongly suggested that they could be critical oncogenes and take important roles in ovarian cancer etiology with advantaged stage.

In order to validate our above hypothesis, we selected some typical downstream prediction targets which have associated with cancer as supporting evidence. ATF3 is a member of the ATF/cyclic adenosine monophosphate response element binding family of transcription factors (17,18). It is expressed at low levels in normal and quiescent cells, but can rapidly and significantly increase in various cancers (19). Previous research supports that overexpression of ATF3 could play an oncogenic role in carcinogenesis. These studies provide correlative evidence that ATF3 expression contributes to the successful propagation of human cancer (17,20-22), and also could promote metastasis, cell adhesion and invasion *in vitro* and *in vivo* (23,24). Furthermore, STMN1 overexpression is associated with polyploidy, tumor-cell invasion, early recurrence and poor prognosis in human hepatoma and ovarian cancer (25-27). MYC was also proven to have a pivotal role as a regulator of tumorigenesis in numerous human cancers of diverse origin (28). In our results, they all increased more in elderly advanced OPSC group, strongly supporting our pathogenic hypothesis for miR-30a*, miR-30e* and miR-505*, and suggesting that OPSC also has aggressive biologic behavior when presented with advanced-stage. Epidemiology results show that incidence and mortality rate of advanced OPSC rise continuously with advancing age. Previous reports have shown that women with OCC have a poorer prognosis compared to

serous ovarian cancer (7,29). However, most of these studies originate from limited research with different age and stage thus yielding different prognosis. In the current study, we found the survival rate of elderly advanced OPSC was significantly shorter than that for patients with OCC. Although, there are no data supporting the concept that elderly women with cancer should receive differential treatment based on age alone, the actual condition is that cancer risk increases with age (4). Pignata and Vermorken (1) already demonstrated that ageing is associated with important changes which can affect pharmacologic properties of cytotoxic agents including pharmacokinetic, pharmacodynamic and toxicity profiles. Therefore, age should be regarded as an important prognostic variable in the pathogenesis and treatment of advanced OPSC. Major questions about ovarian cancer in older-aged women need urgent attention from the research community since the incidence and the prognosis of this population is continuously worsening (1).

The above data of this research supported our hypothesis and strongly suggest that miR-30a*, miR-30e* and miR-505* may be the important pathogenic factors for elderly OPSC patients with advanced stage. This is the first report indicating and validating the differences and significance of miR-30a*, miR-30a* and miR-505*, and their targets (ATF3, STMN1 and MYC) in elderly OPSC with advanced stage. We hope this can improve understanding of molecular underpinnings during EOC development and progression, especially in elderly advanced OPSC patients; and to identify putative targets, including mRNA and proteins, which may open a new field for the understanding of this disease and providing improved diagnostic, prognostic and therapeutic approaches to individual patients, especially to the elderly.

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