Decreased Expression of RNA Interference Machinery, Dicer and Drosha, Is Associated with Poor Outcome in Ovarian Cancer Patients

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Acknowledgments: WMM, YGL, and WAS are supported by the National Cancer Institute - DHHS - NIH T32 Training Grant (T32 CA101642). This research was funded in part by support from the Ovarian Cancer Research Fund, Inc. Program Project Development Grant, The University of Texas M .D. Anderson Cancer Center Ovarian Cancer SPORE (P50 CA083639), the Gynecologic Cancer Foundation, and the Zarrow Foundation. Work also supported by USDOE Contract No. DE-AC02-05CH11231.

Running Header: Dicer/ Drosha Expression in Ovarian Carcinoma

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Abstract

Background: The clinical and functional significance of RNA interefence (RNAi) machinery, Dicer and Drosha, in ovarian cancer is not known and was examined.

Methods: Dicer and Drosha expression was measured in ovarian cancer cell lines (n=8) and invasive epithelial ovarian cancer specimens (n=111) and correlated with clinical outcome. Validation was performed with previously published cohorts of ovarian, breast, and lung cancer patients. Anti-Galectin-3 siRNA and shRNA transfections were used for *in vitro* functional studies.

Results: Dicer and Drosha mRNA and protein levels were decreased in 37% to 63% of ovarian cancer cell lines and in 60% and 51% of human ovarian cancer specimens, respectively. Low Dicer was significantly associated with advanced tumor stage ($p=0.007$), and low Drosha with suboptimal surgical cytoreduction ($p=0.02$). Tumors with both high Dicer and Drosha were associated with increased median patient survival (>11 years *vs.* 2.66 years for other groups; $p<0.001$). In multivariate analysis, high Dicer (HR=0.48; $p=0.02$), high-grade histology (HR=2.46; $p=0.03$), and poor chemoresponse (HR=3.95; $p<0.001$) were identified as independent predictors of disease-specific survival. Findings of poor clinical outcome with low Dicer expression were validated in separate cohorts of cancer patients. Galectin-3 silencing with siRNA transfection was superior to shRNA in cell lines with low Dicer (78-95% *vs.* 4-8% compared to non-targeting sequences), and similar in cell lines with high Dicer.

Conclusions: Our findings demonstrate the clinical and functional impact of RNAi machinery alterations in ovarian carcinoma and support the use of siRNA constructs that do not require endogenous Dicer and Drosha for therapeutic applications.

Introduction

Since the discovery that gene expression can be altered by interfering RNA molecules (RNAi)[1], abundant research has focused on the role of RNAi in human cancer pathogenesis. Targeting specific genes allows investigators to dissect and identify key regulators of angiogenic, proliferative, and survival pathways. In addition, RNAi applications have been tested as potential therapeutic modalities in preclinical cancer studies[2, 3] and may silence specific genes that are not inhibited by current therapeutic agents.

Regulation of gene expression by RNAi occurs through either the micro-interfering (miRNA) or small-interfering (siRNA) pathway. In the nucleus, endogenous RNA segments are processed by the ribonuclease III enzyme Drosha into precursor short hairpin RNA structures (approx 60-70 nt)[4, 5], then translocated to the cytoplasm and processed by Dicer, also an RNA endonuclease, resulting in mature (19-21 nt) double-stranded RNA fragments[6]. Translational repression or degradation of host mRNA occurs following binding of miRNA with the RNA-induced silencing complex, RISC[7, 8]. The production of siRNA molecules occurs in a similar manner, although Drosha processing is not required[9].

Since, components of the RNAi cascade directly affect the processing and maturation of miRNAs[5], we asked whether altered expression of RNAi machinery, Dicer and Drosha, could impact the clinical outcome of patients with ovarian cancer. To address this question, we correlated Dicer and Drosha expression in ovarian cancers with clinical

and pathological outcome variables. Furthermore, the functional relevance of altered Dicer expression was examined *in vitro*. These findings may not only invoke insight into the association of miRNA expression in human cancers, but also support development of novel RNAi therapeutic modalities.

Methods and Materials

Cell lines and culture. The derivation, sources, maintenance of the ovarian cancer cell lines used in this study, HeyA8, SKOV3ip1, A2780-Par, IGROV, EG, 222, OVCAR3, and OVCAR420 have been reported previously [10]. The non-transformed ovarian surface epithelial cell line HIO-180 was a kind gift from Dr. Andrew Godwin at Fox Chase Cancer Center, Philadelphia, PA.

Human samples. Invasive epithelial ovarian cancer specimens (n=111) were obtained for Dicer and Drosha expression analysis from the M. D. Anderson Cancer Center and the Brigham and Women's Gynecologic Oncology Tumor Banks following IRB approval. Benign ovarian epithelial samples (n=11) were obtained from microdissected paraffin-embedded specimens or epithelial scrapings taken following surgical removal. Clinical outcome data were obtained from patient records. Response to initial chemotherapy (sensitive, normalization of CA-125 and/or negative second-look laparotomy with no recurrence within 6 months of completion of initial chemotherapy; refractory/resistant, progression or recurrence within 6 months completing initial chemotherapy) was recorded.

Gene expression profiling of human cancer specimens with microarrays. The relationship between Dicer (212888_at) and Drosha (218269_at) expression and patient survival in ovarian (GEO accession GSE3149)[11], breast (Array Express accession E-TABM-158[12], GEO accession GSE1456[13], GEO accession GSE 4922[14]), and lung (GEO accession GSE3141)[11] cohorts was examined using genome-wide gene expression profiling with either Affymetrix HG U133A or Affymetrix HG U133 Plus 2.0 arrays.

SiRNA and shRNA transfection. Anti-Galectin-3 (target sequence: GTACAATCATCGGGTTAAATT; Dharmacon, Lafayette, CA) and control non-targeting (NT) oligonucleotides (target sequence: UUCUCC GAACGUGUCACGU; Qiagen, Valencia, CA) were used for siRNA and shRNA transfections. ShRNA was prepared using a lentiviral gene transfer vector (containing green fluorescent protein), as previously described[15]. For siRNA and shRNA transfections, 2 x 10^5 cells/well were plated and 5 µg of Galectin-3 or NT siRNA was added per manufacturer's protocol.Transfection was considered optimal if >90% transfection rate was achieved.

Western blot analysis. After protein loading, bands were separated by SDS-PAGE and transferred to nitrocellulose paper. Immunoblotting was performed as previously described[16] using either mouse anti-Dicer (1:1000; Abcam, Cambridge, MA), mouse anti-Drosha (1:500; Abcam), or rabbit anti-Galectin-3 antibodies (provided by Dr. Avraham Raz, Karmanos Cancer Center, Wayne State University, Detroit, MI).

Quantitative real-time PCR analysis. RNA was extracted and quantitative analysis of Dicer and Drosha mRNA expression was performed using the TaqMan gene expression assay kit (Applied Biosystems, Foster City, CA) with either Dicer, Drosha, or 18s RNA primers (Applied Biosystems) as previously described[17, 18]. The final expression values represented ratios of either decreased (0 to 1) or increased (>1) expression relative to normal ovarian epithelium.

Mutational analysis. Genomic DNA was sequenced for *DICER1* (NM_177438) and *RNASEN* (NM_013235) coding exons and their flanking splice sites to assess for potential mutations, as previously described.(REF) All sequence variants identified were verified by manual inspection of the chromatograms by two individuals.

Statistics. To determine the distribution of Dicer and Drosha levels around cutpoints, histograms were created using $\log_2$ of the expression ratio and tested for normality with the Kolmogorov-Smirnov test. The Kruskall-Wallis test was used to compare means of a continuous variable not conforming to the assumptions of normality. Contingency tables and Fisher's exact test were used to statistically evaluate the relationship between death and categorical variables. Kaplan-Meier plots were constructed and a log-rank test was used to determine differences in survival curves. Multivariate analyses with a Cox proportional hazard model were used to examine the effects of Dicer and Drosha expression on death from disease while adjusting for other covariates.

The relationship between Dicer and Drosha expression and survival in microarray data sets was explored for each gene by dichotomizing the cases from each cohort into high and low expression groups using the median expression level of that cohort. The significance of the Cox hazard ratio was assessed using Wald's test (using the package "survival" (v 2.34) in the R language for statistical computing (v 2.6.1))[19]. A p value of <0.05 was considered statistically significant for all analyses. A Bonferroni correction was used in analyses involving multiple comparisons.

Results

Dicer and Drosha expression in ovarian cell lines. Compared to the non-transformed ovarian surface epithelial cells, HIO-180, Dicer and Drosha mRNA expression was increased by 2.01 to 3.41 fold and 1.08 to 1.87 fold, respectively, in half of the ovarian cancer cell lines (Figure 1A). In the other 4 cell lines, mRNA expression of Dicer (2.0 to 12.5 fold) and Drosha (1.1 to 15.3 fold) was decreased.Protein expression was decreased in 5 of 8 cell lines (1.20 to 5.33 fold) for Dicer, and in 3 of 8, for Drosha (1.09 to 2.23 fold; Figures 1B and C).

Dicer and Drosha expression in human ovarian cancer tumors. Based on these differences, we next examined the expression of Dicer and Drosha in 111 human ovarian cancers relative to 11 benign epithelial ovarian specimens by real-time PCR. The distribution of Dicer levels in ovarian cancer specimens was bimodal ($p<0.01$; Kolmogorov-Smirnov test for normality) with two ratio peaks (0.43 and 4.25). The division between these two populations corresponded to a Dicer expression ratio of 1.2.

Drosha levels in cancer specimens followed a normal distribution (p=0.15) with a peak corresponding to a median value of 1. These findings supported our decision to dichotomize Dicer and Drosha levels at 1 for further analyses. Similar to the ovarian cell line analysis, expression varied among cancer specimens, with 59.5% and 51.4% demonstrating decreased expression of Dicer and Drosha, respectively (Supplementary Table 1). Furthermore, 38.7% of specimens expressed decreased levels of both Dicer and Drosha. Relative to benign ovarian epithelium, the median ratio of expression for cancer specimens with decreased Dicer and Drosha was 0.27 (range, 0.01-1.00; three specimens with undetectable levels) and 0.52 (range, 0.02-1.00; one specimen undetectable), respectively. Specimens with increased expression demonstrated a median Dicer ratio of 3.38 (range, 1.13-10.41) and 1.98 (range, 1.02-18.85) for Drosha**.**

Dicer and Drosha expression correlate with clinical/pathological features and mortality. The demographic characteristics of all patients (mean age 62.5 years) with invasive epithelial ovarian carcinoma are listed in Table 1. Among all ovarian cancer patients, most had advanced stage and poorly differentiated tumors, and 77% had undergone optimal primary tumor reductive surgery (residual tumor ≤ 1 cm). The majority of patients (53.2%) had tumors that were sensitive to initial chemotherapy compared to 33.3% with either refractory or resistant disease (data missing for 13.5%). In separate univariate analyses, neither Dicer nor Drosha levels were associated with age, grade, or chemotherapy response (Table 2). However, low Dicer significantly correlated with advanced stage ($p<0.01$), and low Drosha with greater likelihood of suboptimal cytoreductive surgery (p=0.02). In light of these findings, we next evaluated whether

Dicer and/or Drosha were related to patient mortality (Figure 2A and B). Median overall survival was substantially associated with both low tumor Dicer expression (2.33 *vs.* 9.25 years; p<0.001) and low Drosha expression (2.74 *vs.* 7.92 years; p=0.008). Compared to other groups, tumors with both high Dicer and Drosha expression were associated with increased median patient survival time (>11 years [median survival not reached] *vs.* 2.66 years; p<0.001). In univariate analyses, death from disease was associated with both low Dicer and low Drosha expression (p=0.01 and p=0.007, respectively). In multivariate analysis (variables in this model included age, stage, grade, Dicer, Drosha, cytoreduction, and response to initial chemotherapy), poorly differentiated tumors (p=0.03) and resistant/refractory chemoresponse (p<0.001) were predictors of poor survival. Furthermore, increased Dicer expression demonstrated a protective effect in ovarian cancer patients (HR, 0.48; 95% CI, 0.26-0.87; p=0.02). Next, we determined whether increased expression of Dicer and Drosha demonstrated a greater protective effect when paired in an interaction model. Interestingly, increased Dicer and Drosha demonstrated a greater effect toward improved survival (HR, 0.25; 95% CI, 0.11-0.55; p<0.001) than the effects of each gene alone.

To validate our findings, we correlated expression of Dicer and Drosha with patient survival in a previously reported cohort of 132 ovarian cancer patients[11]. Similar to our findings, high expression of Drosha (HR, 0.55; 95% CI, 0.34-0.89; p=0.014) or Dicer (HR, 0.53; 95% CI, 0.33-0.85; p=0.008) was associated with increased survival (Figure 2C).

To examine how robust this association might be across other tumors, we measured Dicer and Drosha relative expression ratios in two separate cohorts of 91 lung cancer[11] and 129 breast cancer patients[12]. High expression of Dicer (HR, 0.43; 95% CI, 0.23-0.80; p=0.008), but not of Drosha (HR, 1.34; 95% CI, 0.74-2.40; p=0.33), was associated with increased survival in the lung-cancer cohort (Figure 2D). Similarly, high expression of Dicer (HR, 0.32; 95% CI, 0.14-0.72; p=0.006) but not of Drosha (HR, 0.93; 95% CI, 0.45-1.92; p=0.84) was associated with increased disease-free survival as well as distant recurrence–free survival and overall survival (data not shown) in the breast cancer cohort. The relationship between high Dicer expression and increased disease-free survival was also observed in two other cohorts of breast cancer patients (HR, 0.33; 95% CI, 0.17-0.66; p=0.002; n=159; Figure 2E)[13] and (HR, 0.64; 95% CI, 0.42-0.97; p=0.036; n=249; Figure 2F)[14].

Mutational analysis of Dicer and Drosha in ovarian cell lines. We next asked whether the variable Dicer and Drosha expression could be explained by gene mutations. Genomic DNA from cell lines with low (HeyA8 and SKOV3ip1) and high (OVCAR3 and A2780-PAR) Dicer and Drosha were analyzed for mutations. Two synonymous single-nucleotide polymorphisms were discovered in both Dicer and Drosha sequencing in all four cell lines. Two different non-synonymous mutations were noted in Drosha in OVCAR3 and A2780-PAR cell lines. A splice-site mutation was discovered for Drosha in the SKOV3ip1 cell line. RT-PCR failed to demonstrate truncation of Drosha in any of the four cell lines examined by mutational analysis (data not shown).

Comparison of in vitro *gene silencing using shRNA or siRNA.* Although low Dicer levels were associated with poor clinical outcomes in ovarian cancer patients, the functional relevance of this relative expression is not known. Therefore, we compared the efficacy of silencing a constitutively expressed gene, Galectin-3, in ovarian cancer cell lines that were characterized by either high or low Dicer using either siRNA or shRNA transfections (Figure 3). Compared to controls, siRNA reduced (78% and 95%) Galectin-3 levels in the HeyA8 and SKOV3ip1 (low Dicer) cells, respectively. In contrast, very poor silencing was noted in these cells with shRNA (8% and 4%, respectively). In the OVCAR3 and 222 cells (high Dicer), 62% to 73% Galectin-3 silencing was observed with both siRNA and shRNA constructs.

Discussion

In this study, we found that Dicer and Drosha expression ratios vary significantly in ovarian cancer cell lines, as well as, invasive epithelial ovarian cancers and are significantly associated with patient survival. The association is robust, being externally validated in independent ovarian, lung and breast cancer patients' data sets. While the precise mechanism for this association is incomplete, our transfection data suggests expression of these key processing enzymes are relevant to the looming field of RNAi-based therapeutics.

The production of mature endogenous interfering RNA involves a cascade of events inextricably linked to Dicer and Drosha function. In cell culture models, silencing of Dicer and Drosha expression significantly reduces the production of precursor and

mature miRNAs[5]. Loss of Dicer in mice is lethal during early development and disrupts embryonic stem cell differentiation[20]. Further, DNA copy–number abnormalities of Dicer and Argonaute 2 (a component of RISC) have been described in human melanoma, breast, and ovarian cancers[21]. It is therefore plausible, that dysregulated gene expression may result from functionally handicapped processing of endogenous silencing mechanisms in some tumors. Our observation of differential Dicer and Drosha expression both in ovarian cancer cell lines and in human tumors supports this contention. Clinically, we suggest this finding is independently represented by ovarian cancer mortality. Not limited to ovarian cancer, decreased Dicer mRNA expression has also been associated with decreased survival in patients with non-small–cell lung cancer[22]. Of interest, Dicer expression appeared to be up-regulated in noninvasive precursor lesions relative to invasive lung adenocarcinoma.[23]

Underscoring the complexity of the RNAi machinery are observations in other tumor types which contradict our findings. High Dicer and Drosha expression correlated with poor prognostic factors in patients with prostate and esophageal carcinoma.[24, 25] Furthermore, reduction of Drosha expression in esophageal cancer cell lines with siRNA significantly reduced cellular proliferation[25]. Nevertheless, these findings suggest that alterations in RNAi machinery play a role in cancer pathogenesis.

Despite growing evidence that Dicer and Drosha expression varies among tumor types, the regulation of these genes remains unclear. Recently, Dicer mutations were reported in *C. elegans*[26], and in humans, Chiosea and colleagues found deletions of the Dicer

locus in a fraction of precancerous and invasive lung adenocarcinomas[23]. Our mutational analysis demonstated that alterations of genomic DNA from cancer cells likely do not account for the variability in Dicer and Drosha levels. Although a splice-site mutation of Drosha was discovered, these findings were not consistent among cell lines examined and did not appear to affect translational processing. However, in breast cell lines, two forms of Dicer exist based on alternative splicing mechanisms which appear to affect protein stability[27]. In addition, DNA methylation of the Dicer gene was not present in a small subset of lung cancer specimens [22]. As the function of miRNAs in tumorigenesis becomes clearer, further studies will be needed to delineate the regulation and stability of the RNAi machinery.

From a developmental therapeutics standpoint, our discovery of the heterogeneous expression profile of Dicer and Drosha in ovarian cancer patients may have specific implications in constructing efficacious RNAi-based treatment. To highlight this point, we demonstrated differential targeting efficiency of a constitutively expressed gene by two strategies of gene silencing; one dependent (shRNA) and one independent (siRNA) of Dicer processing. In the presence of functional Dicer, both strategies silenced Galectin-3 expression; however, only siRNA was efficacious in the cell lines without Dicer. Nevertheless, shRNA-based therapy has been explored in several in vivo models as the imperfect complimentarity to the target gene has the potential to induce robust gene silencing for cancer therapy. However, in one study, Grimm and colleagues reported increased mortality in mice after delivery of multiple shRNA sequences, which was thought to be related to competition between exogenous shRNA

and the production and expression of host miRNAs, thereby overwhelming key components of the RNAi cascade[28]. We previously demonstrated that siRNA-mediated therapy was highly effective in decreasing tumor growth and angiogenesis with no apparent evidence of drug-related toxicity in preclinical mouse cancer models[2, 16]. Since low Dicer characterizes a relevant proportion of ovarian cancers, RNAi-based therapy will have to consider altered integrity of these processing enzymes.

In conclusion, Dicer and Drosha expression was associated with clinical outcome in ovarian carcinoma. As investigators begin to define the role of interfering RNAs in humans, these findings could directly relate to any protective role that miRNAs may play in tumor development and progression. Nevertheless, given the substantial proportion of tumors with low expression of Dicer, siRNA-based therapeutic approaches may be more attractive than RNAi fragments that require Dicer function.

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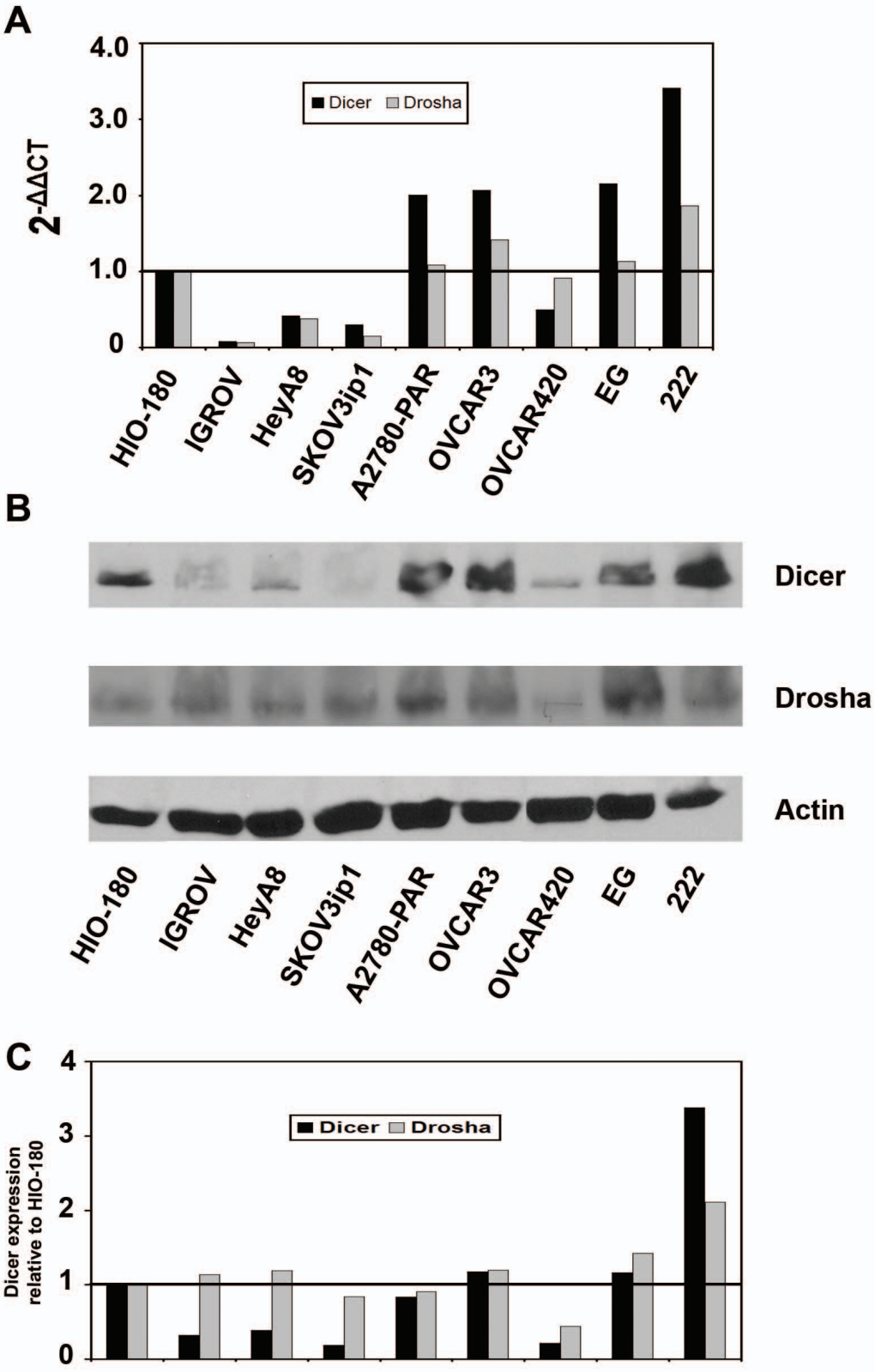
Figure Legends

Figure 1. Dicer and Drosha expression in ovarian cell lines. **A**) Real-time PCR analysis of Dicer and Drosha mRNA expression in a non-transformed ovarian epithelial cell line (HIO-180) and invasive ovarian epithelial cancer cell lines. $2^{-\Delta\Delta CT}$= Ratio of Dicer and Drosha expression relative to that in the HIO-180 cell line [18]. **B**) Western blot analysis of Dicer and Drosha. **C**) Densitometry analysis.

Figure 2. Kaplan-Meier survival curves of patients with invasive epithelial ovarian cancer in relation to tumor expression of **A**) Drosha and Dicer, **B**) Dicer and Drosha combined relative to that in benign ovarian epithelium. Kaplan-Meier survival curves of validation analyses for Dicer and/or Drosha expression in independent ovarian (C), lung (D), and breast (E-F) patient cohorts.

Figure 3. Comparison of siRNA and shRNA transfections targeting Galectin-3 in ovarian cancer cell lines with low Dicer (HeyA8 and SKOV3ip1) and high Dicer (OVCAR3 and 222) expression levels by Western blotting. Densitometry analysis comparing Galectin-3 silencing (normalized to actin loading) to control transfections with non-targeting sequences.

Figure 1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Dicer
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Dicer expression relative to HIO-180
Dicer
Drosha

Supplementary Table 1. Distribution of Dicer and Drosha expression in ovarian cancer tumors

		N (%) (N=111)
Individual	Low Dicer	66 (59.5)
	Low Drosha	57 (51.4)
Joint	Low Dicer and Drosha	43 (38.7)
	High Dicer, Low Drosha	14 (12.6)
	Low Dicer, High Drosha	23 (20.7)
	High Dicer and Drosha	31 (27.9)

Table 1. Demographic features of patients with invasive ovarian cancer

Variable		N (%) N=111
Age*		62.5 (25-96)
Stage	I/II	8 (7.2)
	III/IV	103 (92.8)
Cytoreduction	Optimal	86 (77.5)
	Suboptimal	25 (22.5)
Grade	Low (1 or 2)	16 (14.4)
	High (3)	95 (85.6)
Response to initial chemotherpay	Sensitive	59 (53.2)
	Resistant/ Refractory	37 (33.3)
	Missing	15 (13.5)
Status	Alive with disease	14 (12.6)
	Alive without disease	33 (29.7)
	Dead of disease	64 (57.7)

*Mean (Range)

Table 2. Correlation of clinical and pathological features with Dicer and Drosha expression in invasive epithelial ovarian carcinoma

Variable		Drosha			Dicer		
		Low N (%)	High N (%)	p value	Low N (%)	High N (%)	p value
Age	Mean (SD)	61.2 (12.95)	63.8 (11.66)	0.37	63.1 (11.70)	61.6 (13.34)	0.74
Stage	I & II	2 (3.5)	6 (11.1)	0.15	1 (1.5)	7 (15.6)	<0.01
	III & IV	55 (96.5)	48 (88.9)		65 (98.5)	38 (84.4)	
Grade	Low	7 (12.3)	9 (16.7)	0.59	9 (13.6)	7 (15.6)	0.79
	High	50 (87.7)	45 (83.3)		57 (86.4)	38 (84.4)	
Cytoreduction	Optimal	39 (68.4)	47 (87.0)	0.02	47 (71.2)	39 (86.7)	0.07
	Suboptimal	18 (31.6)	7 (13.0)		19 (28.8)	6 (13.3)	
Response to initial chemotherpay	Sensitive	27 (47.4)	32 (59.3)	0.35	31 (47.0)	28 (62.2)	0.15
	Resistant/ Refractory	20 (35.1)	17 (31.4)		23 (34.8)	14 (31.1)	
	Missing	10 (17.5)	5 (9.3)		12 (18.2)	3 (6.7)	

Figure 2

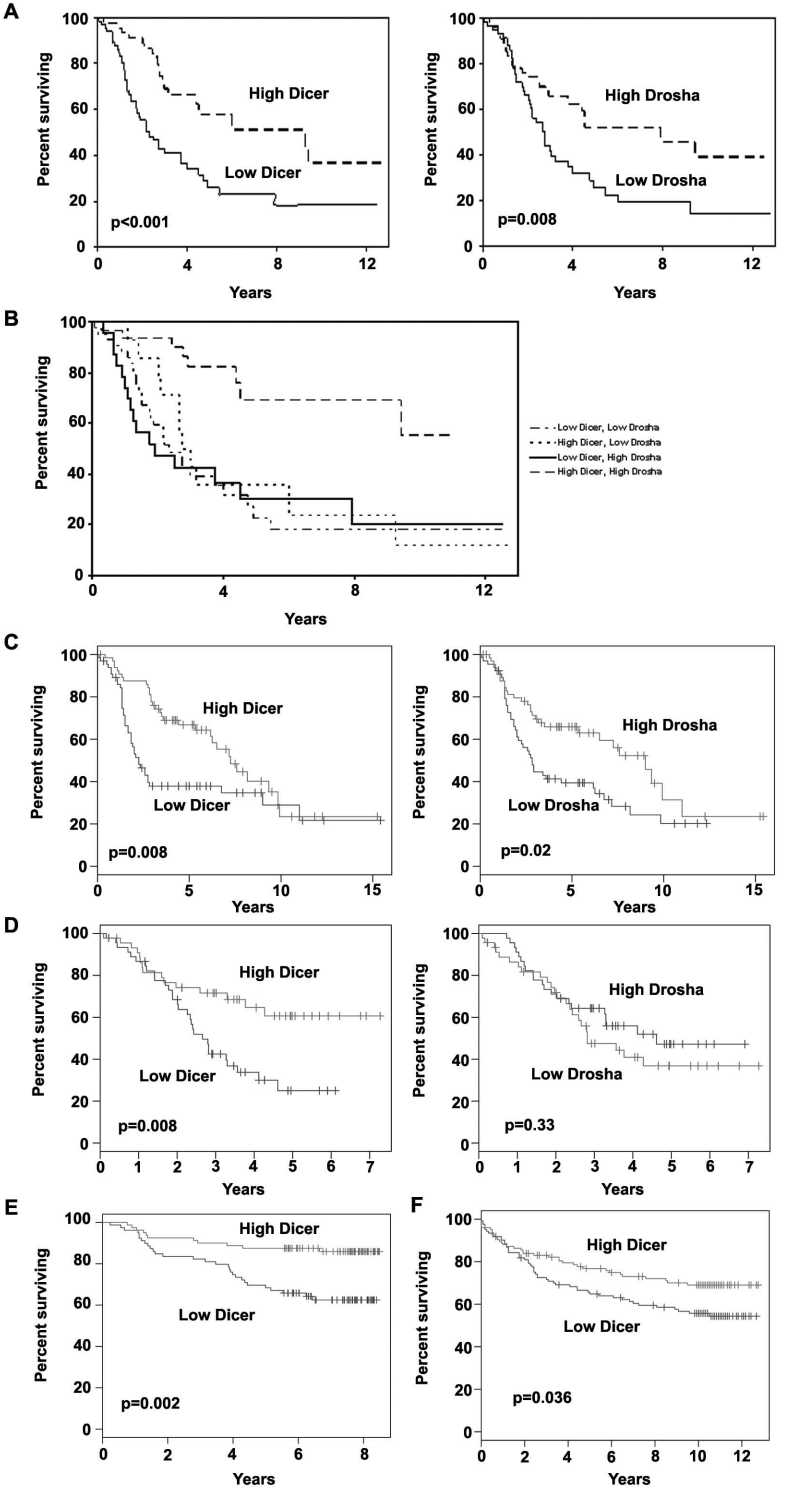
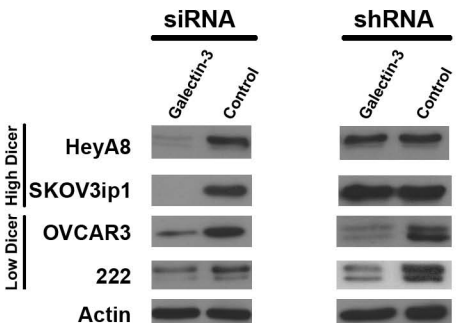
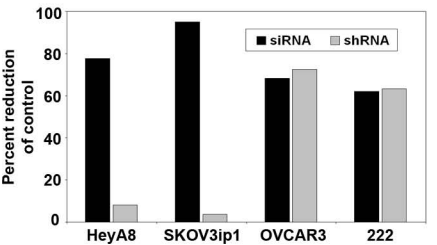

A
Percent surviving
High Dicer
Low Dicer
p<0.001
Years
High Drosha
Low Drosha
p=0.008
B
Low Dicer, Low Drosha
High Dicer, Low Drosha
Low Dicer, High Drosha
High Dicer, High Drosha
C
p=0.008
p=0.02
D
p=0.008
p=0.33
E
p=0.002
F
p=0.036

Figure 3


siRNA
shRNA
Galectin-3
Control
Galectin-3
Control
High Dicer
Low Dicer
HeyA8
SKOV3ip1
OVCAR3
222
Actin


Percent reduction of control
100
80
60
40
20
0
siRNA
shRNA
HeyA8
SKOV3ip1
OVCAR3
222