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MicroRNA let-7a Modifies the Effect of Self-renewal Gene *HIWI* on Patient Survival of Epithelial Ovarian Cancer

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Abstract

Aberrant expressions of self-renewal gene *HIWI* and microRNA let-7a are in epithelial ovarian cancer (EOC). A U-shape association between *HIWI* expression and overall survival is seen in several human cancers but unknown in EOC. HIWI physically binds let-7a, but the clinical relevance of this interaction is yet to be addressed. Here we analyzed *HIWI* and let-7a expressions in 211 primary EOC tissues using quantitative reverse transcription PCR to investigate *HIWI* and its interaction with let-7a in the prognostic significance of EOC. Associations of *HIWI* and its interaction with miRNA let-7a with patient survival were analyzed using the Kaplan-Meier survival curves and Cox proportional hazard regression models. Kaplan-Meier survival curves showed that patients with medium *HIWI* had poorer overall survival than those with low or high *HIWI*. An 89% increased death risk (HR = 1.89, 95% CI: 1.29 – 2.98) was observed in the medium *HIWI* group in multivariate Cox proportional hazard regression analyses. Among patients with high let-7a expression, those with medium *HIWI* had an increased risk of death compared to those with low *HIWI* (HR = 2.62, 95% CI: 1.30 – 5.30), whereas among those with low let-7a, no significant association between HIWI expression and overall survival was observed (HR = 1.63, 95% CI: 0.86 – 3.08). Moreover, *HIWI* expression also affected chemotherapy response. The results suggested that miRNA let-7a could modify the effect of *HIWI* expression on patient survival of EOC, expanding our understanding of the clinical relevance of *HIWI* and let-7a interaction in EOC prognosis.

Introduction

HIWI (also known as piwi-like RNA-mediated gene silencing 1, *PIWIL1*), located on chromosome 12q14.33, is a member of the P-element induced wimpy testis (*PIWI*) gene family in humans [1]. This family is evolutionarily conserved across species; their encoded-proteins are highly homologous, particularly in the carboxy-terminus [1]. The HIWI is a ribonucleoprotein in the length of 861 amino acids, which contains a N-terminal PIWI/Argonaute/Zwille (PAZ) domain and a PIWI domain at the carboxy-terminus [2-4]. The PAZ domain binds to single-stranded small RNA, and the PIWI domain functions as an RNase H endonuclease cleaving target RNA complementarily bound to small RNA [4-6]. The involvement of HIWI proteins in the regulation of gene expression is through their interacting partners of small non-coding RNAs such as namely PIWI-associated RNAs (piRNAs) [7]. Nuclear HIWI controls retrotransposon silencing via affecting DNA methylation [8-10]. Recently, Sequencing results of Immunoprecipitation (IP) against HIWI show that HIWI interacts with miRNAs including let-7a in fining gene expression [11]. Moreover, it has also been reported that HIWI can be a partner interacting with Dicer, a key RNase III endonuclease in miRNA maturation [12-14]. These findings suggest HIWI is also localized in cytoplasm and may participate in the role of miRNAs in gene regulation. The *HIWI* gene is expressed in hematopoietic stem cells and germ cells but not in differentiated cells. It has been evidenced that HIWI plays an important role in maintaining stem cell renewal [1,7,15,16]. In the PIWI mutant models, germline stem cell asymmetric divisions cannot be functionally processed in both male and female flies, leading to a loss of germline stem cells and a reduced number of eggs and sperms during oogenesis or

spermatogenesis [7,17]. In healthy women, those with age younger than 50 years old express HIWI in ovary tissues [18].

Aberrant *HIWI* expression is associated with tumorigenesis and with cancer patient survival. Over-expressed *HIWI* has been observed in seminomas testicular tumors but not in nonsemnomatous, spermatocytic seminomas and somatic types [1,19]. A large number of gastric cancers show increased *HIWI* expression in comparison to normal tissues, and *HIWI* has been involved in the proliferation of gastric cancer cells [20]. Both U-shape and linear associations between HIWI expression levels and cancer patient survival have been observed. Compared to moderate expression, both low and high *HIWI* expression were reported to be associated with increased death in patients with soft-tissue sarcoma, suggesting that *HIWI* may have biphasic effects on tumor progression [21,22]. Recently, Grochola et al. reported that *HIWI* expression increased pancreatic cancer-related death but in a male-specific manner [23]. Prognostic value of *HIWI* has been shown in several human solid tumors including hepatocellular carcinoma [24], colorectal cancer [25], glioma [26], and esophageal carcinoma [27]. Very recently, it has also been shown that the expression of *HIWI* was significantly higher in epithelial ovarian cancer than in normal ovaries and benign tumors, while enforced overexpression of *HIWI* in SKOV3 ovarian cancer cell line led to reduced metastatic capacity [18], suggesting moderate *HIWI* expression may associate with tumor development and progression in ovarian cancer. However, whether HIWI expression has prognostic value in ovarian cancer is yet to be investigated.

MicroRNA let-7a is one member of let-7 family, which is one well-characterized miRNA in controlling gene expression, and plays important roles in cell proliferation, differentiation, apoptosis and metabolism [28-30]. Dysregulated let-7a has been reported in different types of human cancer [31-34], and associates with cell proliferation, chemotherapy response, and patient survival [35-37]. Moreover, the involvement of let-7a has been demonstrated in stemness [38-40]. Our previous study also showed that let-7a was associated with epithelial ovarian cancer survival [37]. Given that cytoplasmic HIWI protein can interact with let-7a directly [11] or indirectly through miRNA maturation-associated protein Dicer, which could form a negative feedback loop with let-7a [41], and that both HIWI and let-7a are involved in the maintenance of stem cells [1,7,15,16,42], we speculate that HIWI and let-7a may orchestrate in executing biological functions. Thus, the purposes of this study were to determine *HIWI* expression and its interaction with let-7a in the prognosis of epithelial ovarian cancer.

Materials and Methods

Patients and tumor samples

In this study, a written informed consent was obtained from each individual of the participants, and all subjects in this study were de-identified for the sake of participants' privacy. With the approval of the University's ethical review committee, 211 women diagnosed with epithelial ovarian cancer were enrolled between October 1991 and February 2000 in the Department of Gynecology and Obstetrics at University of Turin in Italy. Fresh tumor tissues were collected for the study at surgery. Disease stage and

tumor grade were determined for each patient according to the International Federation of Gynecology and Obstetrics Classification (FIGO) and WHO criteria [43,44]. Most of the patients received standard post-operative chemotherapy after cytoreduction surgery and were subsequently followed up for disease progression until June 2001. Median follow-up time was 31 months, ranging from 0.6 to 114 months.

There were four categories of treatment response defined in the study. The definition is as follows: (a) complete response: resolution of all evidence of disease for at least a month, (b) partial response: a decrease of ≥50% in the product of the diameters (maximum and minimum) of all measurable lesions without the development of new lesions for at least a month, (c) stable disease: a decrease of <50% or an increase of <25% in the product of the diameters of all measurable lesion, and (d) progressive disease: an increase of ≥25% in the product of the diameters of all measurable lesions or the development of new lesions. Of the 176 patients with available information on chemotherapy treatment response, 128 (72.7%) who had complete response was considered 'Yes' response in data analysis, while 48 (27.3%) who were in the other three categories were grouped as 'No' response, which included 36 with partial response, 4 with stable disease, and 8 with progressive disease.

Analysis of HIWI and let-7a expression

All tumor samples were examined by two independent pathologists, and the specimens which contained 80-90% tumor cells were used in this study. Total RNAs extracted from the tumor samples were used as templates to make cDNA using the Cloned AMV

First-Strand cDNA Synthesis kit (Invitrogen™, Carlsbad, CA). The primers for *HIWI* and small nucleolar RNA *RNU48* (used for normalization) were designed based on sequences in Genbank (accession number AB274731 for *HIWI* and X96648 for *RNU48*). The primer sequences for *HIWI* and *RNU48* are: *HIWI*-forward, 5'- CCT GGC TTC ACT ACT TCC ATC C; *HIWI*-reverse, 5'- ACG TCA GTG CAG AGC ATG ATG; *RNU48*-forward, 5'AGTGATGATGACCCCAGGTAACTC, and *RNU48*-reverse, 5'- CTG CGG TGA TGG CAT CAG. Real-time PCR was performed to analyze *HIWI* expression using the Chromo4™ Real-time PCR System (MJ Research Inc., Waltham, MA). In the PCR reaction (20 µl), 2 µl of cDNA template was mixed with 10 µl of 2× Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA) and each pair of primers, at final concentrations of 100 nM for both *HIWI* and *RNU48*. PCR amplification included initial incubation at 50°C for 2 minutes, denaturing at 95°C for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at 60°C for one minute. Melting curves were analyzed after each run to verify the size of PCR product.

Analysis of *let-7a* expression in tumor tissue was performed using Taqman® microRNA assay (Applied Biosystems), in which the preparation of miRNA cDNA was made first using a stem-loop method, following the manufacturer's instruction as described in our previous report [45] . Briefly, levels of *let-7a* and RNU48 (an internal control for normalization) expression in the samples were determined with the Taqman® miRNA assay (Applied Biosystems) using the Chromo4 Real-time PCR System. In the PCR reaction (15 µl), 0.3 µl of cDNA template was mixed with 7.5 µl of 2×Taqman® Universal PCR master mix (Applied Biosystems), 0.75 µl of 20× probe/primers

(Applied Biosystems) of either *let-7a* or RNU48, and water. The PCR amplification conditions were the same as for the quantification of *HIWI* gene in this study. Each sample was analyzed in duplicate, and the analysis was repeated for those with CV above 5%.

Statistical analysis

HIWI and let-7a expression was quantified as an expression index (EI), which was calculated based on the formula $1000 \times 2^{(-\Delta Ct)}$, where $\Delta Ct = Ct_{\text{target gene}} - Ct_{RNU48}$. To analyze *HIWI*'s associations with disease features and patient survival, the EI values were grouped into 3 categories, low, medium and high, which were classified as undetectable expression, detectable expression below median and detectable expression equal to or above median, respectively. Let-7a was classified into two groups, low and high, based on the median of let-7a expression distribution as the cutoff when the interaction analysis was performed. Associations between *HIWI* expression and clinicopathologic factors were analyzed by Chi-square statistic or Fisher's exact statistic as appropriate. Survival analysis was performed to assess associations of *HIWI* expression with risks of disease progression and death. Cox proportional hazards regression and Kaplan-Meier survival curves were employed for the survival analyses. Proportional hazards assumption was also tested in Cox proportional hazards regression. All statistical analyses were performed using SAS version 9.2 (SAS Institute, Cary, NC). All p-values are two-sided. Significant values are shown in bold.

Results

Clinical and pathological characteristics of patients

Clinical and pathological features of the 211 participants enrolled in this study were shown in Table 1. These patients underwent surgery at ages between 26 to 82 years, and the median age was 58 years. Based on the International Federation of Gynecology and Obstetrics Classification [43], 52 patients were diagnosed with stage I disease (24.6%), 12 stage II (5.7%), 133 stage III (63.0%), and 14 stage IV (6.6%). Tumor grade and histology were determined according to the WHO guidelines [44]. Thirty-four (16.1%) were grade 1 tumors, 40 (19.0%) grade 2 and 137(64.9%) grade 3. Eighty-five (40.3%) patients had serous tumors, and 126 patients had non-serous tumors which included 16 (7.6%) clear cell, 41 (19.4%) endometrial, 18 (8.5%) mucinous, 14 (6.6%) müllerian, 1 other (0.5%) and 36 (17.1%) undifferentiated histology. Surgical debulking was performed on all the patients. Optimal debulking results were achieved in 108 (51.9%) patients, and 100 (48.1%) had suboptimal outcomes. Ninety-one patients (44.0%) had no residual tumor, and 116 (56.0%) had a residual tumor size greater than 0.

Association of HIWI expression with epithelial ovarian cancer prognosis

Of 211 samples, 82 (38.9%), over a third, had undetectable expression of *HIWI*, and 129 had detectable expression with an average EI of 0.32, ranging from 0.02 to 4.86 (5^{th}-95^{th} percentiles). To investigate the impact of *HIWI* expression on prognosis of ovarian cancer, we classified patients based on their *HIWI* expression into three groups, low, medium and high, using the EI = 0 (undetectable), >0 to <0.32, and ≥0.32 as

cutoffs. The numbers of patients in these groups were 82, 65 and 64, respectively. Kaplan-Meier survival curves showed that patients with low and high *HIWI* did not have substantial differences in progression-free or overall survival, but did have more favorable prognosis than those with medium *HIWI* expression (data not shown). Based on this finding and U-shaped associations with death observed elsewhere [21-23], we combined the low and high groups together, and compared their survival outcomes with those with medium *HIWI* expression. Kaplan-Meier survival curves showed that patients with medium expression had worse overall survival ($p = 0.025$) (Figure 1). However, disease progression-free survival was not different by *HIWI* expression ($p = 0.200$) (data not shown).

To confirm the results of Kaplan-Meier analysis and to adjust for potential confounding factors, we further analyzed the data with the Cox proportional hazards regression. Proportional hazards assumption test for *HIWI* expression did not show significance ($p = 0.59$). The results of univariate and multivariate analyses were shown in Table 2. Here too, medium *HIWI* expression was significantly associated with elevated risk for death in both univariate and multivariate analyses. After adjustment for patient age at surgery, disease stage, tumor grade, residual tumor size, histological type and chemotherapy status, the association remained significant. The adjusted hazard ratio (HR) was 1.89 (95% CI: 1.29 – 2.98) for high or low *HIWI* expression compared to medium expression. In addition, disease stage and residual tumor size showed positive associations with the risk of death, while chemotherapy treatment improved patient survival. Their adjusted HRs were 1.71 (95% CI: 1.08 – 2.71) for disease stage, 5.29

(95% CI: 2.50 – 11.20) for residual tumor size and 0.40 (95% CI: 0.17 – 0.95) for chemotherapy treatment, respectively. However, the associations of tumor grade, histological type and patient age at surgery with the risk of death were not statistically significant. Similarly to the Kaplan-Meier analysis, little evidence of association was found between disease progression and *HIWI* expression. Residual tumor size was positively associated with the risk of relapse. The adjusted HR was 3.00 (95% CI: 1.62 – 5.57).

Associations of HIWI expression with clinical and pathological features in epithelial ovarian cancer

We also analyzed associations of *HIWI* expression with clinical and pathologic characteristics. A significant association was observed between *HIWI* expression and patient response to chemotherapy ($p = 0.002$). Patients with medium expression of *HIWI* had worse response to chemotherapy than those with either low or high expression. The odds ratio (OR) was 3.01 (95% CI: 1.50 – 6.04) (Table 3). However, no associations were found between *HIWI* expression and other clinical and pathologic variables including disease stage, tumor grade, histological type, residual tumor size and debulking results.

Interplay between HIWI and let-7a in epithelial ovarian cancer prognosis

The average EI of let-7a in this study was 4.62 with the range from 0.53 to 35.3 (5^{th} – 95^{th} percentiles).

Previous studies including immunoprecipitation sequencing results suggest that HIWI might interplay with let-7a in regulating biological processes [11,15,41,42]. To examine whether there is any interaction between *HIWI* and let-7a expressions in the patient survival of epithelial ovarian cancer, we used the median of let-7a expression as the cutoff value to classify *let-7a* into two groups, high (EI $\geq$ 4.62, median) and low (EI < 4.62). Then we classified patients into four groups; group 1 with low (EI = 0) or high (EI $\geq$0.32) *HIWI* and low (EI < 4.62) *let-7a*, group 2 with low or high *HIWI* and high *let-7a* (EI $\geq$ 4.62, median), group 3 with medium (0 < EI <0.32) *HIWI* and low *let-7a*, and group 4 with medium *HIWI* and high *let-7a*. Multivariate Cox proportional hazard models showed that the adjusted HRs of death were 1.11 (95% CI: 0.66 – 1.86) for Group 2, 1.64 (95% CI: 0.88 – 3.04) for Group 3, and 2.71 (95% CI: 1.38 – 5.32) for Group 4 (Table 4) after the adjustment for patient age at surgery, disease stage, tumor grade, residual tumor size, histological types and chemotherapy status. However, no significant associations were found in progression-free survival in the multivariate analysis.

We next sought to how *HIWI* and *let-7a* modulate each other the effect on patient survival using stratification analyses (Table 5). When we stratified patients by *HIWI* expression levels, we did not find the associations of *let-7a* expression with overall survival ($p > 0.05$) within the strata of either low/high or medium *HIWI*. In contrast, when we stratified patients by *let-7a* expression levels (low and high), we found among patients with high *let-7a* expression, medium expression of *HIWI* significantly increased the risk of death compared to low/high *HIWI* expression; the adjusted HR was

2.62 (95% CI: 1.30 – 5.30). Among those with low *let-7a* expression, however, no significant association was found between the risk of death and *HIWI* expression levels; the adjusted HR was 1.63 (95% CI: 0.86 – 3.08).

Discussion

This study examined the prognostic value of self-renewal-associated gene *HIWI* expression. *HIWI* expression was detectable in 61% (129 out of 211) epithelial ovarian cancers, similar to the findings of two previous studies where 7 of 10 gastric cancer patients and 40 of 56 pancreatic cancer patients showed detectable expression of *HIWI* using RT-PCR, and 38 of 50 gastric cancers had positive immunohistochemical stains for HIWI protein [20,23]. Moreover, Lim and colleagues reported that significantly upregulated expression of HIWI gene was observed in epithelial ovarian cancer compared to benign tumors [18]. The lack of association between *HIWI* expression and clinical and pathologic variables in our study were in agreement with the investigations of *HIWI* in gastric cancer [20] and cervical cancer [46].

We also investigated the association of *HIWI* expression in epithelial ovarian cancer survival. We found that ovarian cancer patients with medium expression of *HIWI* had shorter overall survival in comparison to those with low or high expression. This association was independent of clinical or pathologic factors of the disease. We also found that patients with medium levels of *HIWI* expression had poor response to chemotherapy compared to those with low or high expression. To our knowledge, this is the first study to examine self-renewal-associated gene *HIWI* expression and survival of patients with epithelial ovarian cancer in a clinical setting. Our study showed that

patients with low or high *HIWI* expression had a more favorable prognosis. This finding was similar to, but in the opposite direction of, observations of several previous clinical studies, which showed that compared to medium, high or low *HIWI* expression was associated with elevated death risks in patients with soft-tissue sarcoma and male patients with pancreatic cancer [21-23]. Our result, however, seems to be more consistent with findings of a very recent report, in which it was shown that significantly increased *HIWI* expression was observed in epithelial ovarian cancer compared to normal ovarian tissues and benign tumor tissues, while enforced overexpression of *HIWI* repressed the invasiveness of ovarian cancer cell SKOV3 [18]. Our findings are also supported by *in vitro* experiments that show similar impacts of loss- and gain-of-function of *HIWI* on cancer cells. Inhibition of *HIWI* expression in gastric cancer cells led to G2/M phase arrest and decreased proliferation index of the cancer cells [20], while high *HIWI* expression appears to induce apoptosis in tumor cell lines of KG1 [15]. G2/M arrest is a critical step in the initiation of apoptosis [47-49]. Thus, both low and high *HIWI* expression may have similar effects on cancer cells.

Interestingly, previous studies on hepatocellular carcinoma [24] , colorectal cancer [25] and esophageal squamous carcinoma [27] have shown direct associations between high HIWI proteins (immunohistochemical staining, IHC) and poor patient survival, but such correlations have been limited to certain subgroups of patients. Zhao and colleagues [24] reported that high HIWI protein was significantly associated with poor prognosis of hepatocellular carcinoma only in patients with alpha-fetoprotein (AFP) less than 300 ng/ml, but not in those with AFP greater than that level. Zeng and colleagues [25]

demonstrated that HIWI protein in tissues adjacent to tumor but not in primary tumor was significantly associated with poor prognosis of colorectal carcinoma. Another study [27] found that patients with high cytoplasmic but not nuclear HIWI protein had poor prognosis in esophageal squamous carcinoma, while no association with prognosis was seen if both cytoplasmic and nuclear HIWI proteins were combined for consideration. Three possibilities may explain the discrepancy between our and previous studies on the association of *HIWI* expression with patient survival. First, translated HIWI from the mRNAs may be aberrant due to premature stop codon-causing truncation or alternative splicing, thereby influencing antigen determinants and IHC results, as well as its function [18]. This aberrant expression of HIWI may result in the phenomenon that it is high at mRNA levels but low at protein levels. Second, different laboratory methods were applied to measure *HIWI* expression (qPCR for mRNA vs IHC for protein); *HIWI* expression at mRNA levels measured by qPCR showed biphasic effects, while *HIWI* expression at protein levels by IHC had monotonic effects [24,25,27]. Finally, the association between *HIWI* expression and cancer prognosis may be tumor or tissue specific.

In this study, we also found a significant interaction between *HIWI* expression and let-7a, increasing the risk of death in epithelial ovarian cancer. Stratification analyses demonstrated let-7a modified the effect of *HIWI* expression on overall survival of EOC. Only at high let-7a levels did patients with medium *HIWI* expression increase the risk of death. This finding supports and extends the previous observation reported by Chen and colleagues [11], suggesting that HIWI protein not only physically binds let-7a, but

also orchestrate each other, executing their biological functions. This also may help to explain the finding that cytoplasmic but not nuclear HIWI positively associated with poor prognosis of esophageal squamous carcinoma [27].

As expected, we found disease stage and residual tumor size, two well-established prognostic risk factors, were unfavorable prognostic indicators, while chemotherapy treatment improved survival. These results suggest that the findings in this study were not observed by chance.

In summary, our study showed that self-renewal gene *HIWI* was associated with overall survival as well as chemotherapy response in a U-shape correlation in epithelial ovarian cancer. Taken together with the recent findings reported by Lim and colleagues [18], *HIWI* expression at a moderate level may associate with the increased risk of death and poor chemotherapy response in epithelial ovarian cancer. We also found the synergetic effect of self-renewal gene *HIWI* and miRNA let-7a on patient survival of epithelial ovarian cancer. These findings suggest that the interplay between self-renewal gene *HIWI* and miRNA let-7a has significant clinical relevance in epithelial ovarian cancer prognosis, and reveal a potential strategy by the modulation of HIWI and let-7a in the management of epithelial ovarian cancer.

Conflict of Interest Statement

The authors declare that they have no conflict of interest**.**

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Figure legends

Figure 1. Kaplan-Meier overall survival curves by levels of *HIWI* expression in epithelial ovarian cancer. Patients with either low (EI = 0) or high (EI ≥ 0.32) *HIWI* expression had better overall survivals than those with medium (0 < EI < 0.32) expression (p = 0.025, log-rank test)

Table 1 Clinicopathological variables and expressions of *HIWI* and let-7a in 211 epithelial ovarian cancer patients

Variables	N	%
Total	211	100
Disease Stage		
I	52	24.6
II	12	5.7
III	133	63.0
IV	14	6.6
Tumor Grade		
1	34	16.1
2	40	19.0
3	137	64.9
Histology (n=178)		
Clear Cell	16	7.6
Endometrial	41	19.4
Mucinous	18	8.5
Müllerian	14	6.6
Undifferentiated	36	17.1
Other	1	0.5
Sub-total (Non-serous)	126	59.7
Serous	85	40.3
Debulking Results		
Optimal	108	51.9
Suboptimal	100	48.1
Residual Tumor Size (cm)		
0	91	44.0
> 0	116	56.0
	N	Median (range)
Age (years)	208	58 (26 – 82)
HIWI expression	211	0.06 (0 – 14.28)
Let-7a expression	211	4.62 (0 – 655)

Table 2. Associations of *HIWI* expression and patient survival in epithelial ovarian cancer

Variable	Relapse	Death
	HR[1] (95% CI[2])	HR (95% CI)
Univariate		
HIWI (Medium vs. Low or High)	1.33 (0.863 – 2.05)	**1.63 (1.06 – 2.52)**
Multivariate[3]		
HIWI (Medium vs. Low or High)	1.38 (0.88 – 2.16)	**1.89 (1.29 – 2.98)**
Age	1.02 (0.99 – 1.04)	1.01 (0.99 – 1.03)
Disease stage	1.28 (0.88 – 1.87)	**1.71 (1.08 – 2.71)**
Tumor grade	1.28 (0.89 – 1.84)	1.45 (0.97 – 2.17)
Residual tumor size (>0 vs 0)	**3.00 (1.62 – 5.57)**	**5.29 (2.50 – 11.20)**
Histological type (Serous vs non-serous)	1.17 (0.75 – 1.82)	0.73 (0.46 – 1.13)
Chemotherapy (yes vs no)	1.86 (0.55 – 6.24)	**0.40 (0.17 – 0.95)**

1. HR: Hazards Ratio.
2. CI: Confidence Interval.
3. Adjusted for age at surgery, disease stage, tumor grade, residual tumor size, histological type and chemotherapy status.

Table 3. Associations of *HIWI* expression with clinical and pathologic variables in epithelial ovarian cancer

Variable	N	Low/ High *HIWI* [1] n (%)	Medium *HIWI* n (%)	OR [2](95% CI[3])	p value
Disease Stage				0.79 (0.42-1.48) [4]	0.459
1-2	64	42 (65.6)	22 (34.4)		
3-4	147	104 (70.8)	43 (29.2)		
Tumor Grade				1.32 (0.71-2.47)	0.382
1-2	74	54 (73.0)	20 (27.0)		
3	137	92 (67.2)	45 (32.8)		
Residual Size (cm)				1.01 (0.56-1.83)	0.967
0	91	63 (69.2)	28 (30.8)		
>0	116	80 (69.0)	36 (31.0)		
Histological Type				1.42 (0.79-2.56)	0.246
Non-serous	126	91 (72.2)	35 (27.8)		
Serous	85	55 (64.7)	30 (35.3)		
Debulking Result				0.82 (0.45-1.47)	0.502
Sub-optimal	100	67 (67.0)	33 (33.0)		
Optimal	108	77 (71.3)	31 (28.7)		
Chemotherapy Response				**3.01 (1.50-6.04)**	**0.002**
Yes	128	98 (76.6)	30 (23.4)		
No	48	25 (52.1)	23 (47.9)		

1. *HIWI* expression: low, EI = 0 (undetectable); medium, 0 < EI <0.32; high, EI ≥0.32.
2. OR: odds ratio obtained from logistic regression analysis
3. CI: confidential interval.
4. In logistic regression analyses, the group of low or high *HIWI* expression was chosen as reference, and the second level of each variable as 'event'.

Table 4. Interaction of *HIWI* and *let-7a* expression in patient survival of epithelial ovarian cancer

Groups[1]	*HIWI*	Let-7a	Relapse		Death	
			Adj-HR [2]	95% CI [3]	Adj-HR	95% CI
Group 1	Low/high	Low	1.00		1.00	
Group 2	Low/high	High	0.94	0.57 – 1.53	1.11	0.66 – 1.86
Group 3	Medium	Low	1.26	0.70 – 2.29	1.64	0.88 – 3.04
Group 4	Medium	High	1.45	0.74 -2.85	**2.71**	**1.38 – 5.32**

1. Groups: group 1, low (EI = 0) or high (EI ≥0.32) *HIWI* and low (EI < 4.62) *let-7a*; group 2, low or high *HIWI* and high *let-7a* (EI ≥ 4.62); group 3, medium (0 < EI <0.32) *HIWI* and low *let-7a*; and group 4, medium *HIWI* and high *let-7a*.
2. Adj-HR: adjusted Hazard Ratio for patient age at surgery, disease stage, tumor grade, residual tumor size, histological type and chemotherapy status.
3. CI: Confidence Interval.

Table 5. Stratification analyses for the associations of *HIWI* and *let-7a* expression with patient survival in epithelial ovarian cancer

Stratified Variable [1]	Comparison Variable	Death		
		Adj-HR [2]	95% CI [3]	P value
HIWI	Let-7a			
Low/high	High vs. low	1.19	0.71 – 2.01	0.511
Medium	High vs. low	1.61	0.75 – 3.43	0.218
Let-7a	HIWI			
Low	Medium vs. low/high	1.63	0.86 – 3.08	0.137
High	Medium vs low/high	**2.62**	**1.30 – 5.30**	**0.007**

1. HIWI: low (EI = 0, undetectable), medium (0 < EI <0.32), high (EI ≥0.32); let-7a: low (EI < 4.62), high (EI ≥ 4.62).
2. Adj-HR: adjusted Hazard Ratio for patient age at surgery, disease stage, tumor grade, residual tumor size, histological type and chemotherapy status.
3. CI: Confidence Interval.

Figure 1. Kaplan-Meier overall survival curves by levels of *HIWI* expression