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

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1 **Abstract**

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

22

23

1 **Introduction**

2 *HIWI* (also known as piwi-like RNA-mediated gene silencing 1, *PIWIL1*), located on
3 chromosome 12q14.33, is a member of the P-element induced wimpy testis (*PIWI*) gene
4 family in humans [1]. This family is evolutionarily conserved across species; their
5 encoded-proteins are highly homologous, particularly in the carboxy-terminus [1]. The
6 *HIWI* is a ribonucleoprotein in the length of 861 amino acids, which contains a N-
7 terminal PIWI/Argonaute/Zwille (PAZ) domain and a PIWI domain at the carboxy-
8 terminus [2-4]. The PAZ domain binds to single-stranded small RNA, and the PIWI
9 domain functions as an RNase H endonuclease cleaving target RNA complementarily
10 bound to small RNA [4-6]. The involvement of *HIWI* proteins in the regulation of gene
11 expression is through their interacting partners of small non-coding RNAs such as
12 namely PIWI-associated RNAs (piRNAs) [7]. Nuclear *HIWI* controls retrotransposon
13 silencing via affecting DNA methylation [8-10]. Recently, Sequencing results of
14 Immunoprecipitation (IP) against *HIWI* show that *HIWI* interacts with miRNAs
15 including let-7a in fining gene expression [11]. Moreover, it has also been reported that
16 *HIWI* can be a partner interacting with Dicer, a key RNase III endonuclease in miRNA
17 maturation [12-14]. These findings suggest *HIWI* is also localized in cytoplasm and
18 may participate in the role of miRNAs in gene regulation. The *HIWI* gene is expressed
19 in hematopietic stem cells and germ cells but not in differentiated cells. It has been
20 evidenced that *HIWI* plays an important role in maintaining stem cell renewal
21 [1,7,15,16]. In the *PIWI* mutant models, germline stem cell asymmetric divisions cannot
22 be functionally processed in both male and female flies, leading to a loss of germline
23 stem cells and a reduced number of eggs and sperms during oogenesis or

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

3 Aberrant *HIWI* expression is associated with tumorigenesis and with cancer patient
4 survival. Over-expressed *HIWI* has been observed in seminomas testicular tumors but
5 not in nonseminomatous, spermatocytic seminomas and somatic types [1,19]. A large
6 number of gastric cancers show increased *HIWI* expression in comparison to normal
7 tissues, and *HIWI* has been involved in the proliferation of gastric cancer cells [20].

8 Both U-shape and linear associations between *HIWI* expression levels and cancer
9 patient survival have been observed. Compared to moderate expression, both low and
10 high *HIWI* expression were reported to be associated with increased death in patients
11 with soft-tissue sarcoma, suggesting that *HIWI* may have biphasic effects on tumor
12 progression [21,22]. Recently, Grochola et al. reported that *HIWI* expression increased
13 pancreatic cancer-related death but in a male-specific manner [23]. Prognostic value of
14 *HIWI* has been shown in several human solid tumors including hepatocellular
15 carcinoma [24], colorectal cancer [25], glioma [26], and esophageal carcinoma [27].

16 Very recently, it has also been shown that the expression of *HIWI* was significantly
17 higher in epithelial ovarian cancer than in normal ovaries and benign tumors, while
18 enforced overexpression of *HIWI* in SKOV3 ovarian cancer cell line led to reduced
19 metastatic capacity [18], suggesting moderate *HIWI* expression may associate with
20 tumor development and progression in ovarian cancer. However, whether *HIWI*
21 expression has prognostic value in ovarian cancer is yet to be investigated.

22

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

15

16 **Materials and Methods**

17 *Patients and tumor samples*

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

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

6
7 There were four categories of treatment response defined in the study. The definition is
8 as follows: (a) complete response: resolution of all evidence of disease for at least a
9 month, (b) partial response: a decrease of $\geq 50\%$ in the product of the diameters
10 (maximum and minimum) of all measurable lesions without the development of new
11 lesions for at least a month, (c) stable disease: a decrease of $< 50\%$ or an increase of
12 $< 25\%$ in the product of the diameters of all measurable lesion, and (d) progressive
13 disease: an increase of $\geq 25\%$ in the product of the diameters of all measurable lesions or
14 the development of new lesions. Of the 176 patients with available information on
15 chemotherapy treatment response, 128 (72.7%) who had complete response was
16 considered 'Yes' response in data analysis, while 48 (27.3%) who were in the other
17 three categories were grouped as 'No' response, which included 36 with partial
18 response, 4 with stable disease, and 8 with progressive disease.

19

20 *Analysis of HIWI and let-7a expression*

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

1 First-Strand cDNA Synthesis kit (Invitrogen™, Carlsbad, CA). The primers for *HIWI*
2 and small nucleolar RNA *RNU48* (used for normalization) were designed based on
3 sequences in Genbank (accession number AB274731 for *HIWI* and X96648 for
4 *RNU48*). The primer sequences for *HIWI* and *RNU48* are: *HIWI*-forward, 5'- CCT GGC
5 TTC ACT ACT TCC ATC C; *HIWI*-reverse, 5'- ACG TCA GTG CAG AGC ATG
6 ATG; *RNU48*-forward, 5' AGTGATGATGACCCCAGGTAAGTC, and *RNU48*-
7 reverse, 5'- CTG CGG TGA TGG CAT CAG. Real-time PCR was performed to analyze
8 *HIWI* expression using the Chromo4™ Real-time PCR System (MJ Research Inc.,
9 Waltham, MA). In the PCR reaction (20 µl), 2 µl of cDNA template was mixed with 10
10 µl of 2× Power SYBR® Green PCR master mix (Applied Biosystems, Foster City, CA)
11 and each pair of primers, at final concentrations of 100 nM for both *HIWI* and *RNU48*.
12 PCR amplification included initial incubation at 50°C for 2 minutes, denaturing at 95°C
13 for 10 minutes, and 40 cycles of denaturing at 95°C for 15 seconds and annealing at
14 60°C for one minute. Melting curves were analyzed after each run to verify the size of
15 PCR product.
16 Analysis of *let-7a* expression in tumor tissue was performed using Taqman® microRNA
17 assay (Applied Biosystems), in which the preparation of miRNA cDNA was made first
18 using a stem-loop method, following the manufacturer's instruction as described in our
19 previous report [45] . Briefly, levels of *let-7a* and *RNU48* (an internal control for
20 normalization) expression in the samples were determined with the Taqman® miRNA
21 assay (Applied Biosystems) using the Chromo4 Real-time PCR System. In the PCR
22 reaction (15 µl), 0.3 µl of cDNA template was mixed with 7.5 µl of 2×Taqman®
23 Universal PCR master mix (Applied Biosystems), 0.75 µl of 20× probe/primers

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

5

6 *Statistical analysis*

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

22

23

1 **Results**

2

3 *Clinical and pathological characteristics of patients*

4 Clinical and pathological features of the 211 participants enrolled in this study were
5 shown in Table 1. These patients underwent surgery at ages between 26 to 82 years,
6 and the median age was 58 years. Based on the International Federation of Gynecology
7 and Obstetrics Classification [43], 52 patients were diagnosed with stage I disease
8 (24.6%), 12 stage II (5.7%), 133 stage III (63.0%), and 14 stage IV (6.6%). Tumor
9 grade and histology were determined according to the WHO guidelines [44]. Thirty-
10 four (16.1%) were grade 1 tumors, 40 (19.0%) grade 2 and 137(64.9%) grade 3.
11 Eighty-five (40.3%) patients had serous tumors, and 126 patients had non-serous tumors
12 which included 16 (7.6%) clear cell, 41 (19.4%) endometrial, 18 (8.5%) mucinous, 14
13 (6.6%) müllerian, 1 other (0.5%) and 36 (17.1%) undifferentiated histology. Surgical
14 debulking was performed on all the patients. Optimal debulking results were achieved
15 in 108 (51.9%) patients, and 100 (48.1%) had suboptimal outcomes. Ninety-one
16 patients (44.0%) had no residual tumor, and 116 (56.0%) had a residual tumor size
17 greater than 0.

18

19

20 *Association of HIWI expression with epithelial ovarian cancer prognosis*

21 Of 211 samples, 82 (38.9%), over a third, had undetectable expression of *HIWI*, and
22 129 had detectable expression with an average EI of 0.32, ranging from 0.02 to 4.86
23 (5th-95th percentiles). To investigate the impact of *HIWI* expression on prognosis of
24 ovarian cancer, we classified patients based on their *HIWI* expression into three groups,
25 low, medium and high, using the EI = 0 (undetectable), >0 to <0.32, and ≥ 0.32 as

1 cutoffs. The numbers of patients in these groups were 82, 65 and 64, respectively.

2 Kaplan-Meier survival curves showed that patients with low and high *HIWI* did not

3 have substantial differences in progression-free or overall survival, but did have more

4 favorable prognosis than those with medium *HIWI* expression (data not shown). Based

5 on this finding and U-shaped associations with death observed elsewhere [21-23], we

6 combined the low and high groups together, and compared their survival outcomes with

7 those with medium *HIWI* expression. Kaplan-Meier survival curves showed that

8 patients with medium expression had worse overall survival ($p = 0.025$) (Figure 1).

9 However, disease progression-free survival was not different by *HIWI* expression ($p =$

10 0.200) (data not shown).

11

12 To confirm the results of Kaplan-Meier analysis and to adjust for potential confounding

13 factors, we further analyzed the data with the Cox proportional hazards regression.

14 Proportional hazards assumption test for *HIWI* expression did not show significance (p

15 $= 0.59$). The results of univariate and multivariate analyses were shown in Table 2. Here

16 too, medium *HIWI* expression was significantly associated with elevated risk for death

17 in both univariate and multivariate analyses. After adjustment for patient age at surgery,

18 disease stage, tumor grade, residual tumor size, histological type and chemotherapy

19 status, the association remained significant. The adjusted hazard ratio (HR) was 1.89

20 (95% CI: 1.29 – 2.98) for high or low *HIWI* expression compared to medium

21 expression. In addition, disease stage and residual tumor size showed positive

22 associations with the risk of death, while chemotherapy treatment improved patient

23 survival. Their adjusted HRs were 1.71 (95% CI: 1.08 – 2.71) for disease stage, 5.29

1 (95% CI: 2.50 – 11.20) for residual tumor size and 0.40 (95% CI: 0.17 – 0.95) for
2 chemotherapy treatment, respectively. However, the associations of tumor grade,
3 histological type and patient age at surgery with the risk of death were not statistically
4 significant. Similarly to the Kaplan-Meier analysis, little evidence of association was
5 found between disease progression and *HIWI* expression. Residual tumor size was
6 positively associated with the risk of relapse. The adjusted HR was 3.00 (95% CI: 1.62
7 – 5.57).

8

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

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

19

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

21 The average EI of let-7a in this study was 4.62 with the range from 0.53 to 35.3 (5th –
22 95th percentiles).

1 Previous studies including immunoprecipitation sequencing results suggest that *HIWI*
2 might interplay with *let-7a* in regulating biological processes [11,15,41,42]. To examine
3 whether there is any interaction between *HIWI* and *let-7a* expressions in the patient
4 survival of epithelial ovarian cancer, we used the median of *let-7a* expression as the
5 cutoff value to classify *let-7a* into two groups, high ($EI \geq 4.62$, median) and low ($EI <$
6 4.62). Then we classified patients into four groups; group 1 with low ($EI = 0$) or high
7 ($EI \geq 0.32$) *HIWI* and low ($EI < 4.62$) *let-7a*, group 2 with low or high *HIWI* and high
8 *let-7a* ($EI \geq 4.62$, median), group 3 with medium ($0 < EI < 0.32$) *HIWI* and low *let-7a*,
9 and group 4 with medium *HIWI* and high *let-7a*. Multivariate Cox proportional hazard
10 models showed that the adjusted HRs of death were 1.11 (95% CI: 0.66 – 1.86) for
11 Group 2, 1.64 (95% CI: 0.88 – 3.04) for Group 3, and 2.71 (95% CI: 1.38 – 5.32) for
12 Group 4 (Table 4) after the adjustment for patient age at surgery, disease stage, tumor
13 grade, residual tumor size, histological types and chemotherapy status. However, no
14 significant associations were found in progression-free survival in the multivariate
15 analysis.

16
17 We next sought to how *HIWI* and *let-7a* modulate each other the effect on patient
18 survival using stratification analyses (Table 5). When we stratified patients by *HIWI*
19 expression levels, we did not find the associations of *let-7a* expression with overall
20 survival ($p > 0.05$) within the strata of either low/high or medium *HIWI*. In contrast,
21 when we stratified patients by *let-7a* expression levels (low and high), we found among
22 patients with high *let-7a* expression, medium expression of *HIWI* significantly
23 increased the risk of death compared to low/high *HIWI* expression; the adjusted HR was

1 2.62 (95% CI: 1.30 – 5.30). Among those with low *let-7a* expression, however, no
2 significant association was found between the risk of death and *HIWI* expression levels;
3 the adjusted HR was 1.63 (95% CI: 0.86 – 3.08).

4 **Discussion**

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

15

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

1 patients with low or high *HIWI* expression had a more favorable prognosis. This finding
2 was similar to, but in the opposite direction of, observations of several previous clinical
3 studies, which showed that compared to medium, high or low *HIWI* expression was
4 associated with elevated death risks in patients with soft-tissue sarcoma and male
5 patients with pancreatic cancer [21-23]. Our result, however, seems to be more
6 consistent with findings of a very recent report, in which it was shown that significantly
7 increased *HIWI* expression was observed in epithelial ovarian cancer compared to
8 normal ovarian tissues and benign tumor tissues, while enforced overexpression of
9 *HIWI* repressed the invasiveness of ovarian cancer cell SKOV3 [18]. Our findings are
10 also supported by *in vitro* experiments that show similar impacts of loss- and gain-of-
11 function of *HIWI* on cancer cells. Inhibition of *HIWI* expression in gastric cancer cells
12 led to G2/M phase arrest and decreased proliferation index of the cancer cells [20],
13 while high *HIWI* expression appears to induce apoptosis in tumor cell lines of KG1
14 [15]. G2/M arrest is a critical step in the initiation of apoptosis [47-49]. Thus, both low
15 and high *HIWI* expression may have similar effects on cancer cells.

16

17 Interestingly, previous studies on hepatocellular carcinoma [24] , colorectal cancer [25]
18 and esophageal squamous carcinoma [27] have shown direct associations between high
19 *HIWI* proteins (immunohistochemical staining, IHC) and poor patient survival, but such
20 correlations have been limited to certain subgroups of patients. Zhao and colleagues
21 [24] reported that high *HIWI* protein was significantly associated with poor prognosis
22 of hepatocellular carcinoma only in patients with alpha-fetoprotein (AFP) less than 300
23 ng/ml, but not in those with AFP greater than that level. Zeng and colleagues [25]

1 demonstrated that HIWI protein in tissues adjacent to tumor but not in primary tumor
2 was significantly associated with poor prognosis of colorectal carcinoma. Another study
3 [27] found that patients with high cytoplasmic but not nuclear HIWI protein had poor
4 prognosis in esophageal squamous carcinoma, while no association with prognosis was
5 seen if both cytoplasmic and nuclear HIWI proteins were combined for consideration.
6 Three possibilities may explain the discrepancy between our and previous studies on the
7 association of *HIWI* expression with patient survival. First, translated HIWI from the
8 mRNAs may be aberrant due to premature stop codon-causing truncation or alternative
9 splicing, thereby influencing antigen determinants and IHC results, as well as its
10 function [18]. This aberrant expression of HIWI may result in the phenomenon that it is
11 high at mRNA levels but low at protein levels. Second, different laboratory methods
12 were applied to measure *HIWI* expression (qPCR for mRNA vs IHC for protein); *HIWI*
13 expression at mRNA levels measured by qPCR showed biphasic effects, while *HIWI*
14 expression at protein levels by IHC had monotonic effects [24,25,27]. Finally, the
15 association between *HIWI* expression and cancer prognosis may be tumor or tissue
16 specific.

17

18 In this study, we also found a significant interaction between *HIWI* expression and let-
19 7a, increasing the risk of death in epithelial ovarian cancer. Stratification analyses
20 demonstrated let-7a modified the effect of *HIWI* expression on overall survival of EOC.
21 Only at high let-7a levels did patients with medium *HIWI* expression increase the risk of
22 death. This finding supports and extends the previous observation reported by Chen
23 and colleagues [11], suggesting that HIWI protein not only physically binds let-7a, but

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

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

8

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

19

20 **Conflict of Interest Statement**

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

22

23

1

2 **References**

- 3 1. Qiao D, Zeeman AM, Deng W, Looijenga LH, Lin H. Molecular
4 characterization of hiwi, a human member of the piwi gene family whose
5 overexpression is correlated to seminomas. *Oncogene* 2002;21(25):3988-3999.
- 6 2. Cerutti L, Mian N, Bateman A. Domains in gene silencing and cell
7 differentiation proteins: the novel PAZ domain and redefinition of the Piwi
8 domain. *Trends Biochem Sci* 2000;25(10):481-482.
- 9 3. Peters L, Meister G. Argonaute proteins: mediators of RNA silencing. *Mol Cell*
10 2007;26(5):611-623.
- 11 4. Farazi TA, Juranek SA, Tuschl T. The growing catalog of small RNAs and their
12 association with distinct Argonaute/Piwi family members. *Development*
13 2008;135(7):1201-1214.
- 14 5. Tolia NH, Joshua-Tor L. Slicer and the argonautes. *Nat Chem Biol*
15 2007;3(1):36-43.
- 16 6. Meister G. Argonaute proteins: functional insights and emerging roles. *Nat Rev*
17 *Genet* 2013;14(7):447-459.
- 18 7. Samji T. PIWI, piRNAs, and germline stem cells: what's the link? *Yale J Biol*
19 *Med* 2009;82(3):121-124.
- 20 8. Siddiqi S, Terry M, Matushansky I. Hiwi mediated tumorigenesis is associated
21 with DNA hypermethylation. *PLoS One* 2012;7(3):e33711.
- 22 9. Brennecke J, Aravin AA, Stark A et al. Discrete small RNA-generating loci as
23 master regulators of transposon activity in *Drosophila*. *Cell* 2007;128(6):1089-
24 1103.
- 25 10. Li C, Vagin VV, Lee S et al. Collapse of germline piRNAs in the absence of
26 Argonaute3 reveals somatic piRNAs in flies. *Cell* 2009;137(3):509-521.
- 27 11. Chen R, Chang G, Zhang Y et al. Cloning of the quail PIWI gene and
28 characterization of PIWI binding to small RNAs. *PLoS One* 2012;7(12):e51724.
- 29 12. Tahbaz N, Kolb FA, Zhang H, Jaronczyk K, Filipowicz W, Hobman TC.
30 Characterization of the interactions between mammalian PAZ PIWI domain
31 proteins and Dicer. *EMBO Rep* 2004;5(2):189-194.
- 32 13. Kolb FA, Zhang H, Jaronczyk K, Tahbaz N, Hobman TC, Filipowicz W. Human
33 dicer: purification, properties, and interaction with PAZ PIWI domain proteins.
34 *Methods Enzymol* 2005;392:316-336.
- 35 14. Megosh HB, Cox DN, Campbell C, Lin H. The role of PIWI and the miRNA
36 machinery in *Drosophila* germline determination. *Curr Biol* 2006;16(19):1884-
37 1894.
- 38 15. Sharma AK, Nelson MC, Brandt JE et al. Human CD34(+) stem cells express
39 the hiwi gene, a human homologue of the *Drosophila* gene piwi. *Blood*
40 2001;97(2):426-434.
- 41 16. Ross RJ, Weiner MM, Lin H. PIWI proteins and PIWI-interacting RNAs in the
42 soma. *Nature* 2014;505(7483):353-359.

- 1 17. Cox DN, Chao A, Lin H. piwi encodes a nucleoplasmic factor whose activity
2 modulates the number and division rate of germline stem cells. *Development*
3 2000;127(3):503-514.
- 4 18. Lim SL, Ricciardelli C, Oehler MK, De Arao Tan IM, Russell D, Grutzner F.
5 Overexpression of piRNA Pathway Genes in Epithelial Ovarian Cancer. *PLoS*
6 *One* 2014;9(6):e99687.
- 7 19. Suzuki R, Honda S, Kirino Y. PIWI Expression and Function in Cancer. *Front*
8 *Genet* 2012;3:204.
- 9 20. Liu X, Sun Y, Guo J et al. Expression of hiwi gene in human gastric cancer was
10 associated with proliferation of cancer cells. *Int J Cancer* 2006;118(8):1922-
11 1929.
- 12 21. Taubert H, Greither T, Kaushal D et al. Expression of the stem cell self-renewal
13 gene Hiwi and risk of tumour-related death in patients with soft-tissue sarcoma.
14 *Oncogene* 2007;26(7):1098-1100.
- 15 22. Taubert H, Wurl P, Greither T et al. Stem cell-associated genes are extremely
16 poor prognostic factors for soft-tissue sarcoma patients. *Oncogene*
17 2007;26(50):7170-7174.
- 18 23. Grochola LF, Greither T, Taubert H et al. The stem cell-associated Hiwi gene in
19 human adenocarcinoma of the pancreas: expression and risk of tumour-related
20 death. *Br J Cancer* 2008;99(7):1083-1088.
- 21 24. Zhao YM, Zhou JM, Wang LR et al. HIWI is associated with prognosis in
22 patients with hepatocellular carcinoma after curative resection. *Cancer*
23 2012;118(10):2708-2717.
- 24 25. Zeng Y, Qu LK, Meng L et al. HIWI expression profile in cancer cells and its
25 prognostic value for patients with colorectal cancer. *Chin Med J (Engl)*
26 2011;124(14):2144-2149.
- 27 26. Sun G, Wang Y, Sun L et al. Clinical significance of Hiwi gene expression in
28 gliomas. *Brain Res* 2011;1373:183-188.
- 29 27. He W, Wang Z, Wang Q et al. Expression of HIWI in human esophageal
30 squamous cell carcinoma is significantly associated with poorer prognosis. *BMC*
31 *Cancer* 2009;9:426.
- 32 28. Johnson SM, Grosshans H, Shingara J et al. RAS is regulated by the let-7
33 microRNA family. *Cell* 2005;120(5):635-647.
- 34 29. Johnson CD, Esquela-Kerscher A, Stefani G et al. The let-7 MicroRNA
35 Represses Cell Proliferation Pathways in Human Cells. *Cancer Res*
36 2007;67(16):7713-7722.
- 37 30. Zhu H, Shyh-Chang N, Segre AV et al. The Lin28/let-7 axis regulates glucose
38 metabolism. *Cell* 2011;147(1):81-94.
- 39 31. Erturk E, Cecener G, Egeli U et al. Expression status of let-7a and miR-335
40 among breast tumors in patients with and without germ-line BRCA mutations.
41 *Mol Cell Biochem* 2014;395(1-2):77-88.
- 42 32. Yanaihara N, Caplen N, Bowman E et al. Unique microRNA molecular profiles
43 in lung cancer diagnosis and prognosis. *Cancer Cell* 2006;9(3):189-198.
- 44 33. Akao Y, Nakagawa Y, Naoe T. let-7 microRNA functions as a potential growth
45 suppressor in human colon cancer cells. *Biol Pharm Bull* 2006;29(5):903-906.

- 1 34. Muller DW, Bosserhoff AK. Integrin beta 3 expression is regulated by let-7a
2 miRNA in malignant melanoma. *Oncogene* 2008;27(52):6698-6706.
- 3 35. Xie YL, Yang YJ, Tang C et al. Estrogen combined with progesterone decreases
4 cell proliferation and inhibits the expression of Bcl-2 via microRNA let-7a and
5 miR-34b in ovarian cancer cells. *Clin Transl Oncol* 2014.
- 6 36. Ruzzo A, Graziano F, Vincenzi B et al. High let-7a microRNA levels in KRAS-
7 mutated colorectal carcinomas may rescue anti-EGFR therapy effects in patients
8 with chemotherapy-refractory metastatic disease. *Oncologist* 2012;17(6):823-
9 829.
- 10 37. Lu L, Schwartz P, Scarampi L et al. MicroRNA let-7a: a potential marker for
11 selection of paclitaxel in ovarian cancer management. *Gynecol Oncol*
12 2011;122(2):366-371.
- 13 38. Golestaneh AF, Atashi A, Langroudi L, Shafiee A, Ghaemi N, Soleimani M.
14 miRNAs expressed differently in cancer stem cells and cancer cells of human
15 gastric cancer cell line MKN-45. *Cell Biochem Funct* 2012;30(5):411-418.
- 16 39. Liu Y, Li H, Feng J et al. Lin28 induces epithelial-to-mesenchymal transition
17 and stemness via downregulation of let-7a in breast cancer cells. *PLoS One*
18 2013;8(12):e83083.
- 19 40. Cairo S, Wang Y, de Reynies A et al. Stem cell-like micro-RNA signature
20 driven by Myc in aggressive liver cancer. *Proc Natl Acad Sci U S A*
21 2010;107(47):20471-20476.
- 22 41. Tokumaru S, Suzuki M, Yamada H, Nagino M, Takahashi T. let-7 regulates
23 Dicer expression and constitutes a negative feedback loop. *Carcinogenesis*
24 2008;29(11):2073-2077.
- 25 42. Worringer KA, Rand TA, Hayashi Y et al. The let-7/LIN-41 pathway regulates
26 reprogramming to human induced pluripotent stem cells by controlling
27 expression of prodifferentiation genes. *Cell Stem Cell* 2014;14(1):40-52.
- 28 43. Shepherd JH. Revised FIGO staging for gynaecological cancer. *Br J Obstet*
29 *Gynaecol* 1989;96(8):889-892.
- 30 44. Scully R, Sobin L, Serov S. *Histological typing of ovarian tumors*. Berlin (NY):
31 Springer; 1999.
- 32 45. Lu L, Katsaros D, de la Longrais IA, Sochirca O, Yu H. Hypermethylation of
33 let-7a-3 in epithelial ovarian cancer is associated with low insulin-like growth
34 factor-II expression and favorable prognosis. *Cancer Res* 2007;67(21):10117-
35 10122.
- 36 46. Liu WK, Jiang XY, Zhang ZX. Expression of PSCA, PIWIL1 and TBX2 and its
37 correlation with HPV16 infection in formalin-fixed, paraffin-embedded cervical
38 squamous cell carcinoma specimens. *Arch Virol* 2010;155(5):657-663.
- 39 47. Concin N, Stimpfl M, Zeillinger C et al. Role of p53 in G2/M cell cycle arrest
40 and apoptosis in response to gamma-irradiation in ovarian carcinoma cell lines.
41 *Int J Oncol* 2003;22(1):51-57.
- 42 48. Manna SK, Bose JS, Gangan V et al. Novel derivative of benzofuran induces
43 cell death mostly by G2/M cell cycle arrest through p53-dependent pathway but
44 partially by inhibition of NF-kappaB. *J Biol Chem* 2010;285(29):22318-22327.

1 49. Zhang Z, Miao L, Lv C et al. Wentilactone B induces G2/M phase arrest and
2 apoptosis via the Ras/Raf/MAPK signaling pathway in human hepatoma
3 SMMC-7721 cells. *Cell Death Dis* 2013;4:e657.
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11 **Figure legends**

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

14 epithelial ovarian cancer. Patients with either low ($EI = 0$) or high ($EI \geq 0.32$) *HIWI*

15 expression had better overall survivals than those with medium ($0 < EI < 0.32$)

16 expression ($p = 0.025$, log-rank test)

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

| Variables | N | % |
|---------------------------------|-----|-----------------------|
| Total | 211 | 100 |
| <u>Disease Stage</u> | | |
| I | 52 | 24.6 |
| II | 12 | 5.7 |
| III | 133 | 63.0 |
| IV | 14 | 6.6 |
| <u>Tumor Grade</u> | | |
| 1 | 34 | 16.1 |
| 2 | 40 | 19.0 |
| 3 | 137 | 64.9 |
| <u>Histology (n=178)</u> | | |
| 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 |
| <u>Sub-total (Non-serous)</u> | 126 | 59.7 |
| Serous | 85 | 40.3 |
| <u>Debulking Results</u> | | |
| Optimal | 108 | 51.9 |
| Suboptimal | 100 | 48.1 |
| <u>Residual Tumor Size (cm)</u> | | |
| 0 | 91 | 44.0 |
| > 0 | 116 | 56.0 |
| | N | <u>Median (range)</u> |
| Age (years) | 208 | 58 (26 – 82) |
| <i>HIWI</i> expression | 211 | 0.06 (0 – 14.28) |
| <i>Let-7a</i> expression | 211 | 4.62 (0 – 655) |

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

| Variable | Relapse | Death |
|--|--|----------------------------|
| | HR ¹ (95% CI ²) | HR (95% CI) |
| <u>Univariate</u> | | |
| <i>HIWI</i> (Medium vs. Low or High) | 1.33 (0.863 – 2.05) | 1.63 (1.06 – 2.52) |
| <u>Multivariate³</u> | | |
| <i>HIWI</i> (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) |

14 1. HR: Hazards Ratio.
 15 2. CI: Confidence Interval.
 16 3. Adjusted for age at surgery, disease stage, tumor grade, residual tumor size,
 17 histological type and chemotherapy status.
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3 **Table 3. Associations of *HIWI* expression with clinical and pathologic variables in**4 **epithelial ovarian cancer**

| Variable | N | Low/ High <i>HIWI</i> ¹ n (%) | Medium <i>HIWI</i> n (%) | OR ² (95% CI ³) | p value |
|------------------------------|-----|---|-----------------------------|--|--------------|
| <u>Disease Stage</u> | | | | 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) | | |
| <u>Tumor Grade</u> | | | | 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) | | |
| <u>Residual Size (cm)</u> | | | | 1.01 (0.56-1.83) | 0.967 |
| 0 | 91 | 63 (69.2) | 28 (30.8) | | |
| >0 | 116 | 80 (69.0) | 36 (31.0) | | |
| <u>Histological Type</u> | | | | 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) | | |
| <u>Debulking Result</u> | | | | 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) | | |
| <u>Chemotherapy Response</u> | | | | 3.01 (1.50-6.04) | 0.002 |
| Yes | 128 | 98 (76.6) | 30 (23.4) | | |
| No | 48 | 25 (52.1) | 23 (47.9) | | |

5 1. *HIWI* expression: low, EI = 0 (undetectable); medium, 0 < EI <0.32; high, EI
6 ≥0.32.

7 2. OR: odds ratio obtained from logistic regression analysis

8 3. CI: confidential interval.

9 4. In logistic regression analyses, the group of low or high *HIWI* expression was
10 chosen as reference, and the second level of each variable as 'event'.

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

| Groups ¹ | <i>HIWI</i> | 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.

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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.

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

