

## miR-7 Expression in Serous Ovarian Carcinomas

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**Abstract.** *Background/Aim:* miR-7 has recently been linked to cancer. Some miR-7 targets, including B-cell lymphoma 2 (BCL2) and epidermal growth factor receptor (EGFR), are involved in ovarian cancer (OC) pathogenesis. The majority of OCs display TP53 mutations, which are critically important for OC development. We aimed to study the expression level of miR-7 and of two of its postulated target genes, BCL2 and EGFR, in serous ovarian carcinomas of different TP53 status and tumour grade. *Materials and Methods:* Gene and miR expression was assessed by real-time reverse transcription polymerase chain reaction in 45 clinical samples of low- (G1+G2) and high- (G3) grade primary serous OC with wild-type (wt) or mutated TP53, as well as in three OC cell lines, each representing a different TP53 status. The results obtained in patients with OC were analysed against their disease-free survival (DFS). *Results:* In high-grade OC with TP53 mutations, the level of miR-7 expression significantly exceeded (by several fold) that in wtTP53 cancer ( $p<0.01$ ). Within the wtTP53 tumour series, the level of miR-7 expression was significantly higher (by over 10-fold) in high-grade than in low-grade OC ( $p<0.01$ ). miR-7 expression was not found to influence DFS. The differences in miR-7 expression depending on TP53 status found in clinical OC samples were not observed in OC cell lines. miR-7 overexpression correlated with diminished BCL2 expression, but there was no relationship between miR-7 and EGFR expression, neither in tumour samples nor in the cell lines. *Conclusion:* There is a link between miR-7 expression and TP53 status and tumour grade in serous OC. Molecular

*mechanisms of these relationships need to be elucidated. Of the two postulated miR-7 target genes we examined, BCL2, but not EGFR, seems to be a possible miR-7 target in OC.*

Over the past decade, microRNAs (miRs) have emerged as important components of carcinogenesis (1). In this context, miR-7 attracted attention only recently. Depending on cancer type and experimental model, it has been suggested that miR-7 plays the role of a tumour suppressor or of an oncogene (2-7). However, decreased miR-7 expression has frequently been observed, and functional studies point to a tumour-suppressor role of miR-7. In ovarian cancer, scarce and inconsistent data are available. Shahab *et al.* showed miR-7 overexpression in ovarian cancer specimens (8), while other researchers observed no alterations in miR-7 expression in ovarian cancer (9-11).

The potential role of miR-7 in carcinogenesis is further supported by the links of the predicted miR-7 targets to cell-to-cell interaction, adhesion, polarity of epithelial cells, proliferation, apoptosis and survival (3, 6, 12-15). Several postulated miR-7 target genes are known to play important roles in oncogenesis, B-cell lymphoma 2 (BCL2) and epidermal growth factor receptor (EGFR) genes among them (7). BCL2 is an inhibitor of apoptosis, and it has been proposed that it has diagnostic, prognostic and predictive value in ovarian cancer (16). EGFR is a cell membrane receptor, which activates mitogen-activated protein kinase, serine-threonine protein kinase and c-Jun N-terminal kinase pathways, crucial to DNA synthesis and cell proliferation. EGFR overexpression in advanced ovarian cancer leads to increased proliferation and impairment of apoptosis, and it correlates with poor prognosis (17).

TP53 is the most frequently mutated gene in different malignancies. In ovarian carcinomas TP53 mutations have a predictive value (18). The highest frequency of TP53 mutations in ovarian cancer is observed in undifferentiated carcinomas, followed by serous, mucinous and endometrioid (19-21). To our knowledge, the relationship between miR-7 expression and TP53 has not been studied either in ovarian or in any other type of cancer.

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Table I. Serous ovarian cancer sample/patient characteristics according to *TP53* mutation status and tumour grade.

	wt <i>TP53</i>		Missense <i>TP53</i> mutation	Null <i>TP53</i> mutation
Tumor grade	G1-G2 (n=13)	G3 (n=11)	G3 (n=11)	G3 (n=10)
Age (years)				
Range	30-74	29-76	33-65	43-66
Median	51	49	54	59
Grade				
1	3	0	0	0
2	10	0	0	0
3	0	11	11	10
Stage <sup>a</sup>				
I	4	1	1	1
II	1	0	1	0
III	6	10	7	7
IV	1	0	2	2
Cancer cell content (%)				
75-79	1	1	0	0
80-89	1	2	3	2
≥90	11	8	8	8
Follow-up				
No. of patients	11	8	10	9
Range (days)	467-2742	147-2122	357-3946	498-2635
Median (days)	1800	792	1140	1196

<sup>a</sup>Data for one patient not available. wt, wild-type; G, grade; n, number of patients

We aimed to study the expression levels of miR-7 and of two of its postulated target genes, *BCL2* and *EGFR*, in serous ovarian carcinomas characterised by different *TP53* status and tumour grade. We analysed ovarian cancer specimens of serous type only, in order to reduce biological heterogeneity related to tumour type. The expression of miR-7, *BCL2* and *EGFR* was also assessed in established ovarian cancer cell lines matching the clinical material in terms of *TP53* status.

## Materials and Methods

**Clinical specimens and cell lines.** Specimens of primary serous ovarian cancer taken during surgery from previously untreated patients were immediately snap-frozen and kept at  $-70^{\circ}\text{C}$  in a biobank of the Maria Skłodowska-Curie Memorial Cancer Centre and Institute of Oncology in Warsaw. All patients gave their informed consent for the specimens to be submitted to the biobank. Adjacent microtome sections of frozen samples were taken for tumour histopathological characteristics based on standard methods, and for the assessment of cancer cell content, *TP53* mutations, and microRNA and gene expression. The specimens included in the study were carefully selected from a larger collection so as to select those with at least 75% cancer cell content (the

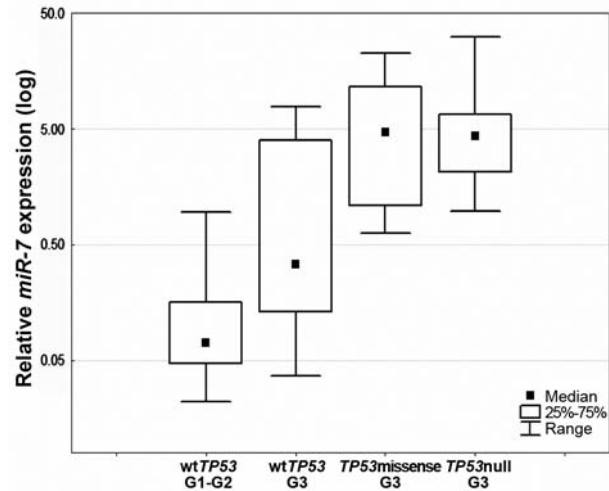


Figure 1. Relative miR-7 expression in clinical samples of primary serous ovarian cancer with different *TP53* status and tumour grade (G). The expression of miR-7 transcript in normal human ovarian surface epithelial cells was set as the reference level (=1).

majority that have been included contained over 90% cancer cells), and to obtain series of samples characterised by different tumour grade and *TP53* gene status [wild type (wt) *TP53* vs. mutated *TP53*, with the assessed missense vs. null mutations]. *TP53* mutational status was determined by polymerase chain reaction-single-strand conformation polymorphism and Sanger sequencing analysis of exons 4-11 of the *TP53* gene (20). Forty-five samples included in the study fell into four categories, low-grade (G1 and G2) with wt*TP53*, high-grade (G3) with wt*TP53*, G3 with missense *TP53* mutation, and G3 with null *TP53* mutation. Patients were followed-up for 467 to 3926 days (median=1226 days), in order to assess disease-free survival (DFS). Patient and sample characteristics are summarised in Table I.

Three ovarian cancer cell lines derived from ovarian carcinomas with different *TP53* status, A2780 (wt*TP53*; purchased from the European Collection of Cell Cultures, Porton Down, Salisbury, UK), TOV112D (missense *TP53* mutation; ATCC® CRL-11731™), and SK-OV-3 (null *TP53* mutation; ATCC® HTB77™), in three biological replicates each, were examined for the expression of miR-7, *BCL2* and *EGFR*.

**Expression quantification.** Total RNA was extracted from frozen tissues and cell lines using mirVana miRNA Isolation Kit (Applied Biosystems, Life Technologies Ltd., Warrington, UK), according to the manufacturer's instructions. The quantity and quality of the RNA samples were analysed using a NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA USA). RNA integrity was checked in a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA), and/or by electrophoresis in a denaturing 1.5% agarose gel. The expression of miR-7 and genes (*TP53*, *BCL2*, *EGFR*) was quantified by reverse transcription real-time polymerase chain reaction with the use of TaqMan MicroRNA Assays, TaqMan Gene Expression Assays, TaqMan® MicroRNA Reverse Transcription Kit, High-Capacity cDNA

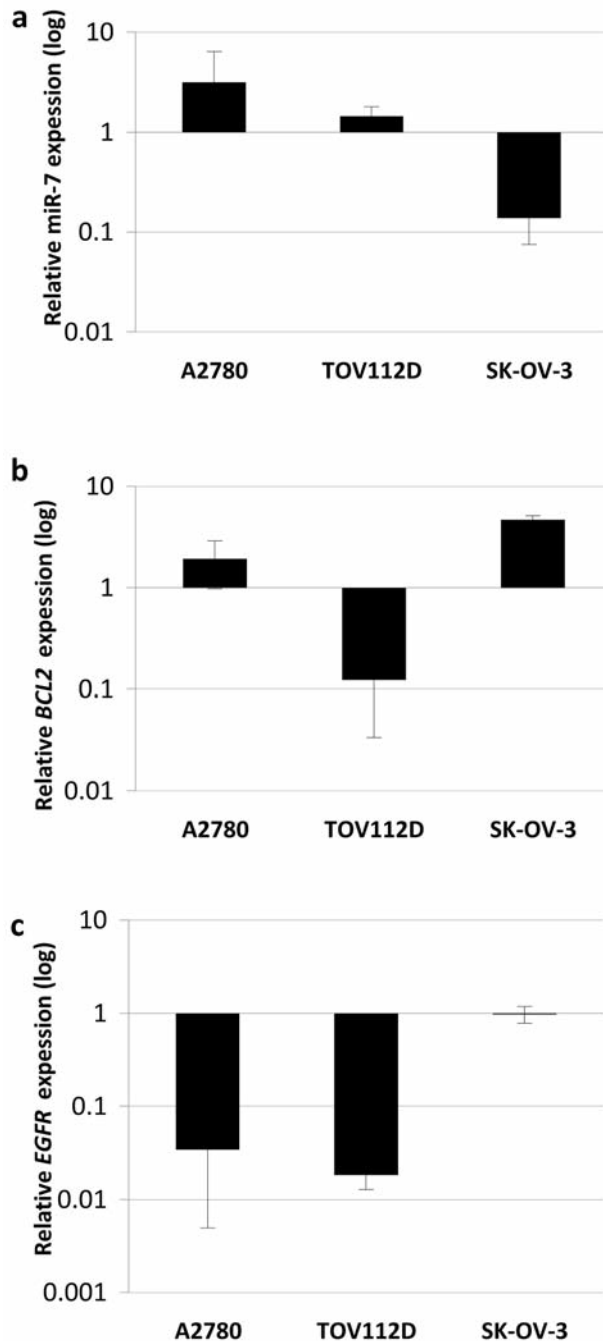


Figure 2. Relative expression of miR-7 (a), B-cell lymphoma 2 (*BCL2*) (b) and epidermal growth factor receptor (*EGFR*) (c) in ovarian cancer cell lines with different *TP53* mutation status. The expression of the respective transcript in normal human ovarian surface epithelial cells was set as the reference level (=1).

Reverse Transcription Kits (Applied Biosystems), following the manufacturer's instructions. Based on the NormFinder algorithm (22) results, small nucleolar RNAs, *RNU6B* and *RNU48*, were chosen as reference points for microRNA expression, and

glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and phosphoglycerate kinase (*PGK*) for gene expression assessment. Relative expression was calculated using the comparative  $-\Delta\Delta C_t$  method, against the calibrator level.

Choosing the appropriate reference tissue for ovarian cancer studies is a challenge. Firstly, the pathogenesis of ovarian cancer is not clear. Ovarian surface epithelium has long been widely regarded as the source of serous ovarian cancer, but very recent evidence strongly suggests the fallopian tube epithelium to be the site of origin [reviewed in: (23)]. Nevertheless, it has been suggested that the two epithelium types are parts of a transitional epithelium of common origin rather than two independent sources of ovarian cancer (24). Secondly, obtaining a sufficient quantity of fresh, reference epithelial cells is difficult. Thus, in ovarian cancer studies, specimens of a normal whole ovary are often used as reference samples (9,10), which, by definition contain different cell types. Viral oncogene- or telomerase-immortalised cells of normal ovarian epithelium or normal ovarian epithelial cells following a short *in vitro* culture provide an alternative, single-cell-type reference material (8, 25, 26). Considering the above, we used normal human ovarian surface epithelium cells (HOSEpiC, cat. no.7310; Scien Cell Res. Lab. Carlsbad, CA, USA) following a short *in vitro* culture (HOSE) as a calibrator.

**Statistical analyses.** The Mann-Whitney U-test was used to examine the differences in microRNA and gene expression. Relationships between miR-7 and gene expression were assessed according to Spearman correlation coefficients. The Cox proportional hazard model was used to assess the relation between miR-7 expression levels and DFS. The significance threshold was set at 0.05.

## Results

**miR-7 expression in tumour samples of primary serous ovarian cancer.** miR-7 expression in high-grade ovarian cancer samples significantly related to the presence *vs.* absence of *TP53* mutations ( $p < 0.01$ ), and in wt*TP53* ovarian cancer samples to tumour grade ( $p < 0.01$ ) (Figure 1). There was no correlation between miR-7 and *TP53* expression at the transcript level.

In high-grade ovarian cancer with *TP53* mutation, miR-7 expression did not differ depending on the *TP53* mutation type (missense *vs.* null).

miR-7 expression was found not to influence the probability of DFS (data not shown).

**miR-7 expression in ovarian cancer cell lines.** The pattern of miR-7 expression in the context of the *TP53* status in ovarian cell lines differed from that in clinical material. Namely, miR-7 expression level was the highest in A2780 ovarian cancer cells, characterised by wt*TP53*. TOV112D ovarian cancer cells, bearing missense *TP53* mutation, exhibited lower miR-7 expression than A2780 cells, and SK-OV-3 ovarian cancer cells, characterised by a null mutation of *TP53*, had the lowest level of miR-7 expression (Figure 2a). *BCL2*. In clinical samples, the level of *BCL2* expression did

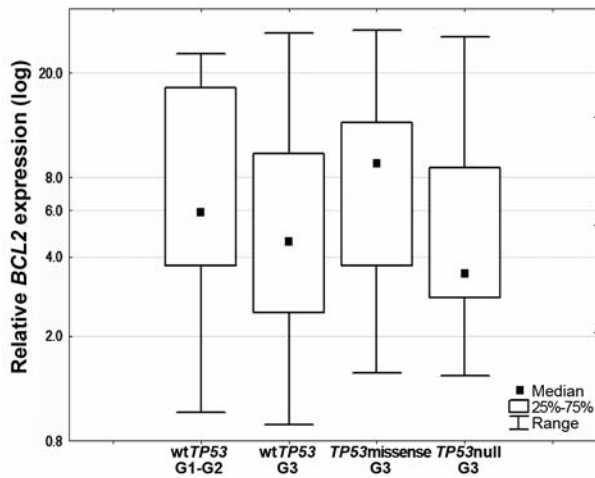


Figure 3. Relative B-cell lymphoma 2 (*BCL2*) transcript levels in clinical samples of primary serous ovarian cancer with different *TP53* status and tumour grade (G). The expression of *BCL2* transcript in normal human ovarian surface epithelial cells was set as the reference level (=1).

not relate to *TP53* mutation status or tumour grade (Figure 3). There was a moderate inverse correlation ( $R=-0.31$ ;  $p<0.05$ ) between the levels of *BCL2* and *miR-7* transcripts. A2780 and SK-OV-3 ovarian cancer cell lines (Figure 2b) expressed several fold higher levels of *BCL2* transcript than did the TOV112D cell line.

*EGFR*. There were no differences in *EGFR* transcript levels depending on *TP53* status or tumour grade (Figure 4). The levels of *EGFR* transcript were found not to correlate with *miR-7* levels. Notably lower levels of *EGFR* mRNA were expressed in A2780 and TOV112D ovarian cell lines than in the SK-OV-3 cell line (Figure 2c).

## Discussion

To our knowledge, this is the first study on *miR-7* expression in serous ovarian carcinomas. We show that in clinical specimens of high-grade serous ovarian cancer, *miR-7* expression relates to the mutation status of the *TP53* gene, with significantly higher *miR-7* levels accompanying *TP53* mutations, but does not depend on the mutation type (missense vs. null). In wt*TP53* tumours, *miR-7* expression significantly related to tumour grade, with higher levels in high-grade tumours. The results suggest that *miR-7* expression increases with tumour malignancy. The levels of *miR-7* did not influence the DFS of patients.

The interplay between *miR-7* and *TP53* needs to be examined in functional studies, but lower *miR-7* levels observed in wt*TP53* cancer raises the possibility that *miR-7*

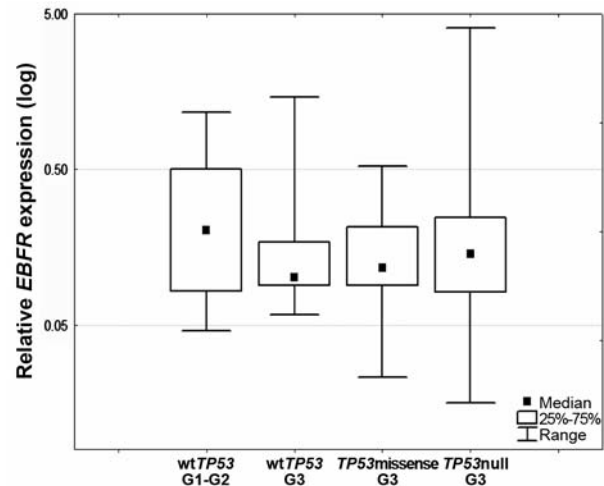


Figure 4. Relative epidermal growth factor receptor (*EGFR*) transcript levels in clinical samples of primary serous ovarian cancer with different *TP53* status and tumour grade (G). The expression of *EGFR* transcript in normal human ovarian surface epithelial cells was set as the reference level (=1).

expression is negatively regulated by the *TP53* pathway. However, the lack of correlation between *miR-7* and *TP53* at the transcript level implies a possible involvement of components of the *TP53* pathway other than *TP53* itself in this relationship.

The relationship between *miR-7* expression and *TP53* status was not reflected in the cell lines. This and other discrepancies between the results obtained in ovarian cancer tissue and the cell lines support the notion that the molecular context of cell lines may be far removed from that of the relevant tumours, which points to the importance of studying clinical specimens, and has serious implications for the relevance of some functional studies considering *TP53* status in ovarian cancer cell lines.

The available data linking *miR-7* and *TP53* status are scarce. Four ovarian cancer cell lines examined by Creighton *et al.*, including one cell line with wt*TP53* (HEY) and one with mutated *TP53* (SK-OV-3, in which the published *miR-7* expression was comparable to our results for this cell line), expressed diverse levels of *miR-7*, inconsistent with regard to the *TP53* status (27). Veerla *et al.* reported that all urothelial G1 and G2 cancer confined to the urothelial mucosa had wt*TP53*, and presented distinctively low levels of *miR-7* (28). Recently, it was demonstrated in colorectal cancer that the mechanism of the tumour-inhibitory role of *miR-7* lies in a direct suppression of oncogenic YY1, a negative regulator of *TP53* and of its downstream effectors, which include p15, caspase and *JUN* proto-oncogene (29). If this is true in ovarian cancer, low *miR-7* expression in wt*TP53*



compared to *TP53*-mutated cancer might result in inactivation or attenuation of the *TP53* pathway. A tumour-suppressor role of miR-7 has been shown in lung, breast and hepatocellular carcinomas and gliomas by ectopic miR-7 expression which resulted in suppression of cell growth and viability, apoptosis induction, migration inhibition, and reduced tumourigenicity. However, most of these cancer types have been shown to exhibit decreased miR-7 expression (4, 6, 7, 13-15, 29-31). Higher expression of miR-7 in *TP53*-mutated and high-grade ovarian tumours does not contradict a possible tumour-suppressor role of miR-7, and may reflect a secondary, context-dependent phenomenon, as discussed in other aggressive types of cancer (32, 33).

In line with our results, Shahab *et al.* have recently found higher miR-7 expression in advanced serous ovarian cancer *vs.* HOSE (8). Wyman *et al.* found a significantly higher miR-7 expression in serous *vs.* clear cell ovarian carcinomas (11). Two other studies that profiled miRs in ovarian cancer did not show miR-7 to be differentially expressed between tumour and normal ovarian tissue (9, 10). Contrary to our results, Iorio *et al.* found no miRs to be associated with tumour grade (9). These discrepancies may result from differences in the reference tissues and fold change (FC) thresholds applied, and in the different histopathological subtypes examined. Nam *et al.* (10) and Iorio *et al.* (9) used whole ovary, not HOSE, as a reference tissue. We examined a series of serous carcinomas, but others studied ovarian carcinomas of different histopathological types. Furthermore, we, as well as Shahab *et al.* (8) and Nam *et al.* (10), analysed data at a threshold FC of >2, while Wyman *et al.* (11) and Iorio *et al.* (9) did so at FC>4 and >3, respectively. In accordance with our results, in patients with breast cancer, the levels of miR-7 positively correlated with tumour grade (33).

The miR-7-*BCL2* relationship has only been shown in a lung cancer cell line (7). Here we found a moderate negative correlation between miR-7 and *BCL2* expression in ovarian cancer samples. A possible relationship between miR-7 and *BCL2* expression was also supported by our results in ovarian cancer cell lines. Our results suggest that miR-7 targets *BCL2* in ovarian carcinoma. *EGFR* has been validated as the target gene of miR-7 in a few studies, including studies on ovarian cancer (4, 6). In our series of ovarian cancer specimens, the expression of miR-7 was found not to correlate with *EGFR* expression, just as in studies on breast carcinoma (34). The lack of correlation between miR-7 and one of its postulated targets exemplifies the phenomenon of an incoherent expression of microRNAs and their presumed targets. Shahab *et al.* studying serous ovarian cancer specimens showed that the expression of only approximately 10% of miR-7 putative targets inversely correlated with miR-7 expression, and approximately 5% correlated positively, while in *miR*-7-

transfected ovarian cancer cell line, these proportions ranged from approximately 8 to 23% (inverse correlation) and from 0 to approximately 1% (positive correlation), depending on the software used for target predictions (8). They obtained similar results for other miRNAs. In addition, on average, only 4% of miR-7 targets that were significantly down-regulated after ectopic expression of miR-7 in the HEY ovarian cancer cell line presented changes in expression that negatively correlated with miR-7 expression in ovarian tumour specimens (8). Nevertheless, the same studies described *EGFR* but not *BCL2* as being down-regulated in *miR*-7-transfected HEY cells. Furthermore, in ovarian cancer specimens, Shahab *et al.* described *EGFR* to correlate negatively with miR-7 expression, but only one out of four *EGFR* Affymetrix probes was down-regulated in tumour tissues, while two out of three *BCL2* probes were down-regulated and one was up-regulated (8). Incoherence between the expression of miRs and their postulated targets may also relate to tissue specificity of the miR-to-target relationship. Furthermore, many gene expression changes may be secondary, because on one hand transfection of a single miR can change the expression of thousands of genes, and on the other, fewer than 20% of the genes affected following miR transfection are the predicted targets, as shown for miR-7 in HEY ovarian cancer cells (8, 35).

In summary, in high-grade serous ovarian cancer, the level of miR-7 expression is significantly increased in *TP53*-mutated tumours, while in *wtTP53* tumours, miR-7 expression significantly rises with increasing tumour grade. miR-7 in primary serous ovarian cancer emerges as a correlate of *TP53* mutation status and grade. Correlation analyses point to *BCL2* but not to *EGFR* as a possible target of miR-7 in ovarian cancer. Functional studies are necessary to elucidate molecular mechanisms linking miR-7 to *TP53* status and tumour grade in ovarian cancer.

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