Circulating U2 Small Nuclear RNA Fragments as a Novel Diagnostic Tool for Patients with Epithelial Ovarian Cancer

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BACKGROUND: Ovarian cancer is the leading cause of death among malignancies in women. Despite advances in treatment, more than half of patients relapse. For disease monitoring, the identification of a blood-based biomarker would be of prime interest. In this regard, noncoding RNAs, such as microRNA (miRNA) or small nuclear RNA (snRNA), have been suggested as biomarkers for noninvasive cancer diagnosis. In the present study, we sought to identify differentially expressed miRNA/snRNA in sera of ovarian cancer patients and investigate their potential to aid in therapy monitoring.

METHODS: miRNA/snRNA abundance was investigated in serum (n = 10) by microarray analysis and validated in an extended serum set (n = 119) by reverse-transcription quantitative PCR.

RESULTS: Abundance of U2-1 snRNA fragments (RNU2-1f) was significantly increased in sera of ovarian cancer patients (P < 0.0001) and paralleled International Federation of Gynecology and Obstetrics stage as well as residual tumor burden after surgery (P < 0.0001 and P = 0.011, respectively). Moreover, for patients with suboptimal debulking, preoperative RNU2-1f concentration was associated with radiographic response after chemotherapy and with platinum resistance (P = 0.0088 and P = 0.0015, respectively). Interestingly, according to the RNU2-1f abundance dynamics, persistent RNU2-1f positivity before surgery and after chemotherapy identified a subgroup of patients with high risk of recurrence and poor prognosis.

CONCLUSIONS: This is the first report to suggest that a circulating snRNA can serve as an auxiliary diagnostic tool for monitoring tumor dynamics in ovarian cancer. Our results provide a rationale to further investigate whether this high-risk patient group may benefit from additional therapies that are directly applied after chemotherapy.

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Ovarian cancer is the leading cause of death among women with gynecologic malignancies in the United States (1) as well as in Europe (2). Standard treatment of this disease comprises primary radical surgery with the aim of macroscopically complete tumor resection and subsequent platinum- and paclitaxel-based chemotherapy (3). So far, residual tumor burden after surgery is believed to be one of the most relevant prognostic factors for ovarian malignancies (4–6). However, despite recent advances in treatment, more than half of all patients will experience recurrence, resulting in a poor overall prognosis (7, 8). Given that the primary tumor tissue is only uniquely available after primary surgery, the identification of a blood-based biomarker for monitoring disease and predicting posttherapeutic outcome is highly desirable. In this regard, noncoding RNAs (ncRNAs)5 in the blood of cancer patients, comprising small nucleolar RNA (snRNA), microRNA (miRNA), piwi-associated RNA, small Cajal body–specific RNA (scRNA), and small nuclear RNA (snRNA), have been suggested as novel biomarkers for noninvasive cancer diagnosis. So far, primarily miRNAs have been studied in this context, because specific and sensitive detection assays are available.

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5 Nonstandard abbreviations: ncRNA, non-coding RNA; snoRNA, small nucleolar RNA; miRNA, microRNA; scaRNA, small Cajal body–specific RNA; snRNA, small nuclear RNA; RNU2-1f, human U2-1 snRNA fragment; RT-qPCR, reverse-transcription quantitative PCR; syn-cel-miR-54, synthetic Candida elegans miR-54; Cq, quantification cycle; FFPE, formalin-fixed paraffin-embedded; AFE, Agilent Feature Extraction; RNU2-1, U2-1 snRNA; AUC, area under the curve; CT, computed tomography; DFS, disease-free survival; OS, overall survival.
Moreover, their high abundance and robust stability in the human circulation have recently been ascertained (9). To date, miRNA-based biomarkers have been proposed for several different cancer types including lung cancer, breast cancer, and gastrointestinal malignancies (10, 11). For ovarian cancer, only 3 pilot studies so far have investigated miRNA candidates that are differentially expressed in ovarian cancer tissue or ovarian cancer cell lines for their presence in the circulation (12–14). Another study compared the expression pattern of exosomal, serum-derived miRNAs with the miRNA pattern of the corresponding primary tumor (15). Notably, these studies primarily aimed at discriminating ovarian cancer patients from healthy controls; however, correlation of circulating ncRNA abundance with the patient's clinicopathological parameters and survival data has not been addressed so far.

Therefore, in the present study, we assessed the abundance pattern of 887 miRNAs, also including 1 snRNA, in serum of ovarian cancer patients in comparison to healthy controls and investigated their potential to aid diagnosis and therapy monitoring. To the best of our knowledge, this is the first report describing clinical relevance for human U2-1 snRNA fragment (RNU2-1f) in ovarian cancer patients and suggesting RNU2-1f abundance dynamics as a diagnostic tool to identify a patient subgroup with high risk of recurrence and poor survival following adjuvant chemotherapy.

Materials and Methods

miRNA EXTRACTION FROM SERUM

Clinical characteristics of study patients, serum preparation, and preanalytical quality control measures are summarized in Supplementary File 1, which accompanies the online version of this article at http://www.clinchem.org/content/vol60/issue1. We extracted miRNA from human serum using the mirVana PARIS kit (Ambion). For microarray discovery analysis, we extracted total RNA from FFPE samples using the miRNeasy FFPE kit (Qia- gen), which has previously been shown to be specific to healthy controls and investigated their potential to aid diagnosis and therapy monitoring. To the best of our knowledge, this is the first report describing clinical relevance for human U2-1 snRNA fragment (RNU2-1f) in ovarian cancer patients and suggesting RNU2-1f abundance dynamics as a diagnostic tool to identify a patient subgroup with high risk of recurrence and poor survival following adjuvant chemotherapy.

miRNA EXTRACTION FROM OVARIAN CANCER TISSUE

Ovarian cancer formalin-fixed paraffin-embedded (FFPE) tissue samples were minidissected under supervision of a pathologist to obtain 3-mm punch biopsies with the highest tumor cell content accessible (usually >60%). In terms of a reference tissue, we prepared punch biopsies from healthy resected ovaries (FFPE) by the same procedure. Subsequently, we extracted total RNA from FFPE samples using the miRNeasy FFPE kit (Qiagen) according to the manufacturer’s instructions; the RNA was eluted in 20 μL RNase-free water.

MICROARRAY ANALYSIS OF SERUM-DERIVED miRNA

Total RNA from 600 μL serum of ovarian cancer patients or healthy controls (n = 5 of each group) was hybridized to the human miRNA Microarray (G4471A Human, Amadid 29297, Sanger 14, Agilent Technologies), carrying 887 individual human miRNA probes. We carried out miRNA labeling, hybridization, and washing steps according to the manufacturer’s instructions. Images of hybridized microarrays were acquired with a DNA microarray scanner (Agilent G2505B), and features were extracted with the Agilent Feature Extraction (AFE) image analysis software version A.10.7.3.1. miRNA expression data from our study have been deposited in the NCBI Gene Expression Omnibus database (acc. no. GSE48485).

DATA ANALYSES

The AFE algorithm generates a single intensity measure for each miRNA, referred to as gTotalGeneSignal, which we used for further data analyses with the GenSpring GX software package version 11.5.1. gTotalGeneSignal measures were normalized by the quantile method. Subsequently, data were filtered on normalized expression values. Only entities where at least 2 of 10 samples had values within the selected cutoff (75th–100th percentile) were further included in the data analysis process.

RT-qPCR

To relatively quantify RNU2-1f in human serum and tissue, we used the hsa-miR-1246 miScript assay (Qiagen), which has previously been shown to be specific for RNU2-1f in serum and for full-length RNU2-1 in a cellular context (16). We carried out all experimental steps according to the manufacturer’s instructions. Briefly, we subjected 2 μL total serum-derived RNA or 10 ng total tissue-derived RNA to poly-A-tailing–mediated universal RT reaction. RT-qPCR was performed with the ABI-7300 system (Applied Biosys-
miRNA microarray

In a discovery cohort of 5 preoperative sera from patients with epithelial ovarian cancer in comparison to age-matched healthy controls by miRNA microarray profiling of circulating miRNA/snRNA was performed.

**Results**

Spike-in normalization with synthetic *C. elegans*-derived cel-miR-54 sequence was performed to allow relative comparison across the analyzed serum samples (9). Absence of syn-cel-miR-54 signal in the human circulation was experimentally verified previously (9), and a previous investigation showed a linear correlation between the logarithm of the amount of syn-cel-miR-54 input and the Cq for syn-cel-miR-54 as well as RNU2-1f (16). From these analyses, we verified the adjusted amount of spike-in (25 fmol per sample) to be in the linear amplification range of the assay (16). We determined a normalized Cq value for RNU2-1f relative to the syn-cel-miR-54 signal [Cq = Cq(syn-cel-miR-54) − Cq(RNU2-1f)]. Accordingly, normalization with small nucleolar RNA, C/D box 44 (SNORD44), was performed, to allow relative comparison of full-length RNU2-1 expression across the tissue samples analyzed. We determined a normalized Cq value for RNU2-1 expression relative to the SNORD44 signal [Cq = Cq(RNU2-1f) − Cq(SNORD44)].

**STATISTICAL ANALYSIS**

Regarding the microarray data, the first step in our analysis was a groupwise comparison of measured miRNA serum concentrations in sera of ovarian cancer patients and healthy controls, respectively. We conducted 2-sided 2-sample Student t-tests per variable, assuming equal variances with GenSpring GX software package version 11.5.1. Furthermore, only miRNA with fold change ≥ 2.0 in the microarray analyses were considered potential candidates for further investigation. All subsequent statistical analyses were performed with GraphPad Prism version 6.01 for Windows (GraphPad Software).

**miRNA/snRNA PROFILING IN SERA OF OVARIAN CANCER PATIENTS**

Profiling of circulating miRNA/snRNA was performed in a discovery cohort of 5 preoperative sera from patients with epithelial ovarian cancer in comparison to 5 age-matched healthy controls by miRNA microarray analysis (G4471A Human, Sanger 14). The microarray carried 887 individual human miRNA probes. Importantly, 2 of these probes (referred to as miR-1246 and miR-1290) were previously shown to be specific for U2-1 snRNA (RNU2-1). Moreover, the putative miR-1246 and miR-1290 precursors were demonstrated to be the result of false mapping, and the proposed circulating mature miR-1246 and miR-1290 sequences in human serum are actually fragmented forms of RNU2-1, detectable by the microarray and the Qiagen miScript assay used herein (16–18).

After array processing, filtering and normalization, pairwise comparison (fold change ≥ 2.0) revealed 22 miRNAs and RNU2-1f to be differentially expressed in the patients’ sera (6 miRNA and RNU2-1f upregulated, 16 miRNA downregulated) (see online Supplementary Table 1). From these differentially expressed miRNA/snRNA, potential biomarker candidates were selected according to the following criteria: 1) upregulation in the malignant situation (with a fold change ≥ 2); and 2) robust signal in the blood (mean normalized gTotalGeneSignal across all samples > 7). According to these criteria, 2 miRNA/snRNA, namely miR-320c and RNU2-1f, were selected as candidates of interest. However, amplification of miR-320c produced unreliable data (abnormal melting curves, data not shown), possibly owing to a high guanine and cytosine content in the mature sequence, and this marker was therefore dropped from our candidate list. As a consequence, RNU2-1f was selected for further analysis in an extended serum set (n = 119), obtained from clinically well-documented ovarian cancer patients.

**PROFILING OF RNU2-1F ABUNDANCE IN SERA OF OVARIAN CANCER PATIENTS**

On the basis of the observation from the discovery cohort that RNU2-1f abundance was significantly increased in sera of ovarian cancer patients, a comprehensive validation analysis of RNU2-1f abundance was conducted. RNU2-1f was quantified by RT-qPCR, and spike-in normalized Cq values are depicted in Fig. 1. RNU2-1f concentration in healthy controls was detectable with a mean Cq value of −2.46 (SD 0.53). Before surgery, RNU2-1f abundance was significantly higher with a mean Cq of −1.31 (SD 1.52) (P < 0.0001). After surgery and subsequent adjuvant chemotherapy, RNU2-1f abundance dropped to a mean Cq of −1.70 (SD 1.13) (P = 0.124), which was still significantly higher than observed in healthy controls (P = 0.0004).

On the basis of ROC curve analysis, we determined a diagnostic cutoff value for circulating RNU2-1f, classifying a given observation as cancerous or healthy (see online Supplementary Fig. 1). Accordingly, a threshold of −1.55 (normalized Cq value) should dichotomize as follows: a score > −1.55 classifies a sample as cancerous...
ous (diagnostic-positive), whereas a score \(< -1.55\) classifies as noncancerous (diagnostic-negative). Following this cutoff, RNU2-1f concentrations at primary diagnosis enabled a discrimination of cancer patients and healthy controls with a sensitivity of 51.61\% (95% CI 38.56\%– 64.50\%) and a specificity of 91.43\% (95% CI 76.94\%–98.20\%), with an area under the curve (AUC) of 0.74.

SERUM RNU2-1f AND CLINICOPATHOLOGICAL FEATURES OF OVARIAN CANCER

Correlation of RNU2-1f abundance with the patient’s clinicopathological parameters was performed. Before surgery, ovarian cancer patients with advanced disease (FIGO III and IV) showed a significantly higher RNU2-1f concentration [Cq \(-0.91 (1.48)\)] than patients with low-stage disease [FIGO I and II, Cq value \(-2.22 (1.18), P = 0.0014\)] (Fig. 2). In reference to the control group of healthy controls, circulating RNU2-1f concentration was predictive for advanced ovarian cancer (FIGO III and IV, \(P = 0.0001\)), but not for low-stage disease (FIGO I and II, \(P = 0.313\)). Moreover, circulating RNU2-1f concentrations after surgery and adjuvant chemotherapy in patients with residual tumor \(>0\) [Cq value \(-1.30 (1.22)\)] were significantly increased, compared to patients with macroscopically complete tumor resection [Cq value \(-2.07 (0.89), P = 0.011\)]. We did not observe a correlation between circulating RNU2-1f concentrations and histological subtype or platinum resistance.

CLINICAL RELEVANCE OF SERUM RNU2-1f IN PATIENTS WITH SUBOPTIMAL DEBULKING

For a subgroup of 15 patients with suboptimal primary debulking (residual tumor \(>0\)), radiographic reports on restaging after the completion of platinum-based chemotherapy were available. In 7 of 15 patients (47\%), restaging by abdominal computed tomography (CT) or positron emission tomography (PET)-CT scanning showed residual abdominal tumor. Kaplan–Meier analysis revealed a clear trend that these patients had a decreased disease-free survival (DFS) and overall survival (OS) (data not shown). However, owing to the low patient number in this subgroup, statistical power of this analysis is limited. Moreover, 5 of 15 (33\%) of these patients were clinically defined to be platinum resistant. Interestingly, preoperative circulating RNU2-1f concentrations significantly correlated with the presence of residual abdominal tumor mass after adjuvant chemotherapy (\(P = 0.0088\)) (Fig. 3A) and platinum resistance (\(P = 0.0015\)) (Fig. 3B).
PROGNOSTIC RELEVANCE OF CIRCULATING RNU2-1f IN SERA OF OVARIAN CANCER PATIENTS

Subsequently, prognostic relevance of the RNU2-1f diagnostic score was analyzed by Kaplan–Meier analysis. After chemotherapy, RNU2-1f abundance across the entire patient cohort tended to correlate with a decreased OS, but this observation did not reach statistical significance (log-rank test: $P = 0.111$; Gehan–Breslow–Wilcoxon test: $P = 0.075$) (see online Supplementary Fig. 2). Consequently, to enable a more differentiated analysis, we classified each individual patient according to her RNU2-1f abundance dynamics, represented by the RNU2-1f concentration changes after chemotherapy in reference to her diagnostic score observed at primary diagnosis. This subgroup analysis was possible for a total of 50 patients, for which paired serum samples before surgery and after adjuvant chemotherapy were available (see online Supplementary Fig. 3). In 8 of 50 patients (16%), persistent RNU2-1f positivity was observed before surgery and after adjuvant chemotherapy (pos-pos), whereas 12 of 50 patients (24%) were initially diagnostically negative and became newly RNU2-1f positive after therapy (neg-pos). Moreover, in 17 of 50 patients (34%), we observed RNU2-1f positivity before surgery, changing to negativity after chemotherapy (pos-neg). Last, in 13 of 50 patients (26%), RNU2-1f concentrations never passed our diagnostic threshold (neg-neg).

Interestingly, Kaplan–Meier analysis revealed that patients who were persistently RNU2-1f positive at primary diagnosis and after chemotherapy (pos-pos) exhibited a significantly shorter DFS and OS than all other patients with pos-neg, neg-pos, and neg-neg status together ($P = 0.022$, $P = 0.013$, respectively) (Fig. 4). To further delineate the negative prognostic impact of the pos-pos group, we separately compared survival of the pos-pos group to each of the other groups alone. Kaplan–Meier analyses revealed the trend that patients in the pos-pos group had a significantly shorter DFS than the neg-pos group alone ($P = 0.038$) and had a shorter OS than the pos-neg or the neg-neg group alone ($P = 0.023$ and $P = 0.046$, respectively) (data not shown). However, statistical power of this detailed analysis was limited, owing to the low total number of patients for each Kaplan–Meier analysis.

FULL-LENGTH RNU2-1 EXPRESSION IN OVARIAN CANCER TISSUE

Previous reports have shown that the Qiagen miR-1246 assay, when used for lysates of tumor cell lines, produces an amplicon of approximately 180 bp in size, corresponding to full-length RNU2-1 and not to its fragmented counterpart found in human serum (RNU2-1f) (16). We could confirm the formation of a 180-bp amplicon by this assay, when applied to ovarian cancer tissue (data not shown). To inquire whether the increased abundance of RNU2-1f in patient serum corresponds to an upregulation of full-length RNU2-1 in primary tumor tissue, we comparatively evaluated expression of RNU2-1 in tumor tissue of 15 ovarian cancer patients and 15 healthy ovarian tissues (see online Supplementary Fig. 4). RNU2-1 expression data were normalized to SNORD44 expression, previously shown
to be a suitable reference gene (19, 20). Normalized RNU2-1 expression values were shown to be upregulated in the tumor tissue [Cq -5.31 (1.36)], compared with tissue of healthy ovaries [Cq -6.62 (0.84), P<0.0047].

Discussion

In the present study, we identified circulating noncoding RNU2-1f as significantly increased in the blood of ovarian cancer patients and showed that persistent RNU2-1f positivity in the blood indicates high risk of recurrence and poor prognosis.

Recent independent profiling studies found circulating tissue-associated miR-1246 to be differentially expressed in several malignancies (21–23). Importantly, given that the putative miR-1246 is very likely a result of false mapping of RNU2-1f, all of the results reported herein concern circulating RNU2-1f rather than miR-1246 (16, 18). In the present investigation, we observed significantly increased RNU2-1f concentrations in the patients’ blood. This is in accordance with previous studies in serum from colorectal, pancreatic, esophageal, and lung cancer patients, likewise reporting RNU2-1f concentrations to be increased in the circulation of cancer patients (16, 18, 21). Consequently, an increased abundance of RNU2-1f in the blood of cancer patients seems to be a more general phenomenon, and its clinical utility in terms of a blood-based biomarker thereby may apply to a broader spectrum of malignancies of different origin.

In our present study, ROC analysis showed a moderate discrimination between cancerous and healthy conditions, based on RNU2-1f diagnostic score (AUC 0.74). This finding is comparable to a previous study on esophageal squamous cell carcinoma, showing a discrimination of tumor patients and healthy controls with an AUC value of 0.75 (21). However, for patients with gastrointestinal or lung cancer, circulating RNU2-1f enabled more accurate discrimination (AUC 0.97 and 0.87, respectively) (16, 18). On the basis of our present findings, we consider serum RNU2-1f a suboptimal candidate for an ovarian cancer screening tool.

The fact that circulating RNU2-1f abundance in patients with advanced disease was significantly higher than in patients with locally restricted cancer is in agreement with results for esophageal and gastrointestinal malignancy (16, 21). This implies that RNU2-1f abundance, paralleling the tumor size, is also a general feature of circulating RNU2-1f and that the rise in serum RNU2-1f abundance in large part is derived from the tumor (16).

The subgroup of patients with suboptimal debulking (R >0) and residual tumor after chemotherapy trended to show a poor prognosis. This finding is consistent with the fact that the residual tumor burden is one of the most important prognostic factors for ova-
Pain recurrence (4). Moreover, many of these patients (33%) were platinum resistant. Remarkably, preoperative RNU2-1f paralleled the presence of residual abdominal tumor following adjuvant chemotherapy and platinum resistance. These interesting findings may suggest that RNU2-1f has possible clinical utility as a biomarker to predict efficiency and response to platinum-based chemotherapy in patients with suboptimal debulking. Generally, advanced ovarian cancer is a comparatively chemosensitive tumor with overall clinical response rates of 70%–80% (8). However, the overall proportion of platinum-resistant patients in our study was too limited to allow a statistically substantiated conclusion regarding whether RNU2-1f was predictive for platinum resistance across the entire patient cohort (also comprising patients with macroscopically complete tumor resection). Moreover, given that radiographic data were available only for a small patient subgroup with suboptimal debulking (n = 15), it was not possible for us to directly evaluate whether RNU2-1f abundance was more sensitive in detecting disease recurrence than conventionally applied abdominal CT or PET-CT scanning.

RNU2-1f diagnostic score across the entire patient cohort tended to be indicative of a decreased OS, but this observation did not reach statistical significance. However, a more differentiated subgroup analysis revealed that persistent RNU2-1f positivity before surgery and after chemotherapy is of prognostic relevance by indicating high risk of relapse and profoundly impaired OS. Although a majority of patients respond to platinum-based chemotherapy, more than half of all patients are expected to experience recurrence (7, 8). Given this clinical challenge, RNU2-1f abundance dynamics in the patient’s blood might be of particular clinical interest in terms of therapy monitoring. RNU2-1f detection could provide a diagnostic tool for ovarian cancer, identifying a subgroup of patients with poor prognosis and high risk of relapse. Following this assumption, persistent RNU2-1f positivity might reflect a phenotype of aggressive tumor dynamics and could be due to the presence of occult metastatic tumor cells, such as circulating tumor cells in the bloodstream or disseminated tumor cells in the bone marrow, which might survive in a platinum-resistant condition, even in clinically defined platinum-sensitive patients. Surprisingly, patients with newly acquired RNU2-1f positivity after chemotherapy (24%) were stratified into a relatively favorable risk group, principally comparable with the neg-neg and pos-neg subgroups. Assuming that RNU2-1f positivity might predict an unfavorable tumor condition, an extended follow-up period might be necessary to study the long-term prognostic relevance of RNU2-1f positivity acquired after adjuvant treatment.

Full-length cellular RNU2-1 constitutes a functional component of the spliceosome complex (24). Different hypotheses exist regarding the mechanism of how RNU2-1f is released into the circulation. One hypothesis supposes RNU2-1f to be encapsulated into apoptotic bodies and to be unspecifically released into the bloodstream in the course of apoptotic degradation (16). Conversely, an alternative processing mechanism has recently been discussed, producing full-size RNU2-1 on the one hand and RNU2-1f on the other hand (18). Following this theory, RNU2-1f is supposed to be stably and selectively transported into the bloodstream (18, 21, 25). Moreover, the fact that circulating RNU2-1f concentration is highly increased in tumor-bearing patients might be functionally connected to a concomitant upregulation of full-length RNU2-1 in the primary tumor, which we confirmed in the present study.

To the best of our knowledge, this is the first report to introduce a serum-derived snRNA as a blood-based biomarker candidate for ovarian cancer. As our key finding, we suggest RNU2-1f abundance dynamics in the patient’s blood as a diagnostic tool to identify a patient subgroup with high risk of recurrence and poor prognosis. Our results also provide a rationale to further investigate whether this high-risk patient group may benefit from additional therapies that are directly applied after chemotherapy. A microarray analysis in a larger patient cohort might have resulted in a higher number of biomarker candidates; however, we do not consider this negative for the impact of our study, as our findings suggest a potential clinical implication of RNU2-1f. Moreover, a direct comparison between RNU2-1f and CA-125 in terms of therapy monitoring was not possible, owing to the lack of corresponding posttherapeutic CA-125 values. Nevertheless, considering the variety of different noncoding RNA species, we believe that analyzing their complex expression patterns may enable the discovery of novel biomarkers, which may be complementary or even superior to CA-125.

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