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Cytokine mRNA and protein expression by cell cultures of epithelial ovarian cancer—Methodological considerations on the choice of analytical method for cytokine analyses

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Problem: To get a comprehensive picture of cytokine expression in health and disease is difficult, cytokines are transiently and locally expressed, and protein analyses are burdened by biological modifications, technical issues, and sensitivity to handling of samples. Thus, alternative methods, based on molecular techniques for cytokine mRNA analyses, are often used. We compared cytokine mRNA and protein expression to evaluate whether cytokine mRNA profiles can be used instead of protein analyses.

Method of study: In kinetic experiments, cytokine mRNA and protein expression of IL-1β, IL-6, IL-8, TNF-α, and TNF-β/LTA were studied using real-time RT-qPCR and Luminex® microarrays in the ovarian cancer cell lines OVCAR-3, SKOV-3 and the T-cell line Jurkat, after activation of transcription by thermal stress. In addition, we analyzed IL-6 and IL-8 mRNA and protein in a small number of ovarian cancer patients.

Results: Ovarian cancer cells can express cytokines on both mRNA and protein level, with 1-4 hours' time delay between the mRNA and protein peak and a negative Spearman correlation. The mRNA and protein expression in patient samples was poorly correlated, reflecting previous studies.

Conclusion: Cytokine mRNA and protein expression levels show diverging results, depending on the material analyzed and the method used. Considering the high sensitivity and reproducibility of real-time RT-qPCR, we suggest that cytokine mRNA profiles could be used as a proxy for protein expression for some specific purposes, such as comparisons between different patient groups, and in defining mechanistic pathways involved in the pathogenesis of cancer and other pathological conditions.

KEYWORDS

cytokine, mRNA, ovarian cancer, protein, protein microarray, real-time polymerase chain reaction

Ulrika Ottander and Lucia Mincheva-Nilsson are shared authorship.

1 | INTRODUCTION

Recently, the involvement and importance of the immune system in cancer and its treatment have righteously and finally been acknowledged as an essential factor, playing a decisive role on the curability, prognosis and outcome of this disease. The research field of oncoimmunology, studying the interactions of tumor cells and the immune system, has achieved a great progress in understanding the pathophysiology of cancer and has developed novel immunotherapeutic strategies, including inhibition of immune checkpoints, with promising results and increasing clinical use.

It is well established that the local cytokine microenvironment plays an important role in tumorigenesis, tumor progression and development of resistance to oncotherapy. Cytokines have an essential role in promoting various immune responses depending on the local settings and have attracted more and more attention in clinical conditions, including cancer.[1,2] Cytokine production in tumor disease is studied for three main purposes: (a) to understand and reveal mechanisms behind tumorigenesis and immune escape, (b) to find biomarkers for cancer diagnosis, prognosis and clinical follow-up, and (c) to evaluate them as possible targets for immunotherapy in cancer treatment.[3] High-grade serous carcinoma of tubo-ovarian origin (HGSC) is an example of a cancer type where the patient's immune system is deranged and its operational mechanisms, including cytokine production, are hijacked or suppressed by the tumor, thus playing a prominent role in modifying the disease course.

Cytokines are produced and secreted by a variety of cells to coordinate the immune response and provide growth-, differentiation-, inflammatory-, and immunosuppressive signals. The released cytokine/group of cytokines following a certain stimuli determine(s) the subsequent immune response.[4] The cytokine signal is potentiated and diversified by cascades—a cytokine stimulates the production of the same or other cytokines and/or cytokine receptors, further potentiating and forwarding the prevailing immune response.[3] It has been shown that different cytokine mRNA profiles, designated Th1, Th2, Th3/Tr1, and Th17, are associated with the ability to mediate and regulate immunity and inflammation, promote or halt growth, movement or immune responses. Thus, a cytokine profile dominated by IFN-γ, IL-12, and IL-15 (Th1) promotes cytotoxicity, a cytokine profile dominated by IL-4, IL-5, and IL-13 (Th2) promotes humoral immunity, IL-1β, IL-6, IL-8, IL-17, TNF-α, and TNF-β/LTA promote inflammation and TGF-β1 and IL-10 (Th3/Tr1) promote immunosuppression, and innate and adaptive T regulatory cell development.[5]

Since cytokines are produced and act locally, with a short half-life,[5] they are often only transiently present in small amounts and/or absent, as proteins, at a given moment in a biological system. Because of this, a need for alternative methods for revealing cytokine presence has emerged. Real-time quantitative RT-PCR is one of the methods often used as a proxy to cytokine protein analyses.[6-12] It has also been an important technique in identifying different diagnostic tests and predicting outcomes for different malignant and non-malignant diseases.[13] Recently, we used this method to gain knowledge on the cytokine mRNA expression profile in paired ovarian cancer tissue samples and peripheral blood mononuclear cell (PBMC) samples of women suffering from HGSC.[6] Our results indicated elevated cytokine mRNA levels in the ovarian cancer microenvironment compared with non-malignant conditions and normal ovarian tissue, where inflammation, immune suppression, and promotion of T reg cells prevailed.

It is well known and proven that not all mRNA signals are translated to proteins.[14,15] Assessing mRNA levels exclusively will only give information on transcribing DNA to mRNA and not on the further protein production and secretion. In addition, cytokines have a diverse range of action, some showing de novo synthesis and others being stored intracellularly and released immediately upon stimulation. Considering the instability of cytokine proteins in serum, it could be of interest to study if it is sufficient to analyze cytokine mRNA profiles to gain knowledge on a specific immune response, and in what instances cytokine mRNA could be used as a proxy for protein analysis.

Here, we compared the mRNA expression for a set of cytokines with the corresponding extracellular protein expression, using quantitative RT-PCR and multiplex protein analysis (Luminex®) in kinetic experiments. Traditionally, enzyme-linked immunosorbent assay (ELISA) has been used for cytokine protein analyses. The advantage of multiplex bead array assays, such as Luminex®, is its efficiency as a high throughput multiplex method analyzing several cytokines at the same time. The sensitivity for each method is comparable.[16,17] In kinetic experiments, we assessed cytokine mRNA and protein expression in three cell lines after activation by thermal stress—the epithelial ovarian cancer cell lines OVCAR-3 and SKOV-3, and Jurkat, a T-cell–derived cell line that was used as a control to the ovarian cancer cell lines. Time points were chosen to capture cytokine mRNA and protein production peaks.[18] Working with cell lines presents a unique opportunity to control the experimental conditions. The correlation between cytokine mRNA and protein expression in kinetic experiments has to our knowledge not been previously investigated in ovarian cancer cells.

2 | MATERIALS AND METHODS

2.1 | Cell lines and cell culture

Epithelial ovarian cancer cell lines OVCAR-3 (HTB-161) and SKOV-3 (HTB-77), and acute T-cell leukemia cell line Jurkat (TIB-152), all purchased from ATCC, were cultured according to ATCC recommendations. OVCAR-3 and Jurkat were cultured in RPMI1640 and SKOV-3 in McCoy medium. The media were supplemented with 10% heat-inactivated FCS, 0.01 mol/L HEPES, 2 mmol/L L glutamine, and penicillin and streptomycin. The adhesive cell lines (OVCAR-3 and SKOV-3) were cultured to confluence, and Jurkat was cultured at a concentration of 1×10^6 cells/mL. Twelve hours after the last media

change, cells and used media were collected for time point 0 in kinetic experiments.

2.2 | Activation of cytokine gene expression by thermal stress

Confluent OVCAR-3 and SKOV-3 cultures in 25 cm^2 culture flasks (VMR international), and 3 × 10^6/mL Jurkat cells were incubated at 42°C for 1 hour to stimulate cytokine expression. Successful heat shock effect was confirmed by assessing increased mRNA levels of HSP4A, MICA, and MICB (Figure 1) by RT-qPCR. Cell culture supernatant and cells were collected for further analyses at seven consecutive time points; at starting point 0, before heat shocking, and at 1, 3, 4.5, 6, 9, and 24 hour post-heat shocking of the cells.

2.3 | Patient samples

Serum samples for cytokine protein assessment of 14 women suffering from HGSC were retrieved from the Ovarian Cancer Biobank at Norrland's university hospital. The samples were collected after

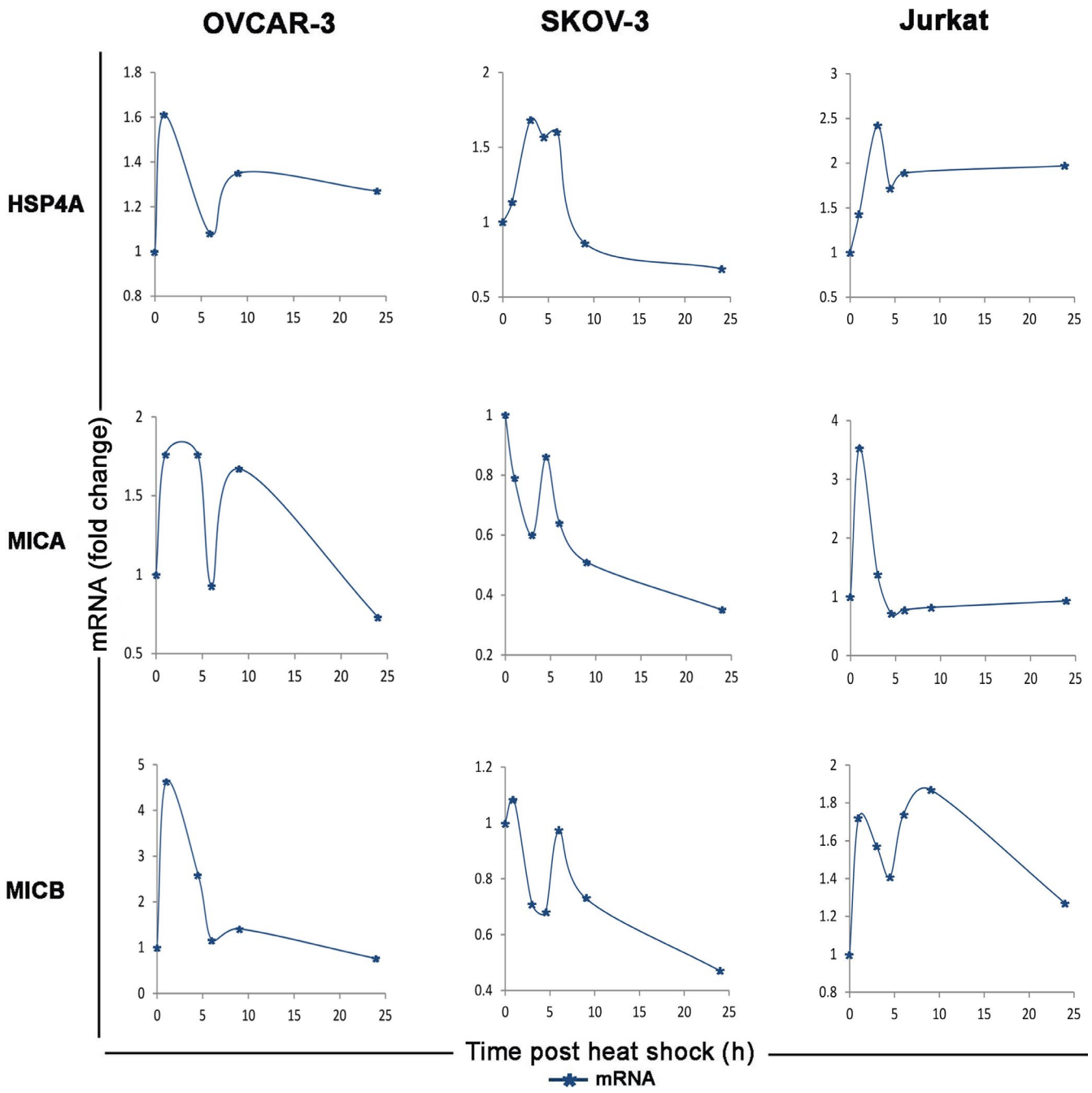

FIGURE 1 Effect of heat shock on thermal stress-inducible genes HSP4A, MICA, and MICB. mRNA upregulation at seven consecutive time points for the ovarian cancer cell lines OVCAR-3 and SKOV-3, and the T-cell line Jurkat, used as a control

ethical permission and informed consent. Cytokine mRNA expression in ovarian tumor tissue from the same patients, previously studied and published,[6] was used as comparison to the protein cytokine assessment.

2.4 | RNA extraction and real-time reverse transcription-quantitative polymerase chain reaction (real-time RT-qPCR)

Total RNA was extracted from approximately 3×10^6 cells from each cell line at each time point, using RNeasy Mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. RNA yield (for Jurkat on average 182.7 ± 37.5 ng/μL, for OVCAR-3 67.8 ± 30.7 ng/μL and for SKOV-3 16 ± 12.8 ng/μL) and RNA purity (for Jurkat on average A_{260}/A_{280} = 2.1 ± 0, for OVCAR-3 2.1 ± 0.05 and for SKOV-3 1.9 ± 0.3) were assessed by spectrophotometry (NanoDrop, ThermoScientific).

For each cell culture sample, 400 ng total RNA in a final volume of 20 μL was transcribed to cDNA by using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher), according to the manufacturer's description. After cDNA synthesis, the volume was adjusted by adding 60 μL sterile Milli-Q water to reach the working cDNA concentration equivalent to 5 ng/μL total RNA.

Multiplex qPCR tests were performed on a QuantStudio 5 Real-Time PCR System (ThermoFisher), detecting the target gene and 18S rRNA as an endogenous control. One μL cDNA/well in 20 μL reaction volume was used in all tests and run for 40 cycles with factory default settings for TaqMan Gene Expression Master Mix (#4369016) and TaqMan® FAM/MGB probe Assays (all purchased from ThermoFisher). The following cytokines were assessed: IL-1β, IL-6, IL-8, TNF-α, and TNF-β/LTA. Their assay ID are as follows: IL-1β (Hs01555410_m1), IL-6 (Hs00985639_m1), IL-8 (Hs00174103_m1), TNF-α (Hs00174128_m1), and TNF-β/LTA (Hs04188773_g1). The constancy of Ct values for the endogenous, house-keeping gene 18S rRNA can be seen in Table 1. Cytokine mRNA amplifications of PMA-ionomycin stimulated PBMCs from healthy donors were used as positive assay controls and template omission as a negative control. Tests were analyzed with QuantStudio Design and Analysis Software 1.4.1. The raw data were evaluated with the $\Delta\Delta C_t$ method resulting in relative quantities (RQ). For each cell line, the RQ for each time point was compared with baseline (time point 0), producing a fold difference.

2.5 | Multiplex cytokine assay Luminex® of culture supernatants and serum samples

Cytokine protein concentrations were assessed by a multiplex magnetic Luminex® assay (R&D systems) according to the manufacturer's instructions. The following cytokines were analyzed: IL-1β, IL-6, IL-8, TNF-α, and TNF-β/LTA. Cell culture supernatant samples were used without dilution. Additionally, an experiment was performed after concentrating the cell culture supernatant six times using Amicon® Ultra-0.5 centrifugal filter devices (Merck KGaA). Serum samples were centrifuged at 16 000*g* for 4 minutes and diluted 2-fold according to the manufacturer's recommendations.

TABLE 1 Average Ct values ± standard deviation for the mRNA expression of the cytokines and thermal stress-inducible genes, and the endogenous control gene 18S rRNA, in OVCAR-3, SKOV-3 and Jurkat, before and at different time points after thermal stress (n = 3)

		Time after heat shock (h)							
Cell line	Cytokine	0	1	3	4.5	6	9	24	18s rRNA
OVCAR-3	Hsp4A	26.5 ± 0.05	25.7 ± 0.33	25.6 ± 0.24	25.3 ± 0.72	26.7 ± 0.56	26.4 ± 0.06	26.4 ± 0.14	13.7 ± 0.59
	MICA	28.6 ± 0.01	26.9 ± 0.49	27.4 ± 0.49	27.7 ± 0.74	28.3 ± 0.34	28.6 ± 0.15	28.6 ± 0.12	14.4 ± 0.76
	MICB	30.0 ± 0.95	26.8 ± 1.49	27.5 ± 2.01	28,7 ± 1.49	29.6 ± 1.01	29.9 ± 0.99	30.0 ± 0.95	13.9 ± 0.66
	IL-6	32.3 ± 0.29	29.8 ± 0.28	31.0 ± 0.92	32.6 ± 0.31	32.0 ± 0.71	32.9 ± 0.11	33.1 ± 0.18	15.8 ± 0.72
	IL-8	28.3 ± 0.69	27.6 ± 0.74	28.2 ± 0.58	29.2 ± 0.82	29.2 ± 1.07	29.1 ± 0.69	29.5 ± 0.56	14.4 ± 1.02
	TNF-α	32.7 ± 0.37	31.5 ± 0.25	32.8 ± 0.46	33.3 ± 0.52	33.3 ± 0.12	33.5 ± 0.06	33.6 ± 0.32	15.2 ± 0.71
SKOV-3	Hsp4A	26.6 ± 0.19	25.8 ± 0.12	25.1 ± 0.06	25.3 ± 0.13	25.6 ± 0.08	26.1 ± 0.11	26.6 ± 0.18	12.1 ± 0.23
	MICA	28.7 ± 0.18	28.3 ± 0.18	28.5 ± 0.06	28.7 ± 0.06	29.1 ± 0.14	29.2 ± 0.18	29.1 ± 0.13	13.1 ± 0.38
	MICB	30.6 ± 0.92	29.9 ± 1.00	30.2 ± 1.13	30.4 ± 0.90	30.7 ± 0.73	30.6 ± 0.79	30.9 ± 1.00	12.4 ± 0.29
	IL-6	34.5 ± 0.17	32.5 ± 0.36	34.7 ± 0.35	34.3 ± 0.69	34.7 ± 0.23	34.4 ± 0.46	33.6 ± 0.61	14.3 ± 0.24
	IL-8	30.5 ± 0.67	29.8 ± 0.56	29.6 ± 0.52	29.6 ± 0.62	30.1 ± 0.63	30.4 ± 0.51	29.3 ± 0.60	11.7 ± 0.80
Jurkat	Hsp4A	25.4 ± 0.10	24.9 ± 0.04	24.6 ± 0.14	24.8 ± 0.07	25.2 ± 0.03	25.2 ± 0.37	25.4 ± 0.21	12.6 ± 0.59
	MICA	31.8 ± 0.16	29.6 ± 0.07	30.6 ± 0.19	32.1 ± 0.29	32.6 ± 0.17	31.9 ± 0.39	31.8 ± 0.10	13.0 ± 0.32
	MICB	28.0 ± 0.97	27.3 ± 0.96	27.4 ± 0.97	27.8 ± 0.97	28.0 ± 0.94	27.6 ± 1.52	28.0 ± 0.92	12.3 ± 0.43
	IL-8	39.4 ± 1.44	33.8 ± 0.84	38.7 ± 1.61	35.5 ± 0.68	35.5 ± 0.24	39.2 ± 0.27	38.7 ± 0.97	13.8 ± 0.35
	LTA	29.4 ± 0.42	28.7 ± 0.42	28.6 ± 0.40	29.3 ± 0.36	29.6 ± 0.43	29.2 ± 0.15	30.1 ± 0.80	12.6 ± 0.31

2.6 | Statistical analyses

Spearman's rank order correlation was used to assess the correlation between cytokine mRNA and protein expression at the different time points. The IBM SPSS software version 25 was used for these analyses. Since the time points are not more than seven, the *P*-values are not given.

3 | RESULTS

3.1 | The experimental thermal stress conditions were sufficient for mRNA upregulation

In our kinetic experiments, we chose to use the ovarian cancer cell lines OVCAR-3 and SKOV-3, and the T-lymphocyte cell line Jurkat as a control. To mimic biological stress, we chose to expose the cell lines to thermal stress at 42°C for 1 hour. The efficiency of the chosen thermal stress conditions was analyzed by assessment of the upregulation of HSP4A and the stress-inducible molecules MICA and B that have heat shock elements in their promoter regions, and are thus upregulated by thermal stress.[19] In Figure 1, the kinetics of mRNA expression for MICA, MICB, and HSP4A in the cell lines OVCAR-3, SKOV-3, and Jurkat are shown. The fold change in mRNA levels was calculated for the time points 1, 3, 4.5, 6, 9, and 24 hour in relation to the mRNA level at the starting point 0 hour (=1). There was an increase in HSP4A, MICA and MICB mRNA expression in the three cell lines tested as shown in the mRNA expression curves (Figure 1). Characteristic for the majority of the curves was a bi-polar pattern of mRNA expression, the highest peak coming at about 1 hour post thermal stress, in most cases followed by a lower peak 6-9 hour post-thermal stress. This was excepted by MICB expression in Jurkat, where the second peak at 9 hour was higher than the initial peak at 1 hour and MICA in SKOV-3, where the highest value was seen at starting point. From these experiments, we concluded that the experimental set-up of thermal stress conditions gave expected and satisfactory results and we could proceed with the cytokine analyses.

The mean individual Ct values and SD of each cell line, internal control, type of cytokine, and time point can be seen in Table 1.

3.2 | Ovarian cancer cells express cytokine mRNA and translate it into protein

Both cytokine mRNA and protein were expressed in ovarian cancer cell lines and Jurkat as illustrated in Figure 2, where the correlation between cytokine mRNA and protein is shown. The calculated RQ fold change values of the mRNA expression in each cell line were correlated to the protein concentration of the corresponding cell line culture supernatant (Figure 2B-D). Not all cytokines were initially detected at protein level in the used culture supernatant. The detection range of the Luminex® assay is shown in Table S1. To test whether the cytokine in question was present but diluted under the detection level, we concentrated the used supernatant six times. In OVCAR-3 (Figure 2B), although the mRNA levels for IL-6 and IL-8 were different, they followed a similar pattern with an early peak followed by a decrease of mRNA with the lowest amount detected at 24 hour post-heat shock. There was a trend toward protein accumulation for IL-6 and IL-8, with the highest protein concentration detected 24 hour post-heat shock. The concentration of IL-6 ranged from 9.6 pg/mL to 18 pg/mL and of IL-8 from 548.7 to 932.4 pg/mL. There was a negative correlation between mRNA and protein production (Spearman's correlation ρ = −.75 for IL-6 and for ρ = −.83 for IL-8). The TNF-α

TABLE 2 Average cytokine protein concentration (pg/mL) in OVCAR-3, SKOV-3, and Jurkat, ±standard deviation, calculated for the seven time points presented in Figure 2 (n = 3)

		Time after heat shock (h)						
Cell line	**Cytokine**	**0**	**1**	**3**	**4.5**	**6**	**9**	**24**
OVCAR-3	IL-6	10.2 ± 8	9.6 ± 7	12.3 ± 3	15.1 ± 11	15.3 ± 11	14.8 ± 6	18.0 ± 7
	IL-6[a]	61.0 ± 10	74.1 ± 20	81.5 ± 8	83.2 ± 11	81.0 ± 7	92.0 ± 8	120.0 ± 14
	IL-8	548.7 ± 116	529.6 ± 213	627.2 ± 83	750.3 ± 220	742.5 ± 300	760.0 ± 207	932.4 ± 169
	TNF-α	ND[b]	ND	ND	ND	ND	ND	ND
	TNF-α[a]	10.3 ± 1	11.7 ± 2	11.9 ± 1	12.0 ± 2	11.0 ± 1	12.6 ± 2	14.9 ± 5
SKOV-3	IL-6	9.5 ± 3.5	ND	ND	5.0 ± 1.2	5.1 ± 1.5	5.0 ± 1.5	5.0 ± 2.5
	IL-6[a]	138.1 ± 90	59.5 ± 2	59.2 ± 5	65.2 ± 5	53.0 ± 3	72.1 ± 40	57.7 ± 4
	IL-8	1230.8 ± 44	267.9 ± 8	242.2 ± 42	303.7 ± 45	331.1 ± 17	331.3 ± 25	422.3 ± 230
Jurkat	IL-8	ND	ND	ND	ND	ND	ND	ND
	IL-8[a]	28.8 ± 5	20.8 ± 0	22.8 ± 3	23.6 ± 2	25.8 ± 7	23.5 ± 2	25.0 ± 4
	TNF-β	ND	ND	ND	ND	ND	ND	ND
	TNF-β[a]	19.9 ± 0	26.6 ± 1	30.1 ± 0	30.2 ± 1	32.7 ± 2	35.6 ± 3	44.4 ± 1

[a]After 6 × concentration of the cell line supernatant.

[b]ND = Not detected.

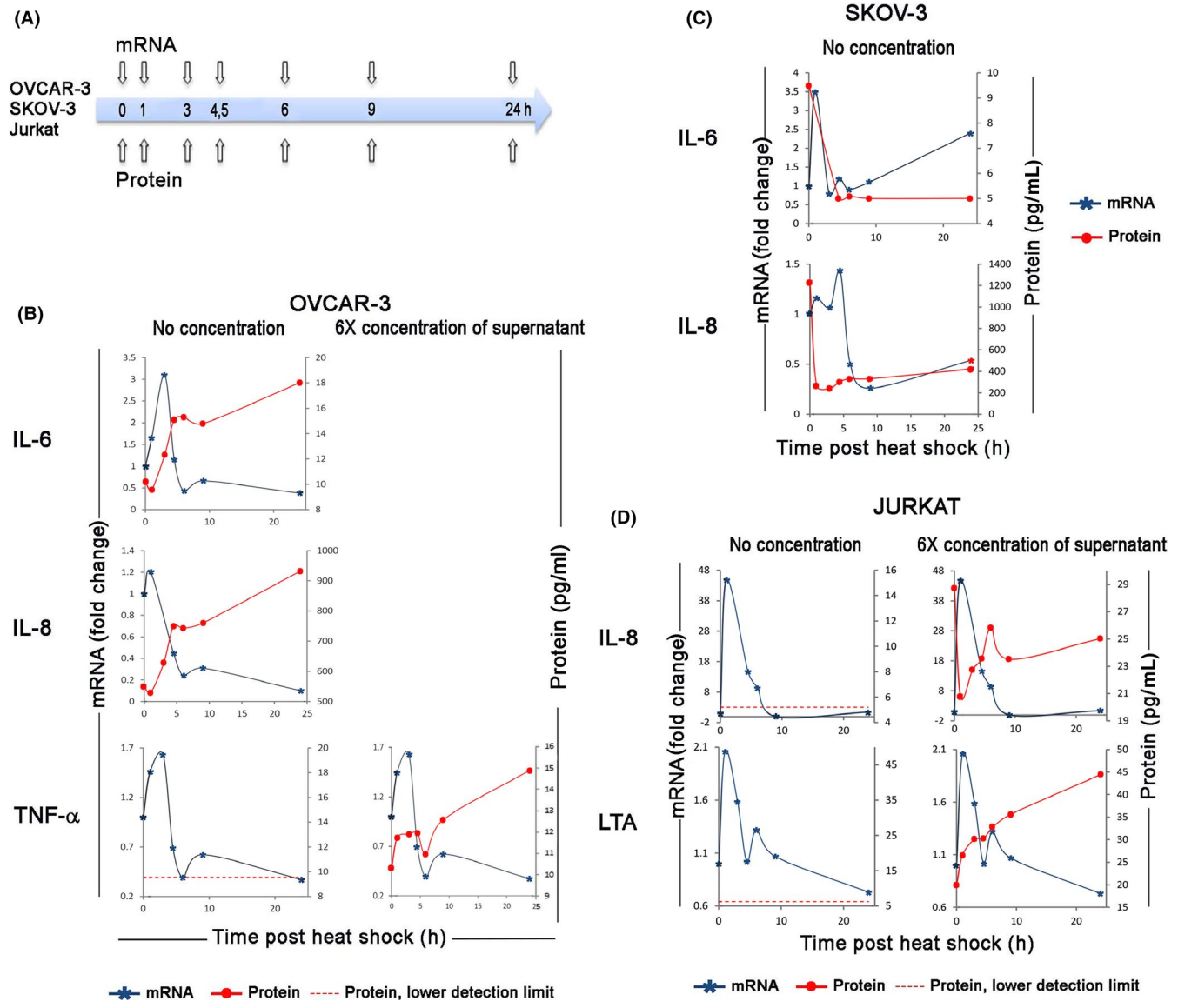

FIGURE 2 Cytokine mRNA and protein expression profiles in kinetic experiments with ovarian cancer cell lines OVCAR-3 and SKOV-3, and the T-cell line Jurkat, used as a control. A. Schematic presentation of the experimental design. B, C, D. Relative mRNA expression (fold change, blue color) and corresponding protein expression (pg/mL, red color) assessed at seven consecutive time points for the cytokines IL-6, IL-8, TNF-α, and TNF- β/LTA, specifically expressed by T cells. Note that TNF-α in OVCAR-3 and IL-8 and TNF-β/LTA in Jurkat were only detected after 6× concentration of the culture supernatant. TNF-α protein expression was absent in SKOV-3, as well as IL-6 protein expression in Jurkat

mRNA level showed a peak at 3 hour post-heat shock and decreased thereafter except for a smaller peak at 9 hour post-heat shock. The secreted protein levels of TNF-α in OVCAR-3 could only be detected after 6× concentration of the cell culture supernatant. The protein level showed signs of accumulation with an increase in concentration over time, from 10.3 to 14.9 pg/mL. Spearman's correlation between mRNA and protein production was $\rho = -.43$.

The SKOV-3 cytokine mRNA expression kinetics for all tested cytokines were somewhat different compared with OVCAR-3 (Figure 2B and C). As can be seen, the initial high peak was followed by a deep fall at 3-9 hours. After that, a continuous upregulation of the mRNA signal was seen, but at 24 hour not reaching the level of the initial peak. Both IL-6 and IL-8 were detected at protein level in SKOV-3 culture supernatants (Figure 2C). Both cytokines exhibited the highest protein concentration at starting point, 9.5 pg/mL for IL-6 and 1230.8 pg/mL for IL-8, and then decreased and reached a steady state at about 5 pg/mL and 400 pg/mL, respectively. (Spearman correlation was $\rho = -.78$ for IL-6 and $\rho = -.54$ for IL-8). In contrast to OVCAR-3, protein concentrations of TNF-α in SKOV-3 supernatants did not reach a detectable level even after 6× concentration (not shown). Secretion of IL-1β, and TNF-β/LTA, a cytokine specifically produced by T lymphocytes, was below detection level in both ovarian cancer cell lines (not shown).

For Jurkat (Figure 2D), the mRNA level for IL-8 and TNF-β/LTA was highest 1 hour post-heat shock for both cytokines, thereafter the level decreased except for a smaller peak at 6 hour for TNF-β/

LTA. Protein expression of IL-8 and TNF-β/LTA was initially not detectable but both cytokines reached detectable levels after 6× concentration of the culture supernatant. The protein level of IL-8 was highest at the beginning (28.8 pg/mL) and reached its lowest value 1 hour post-heat shock (20.8 pg/mL) and increased after this with another peak at 6 hour (25.8 pg/mL). Protein levels of TNF-β/LTA showed a steady increase over time (concentration from 19.9 pg/mL to 44.4 pg/mL). IL-6, TNF-α, and IL-1β were out of range in Jurkat supernatant, with a protein concentration below detection level also after 6× concentration of the supernatant. IL-1β, IL-6, and TNA-α are cytokines not specific for T lymphocytes, which is probably reflected in the Jurkat T-lymphocyte cell line. Spearman's rank order correlation coefficient was for IL-8 ρ = −.37 and for TNF-β/LTA ρ = −.36.

The mean and SD of the Ct values for mRNA transcription in the different cell lines at the chosen time points, as well as the mean Ct values and SD for the internal control, are summarized in Table 1. The mean and SD of the protein expression analyzed by Luminex® are shown in Table 2.

3.3 | Translation of cytokine mRNA to proteins can be missed due to dilution under the protein detection limit of the assay

The detection of cytokine proteins in serum can be biased due to the fact that they are produced and act locally in small amounts, it is only during a high production they will "spill over" to the peripheral blood. Reaching the blood and or other body fluids, they would be diluted and, in some instances, would not be detected if they are below detection level of the analytical method used. The result can thus be wrongly interpreted as a failure to translate cytokine mRNA to protein. The dilution effect was also illustrated in our kinetic experiments. Thus, TNF-α protein expression by OVCAR-3 was revealed after 6× concentration of the used culture supernatant (Figure 2B). The same procedure was done to reveal IL-8 and TNF-β/LTA protein expression in supernatants from Jurkat cultures (Figure 2D). To test whether changing the concentration of the used supernatant could alter the correlation between mRNA signal and protein expression, we analyzed IL-6 in OVCAR-3 and SKOV-3 in non-concentrated and 6× concentrated used supernatants (Figure 3) and found a similar curve pattern for protein expression.

3.4 | Comparison of IL-8 and IL-6 mRNA and protein analyses in paired tumor and serum samples in HGSC patients

We analyzed, in paired samples, the protein expression of IL-1β, IL-6, IL-8, TNF-α, and TNF-β/LTA in the serum of 14 women, suffering from HGSC, and compared it to previously analyzed mRNA expression in biopsies from their tumors.[6] The results are summarized in Table 3. Eight serum samples had detectable protein levels of IL-8 and three of IL-6. Protein levels of IL-1β, TNF-α, and TNF-β/LTA were not detected (not shown). Spearman's correlation coefficient was .52 for IL-8 and −.50 for IL-6.

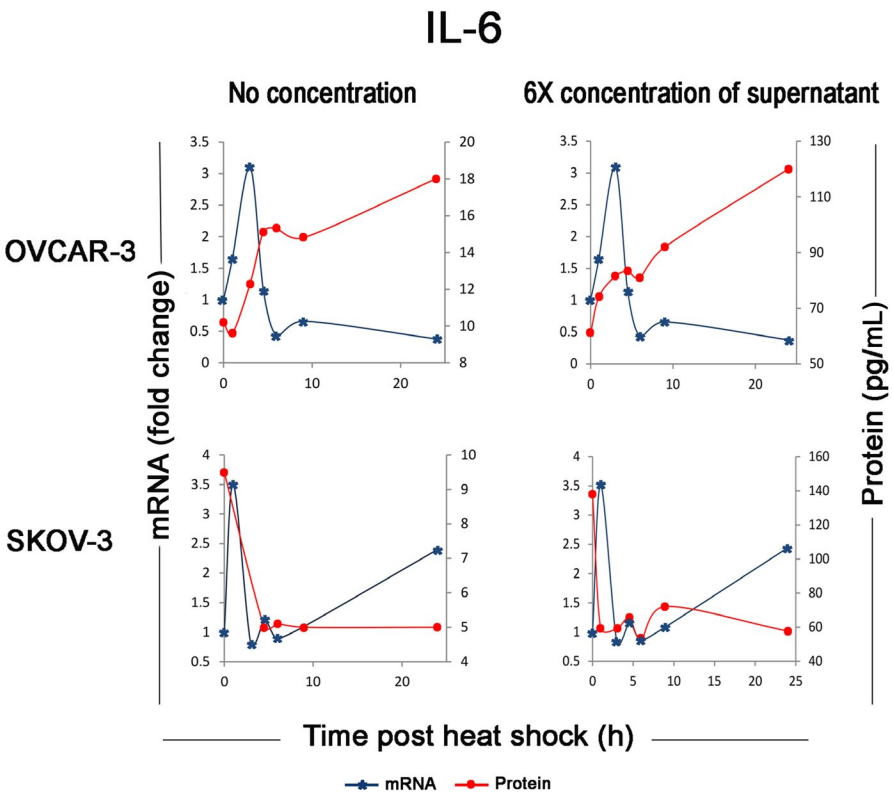

FIGURE 3 IL-6 mRNA expression (fold change, blue color) compared with protein expression (pg/mL, red color) in OVCAR-3 and SKOV-3 before and after a 6× concentration of the corresponding culture supernatant. Note the similar protein curve pattern

TABLE 3 Cytokine mRNA and protein expression in paired samples of tumor tissue and serum in women suffering from high-grade serous cancer of tubo-ovarian origin

	Cytokine					
	IL-8			IL-6		
Sample number	mRNA (Ct)	mRNA (RQ[a])	Protein (pg/mL)	mRNA (Ct)	mRNA (RQ)	Protein (pg/mL)
1	31.1	1	23	35.0	1	ND[b]
2	27.6	21.2	64.1	32.7	4.1	ND
3	34	2.7	ND	33.8	27.4	ND
4	33	0.6	35.2	36.7	1	451.7
5	33.1	0.2	ND	35.7	0.4	36.9
6	33.3	2.5	ND	36.5	2.5	ND
7	30.9	3.1	15.4	29.8	91.7	ND
8	27.8	8.3	10.2	34	1.3	ND
9	33.1	0.3	16.4	37.1	0.3	ND
10	29.5	15.2	ND	35.9	1.7	ND
11	30	4.8	ND	31	18.5	ND
12	26.9	31.1	71.3	30.8	18.8	ND
13	24.9	102.8	ND	29	72.9	9.5
14	25.3	286.8	38.9	29.9	110	ND

[a]RQ = Relative quantity.

[b]ND = Not detected.

4 | DISCUSSION

Studies of cytokines in health and various diseases[1,2] have gained more and more attention, not only due to their significant biological role, but also in the search of biomarkers for diagnosis and treatment.[1,3,20] Despite the fact that the biological effects of cytokines are driven by their protein/peptide form, the assessment of cytokine mRNA expression signatures for defining functional cytokine pathways of pathogenic importance has gained a wide use.[6-12] Factors influencing the cytokine mRNA and protein correlation are many, both biological and experimental, ranging from gene transcription to protein translation and degradation.[21,22] The difficulties in analyzing cytokines at protein level have to be considered. Cytokines are small signaling glycoproteins/peptides that act locally and are unstable with a short half-life and a high susceptibility to post-sampling handling.[5] They exert biological functions by a transient protein expression in complex body fluids, thus the location of cytokine production is of high importance. Samples taken from patients show a "snapshot" of cytokine levels at an unknown time point in the process from transcription of the gene to degradation of the protein. Several studies have been performed to seek for correlation between protein abundance and mRNA expression in human material, with varying results and opposing views.[13,18,21-26] Diverging results are to be expected considering methods used for each analysis, what material is analyzed and the handling of this material, making obtained results specific for a given system.

Here, we investigated cytokine mRNA and protein expression in kinetic experiments using ovarian cancer cells subjected to thermal stress. In addition, a smaller pilot study was performed, where we analyzed the cytokine mRNA and protein expression in paired tumor tissue and serum samples from patients suffering from HGSC.

Our results can be summarized as follows: (a) Ovarian cancer cells can translate mRNA to protein, as shown in the kinetic experiments for IL-6, IL-8, and TNF-α. We found a negative correlation between mRNA and protein expression with a Spearman's coefficient ranging from −.83 to −.43. (b) Concentrating the cell line supernatants, we could reveal protein expression of TNF-α in OVCAR-3 and of IL-8 and TNF-β/LTA in Jurkat, implying that analyzing cytokines on protein level could render false negative results due to technical reasons such as levels below detection limit. Concentrating the supernatant in our kinetic experiments did not alter the relationship between mRNA and protein expression, visualized by obtaining similar pattern of protein expression curves (Figure 3). (c) In paired tumor tissue and serum samples from HGSC patients, the results of the protein analyses in serum varied from no detection, as in TNF-α, to detection of IL-6 in some samples and detection of IL-8 in the majority of samples (Table 3).

Since the investigated time points are few in the kinetic experiments, and the serum samples are taken at only one occasion, no conclusions can be drawn on statistical significance.

As stated above, analyses of cytokine mRNA and proteins are influenced by a complex system with several parameters affecting the outcome of the analyses in an unpredictable way, as reviewed in 22. In an attempt to control and simplify the experimental conditions, we performed kinetic experiments with the ovarian cancer cell lines OVCAR-3 and SKOV-3. Five cytokines, IL-1β, IL-6, IL-8, TNF-α and TNF-β/LTA, all priming inflammatory immune response, were chosen for the kinetic studies based on

our previous assessment of cytokine mRNA expression profiles in HGSC tumors that showed high abundance of mRNA signals for these cytokines, locally in tumor tissue and systemically in PBMCs.[6] Tumor biopsy samples comprise many different cells in the tumor microenvironment. We chose to use ovarian cancer cells in the kinetic in vitro experiments to see whether the tumor cells themselves could translate the cytokine mRNA into protein and whether there is a correlation. Cytokine upregulation in our experimental set-up was achieved by a simple procedure of thermal stress followed by 24 hours' sampling at different time points of cells for mRNA expression analysis and culture supernatants for protein analysis by Luminex® microarrays. Thermal stress was chosen for transcriptional activation since it is simple to perform and easy to control and we have previous experience of the method.[27] Furthermore, the stress-inducing temperature we chose is the temperature used in treatment of different cancers.[28] The efficacy of the thermal stress hit was measured by assessment of the upregulation of the stress-inducible proteins HSP4A and MICA/B. mRNA transcripts were detected for all cytokines in all cell lines and in all tumor samples of the HGSC patients. On the other hand, the results of the cytokine protein analyses were diverse. IL-6, IL-8, and TNF-α protein expression were detected in the kinetic experiments, but the results of the protein analyses of HGSC serum samples varied. The detectability of TNF-α protein in the cell line supernatant was dependent on its supernatant concentration, thus demanding concentration of the supernatant before protein analysis. This implies that mRNA detection by real-time RT-qPCR was easier to assess due to its unique ability to enumerate small amounts, even one molecule, to detectable levels, due to the powerful specific amplification step in the method. A non-detected cytokine protein could be caused not only by biological modifications but also due to technical factors such as a narrow detection span in the chosen method, requiring multiple handling and testing of the samples. That is, when stating that a protein in a patient sample (serum, ascites, lavage etc) is non-detected, this could be caused by a concentration below (or above) the detection level of that method. Our results indicate that it is of importance to investigate different sample dilutions/concentrations before a conclusion about detection of a cytokine protein can be drawn. Furthermore, the pre-analytical handling of the serum samples for cytokine protein analysis is of great importance. Cytokines are unstable proteins susceptible to action of serum metalloproteases and are easily degraded at room temperature and by multiple freezing/thawing. Moreover, shown in our kinetic experiments, there was a delay between transcriptional activation and protein synthesis and secretion. Consistent with previous results,[22] this illustrates that there is a time delay between the mRNA peak and protein peak and implies that the time point for sampling is crucial. Despite the influence of these factors, we show in vitro, in kinetic experiments, that cytokine mRNA in ovarian cancer cells can be upregulated and further translated into protein.

We calculated the correlation between the cytokine mRNA and corresponding detectable protein in ovarian cancer cell lines using Spearman's rank correlation test. We obtained a negative correlation coefficient suggesting that the translation of mRNA to protein was not dependent on mRNA abundancy but probably on other mechanisms. In contrast to the findings in our kinetic experiments, a much variable but positive correlation coefficient of 0.29 to 0.71 was found in a study of cytokine mRNA and protein expression by human PBMCs, where similarly to our protein analyses, a multiplex Luminex® methodology was used.[24] The reason is not known, and several biological and technical factors could have contributed to this discrepancy. For example, different cellular sources were used—PBMCs, a natural cellular source for induction and production of cytokines vs our study where ovarian cancer cells were used, as well as different methods for mRNA detection—we used RT-qPCR and they used microarrays. The overall conclusion of the authors [24] was that it is insufficient to predict protein abundance from quantitative mRNA data. Results consistent with our findings, that is negative correlation coefficient, were found in a study of mRNA and protein expression for a subgroup of proteins including several HSP types, haptoglobin, various cytokeratins, Rab proteins, and others in 76 cases of lung adenocarcinomas.[25] It was suggested that the opposite correlation between mRNA and protein may reflect a negative feed-back on the mRNA or protein expression, or a presence of other, currently unknown, regulatory influences.[25] This suggestion might also be an explanation to our findings. Features in our study that could partially explain the similar findings of a negative Spearman's coefficient [25] could be that (a) the cellular sources were similar—both studies were done with tumor cells and (b) by choosing thermal stress for cytokine induction, we upregulated HSP genes—these genes constituted a majority in the subgroup of mRNA and proteins with negative correlation coefficient found in the 76 lung adenocarcinoma tumors. In other subgroups of proteins in this investigation,[25] no correlation between mRNA and protein expression was found. There are several more studies confirming that the abundancy of protein expression and mRNA levels is poorly correlated.[13,22-26]

In our comparison of IL-1β, IL-6-, IL-8, TNF-α, and TNF-β/LTA mRNA levels in tumor tissue to corresponding protein levels in serum of patients suffering from HGSC, two cytokines were detectable at protein level in the serum samples, IL-6 and IL-8. As we have previously shown,[6] these cytokines had one of the highest expression in HGSC tissue when comparing to ovarian tissue from women with benign cystadenomas and healthy ovaries. It is highly likely that the detectable protein levels in the serum of cancer patients are due to an outflow of cytokines in the blood, produced locally by the tumor and adjacent cells in the tumor microenvironment. Considering cytokine mRNA vs protein expression in HGSC patients, it is not possible to draw conclusions from the current comparison since only two cytokines where expressed and by a maximum of eight patient samples (IL-8). A broader comparison has to be made on a much larger patient material before

conclusions can be drawn on replacing cytokine protein analysis by mRNA analysis when used specifically for gaining knowledge on protein level and function. But, for comparisons between sample groups (for example benign vs malignant) where the emphasis is on the difference between groups and not in an exact individual protein level, we conclude that analyzing cytokine mRNA profiles by RT-qPCR is more reliable in detecting very low abundance of biological molecules, with a wider detection span, and less affected by post-sampling handling. In addition, if no concrete protein values are needed, immunohistochemistry could be considered as a useful complement to mRNA expression analysis to provide a semi-quantitative picture of protein levels.

In conclusion, so far, the comparisons between cytokine mRNA and protein expression levels performed on human material show very diverging results, often specific to a certain material (PBMC/tissue/serum/plasma) and method (PCR/microarray/ELISA/flow cytometry/multiplex immunoassay/polyacrylamide gel electrophoresis) making it difficult to apply outside the investigated system.[23-26] Considering the very high sensitivity and reproducibility of the real-time quantitative RT-PCR method, we would like to suggest that determination of cytokine mRNA profiles could be used as a proxy for protein-mediated functions for some specific purposes, such as comparisons between different patient groups and in defining mechanistic pathways involved in the pathogenesis of cancer and other pathological conditions.

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CONFLICT OF INTEREST

None.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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