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Interferon-γ expression is an independent prognostic factor in ovarian cancer

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KEY WORDS
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Background: Epithelial ovarian cancer prognosis is improved by the presence of intratumoral $CD3^+$ T cells, which are known to produce interferon-γ. We therefore speculated that interferon-γ expression in ovarian cancer-infiltrating T-lymphocytes might cause better prognosis.
Patients and methods: Reverse transcriptase polymerase chain reaction was performed to measure the expression of interferon-γ and other related genes in normal ovaries (n = 19) and in ovarian cancer specimens (n = 99). Median follow-up of patients was 5.8 years.
Results: Interferon-γ and CD-3 expression did not significantly differ in normal and malignant tissue. Patients with high levels of interferon-γ expression had significantly longer progression-free and overall survival. Median time to progression was 10 and 29 months for patients with low and high interferon-γ expression, respectively ($P = .039$). Corresponding survival times were 29 and 44 months ($P < .032$). Application of multivariate Cox regression analysis showed interferon-γ expression to be an independent prognostic factor for progression-free and overall survival.
Conclusion: Elevated interferon-γ expression correlates with improved clinical outcome in patients with ovarian cancer.
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Epithelial ovarian cancer is the leading cause of death from gynecologic malignancies. Most of these tumors are diagnosed at an advanced stage; more than half of such patients achieve remission after surgical debulking and primary chemotherapy, but overall 5-year survival remains less than 40%.[1] Although tumor stage, residual disease after surgical debulking, and response to chemotherapy affect the outcome of ovarian carcinoma, variability in progression-free and overall survival among patients with similar clinical and pathologic characteristics makes it difficult to reliably predict outcome. One reason for this difference could be that ovarian carcinoma is recognized and attacked by the immune system. The tumors are frequently infiltrated by lymphocytes, which exhibit oligoclonal expansion, recognize tumor antigens, and display tumor-specific

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Table I Clinical characteristics of patients with epithelial ovarian cancer

	All	IFN-γ low	IFN-γ high	*P* value
Number	99	36	63	
Age (y; median min-max)	66 (31-87)	67 (48-87)	65 (31-82)	.476
FIGO stage, n (%)				
I	18 (18)	7 (19)	11 (18)	.487
II	4 (4)	0 (—)	4 (6)	
III	62 (63)	23 (64)	39 (62)	
IV	15 (15)	6 (17)	9 (14)	
Grade, n (%)				
1	3 (3)	2 (6)	1 (2)	.016
2	56 (57)	26 (72)	30 (47)	
3	40 (40)	8 (22)	32 (51)	
Histopathologic classification, n (%)				
Serous	37 (37)	9 (25)	28 (44)	.005
Mucinous	30 (30)	18 (50)	12 (19)	
Other	32 (32)	9 (25)	23 (37)	
Residual disease, n (%)				
None	37 (37)	14 (39)	23 (36)	.969
<2 cm	23 (23)	8 (22)	15 (24)	
>2 cm	39 (40)	14 (39)	25 (40)	
First-line chemotherapy, n (%)				
No primary chemotherapy	8 (8)	3 (8)	5 (8)	.621
Cislatin plus cyclophosphamide	41 (41)	13 (37)	28 (44)	
Carboplatin plus paclitaxel	43 (43)	18 (51)	25 (40)	
Carboplatin single drug	7 (7)	2 (6)	5 (8)	
Median PFS (d)	652	319	876	.039
Median OS (d)	1141	856	1351	.032

cytolytic activity in vitro.[2-5] Zhang et al[6] recently reported that the presence of intratumoral $CD3^+$ T cells correlates with improved clinical outcome. These results were interpreted such that the T cell—mediated immunologic tumor-host interaction might be responsible for improved survival. However, tumors infiltrated by T cells have also shown elevated levels of interferon-γ (IFN- γ). In addition to stimulating immune-effector cells, this cytokine is known to exert a pleiotropic influence, including direct antiproliferative activity on ovarian cancer cells.[7,8] A possible explanation for inhibition of ovarian cancer cell proliferation might be the ability of IFN-γ to regulate several genes related to apoptosis or proliferation. In ovarian cancer cells, IFN-γ has been shown to downregulate the message and encode protein of the proto-oncogene HER-2/*neu* or to induce the class II tumor suppressor gene *H-REV107-1*.[9,10] Moreover, epigenetic silencing of multiple interferon pathway genes is associated with immortalization.[11] These findings indicate an important role of IFN-γ in ovarian cancer. Recent clinical trials confirmed that treatment of ovarian cancer patients with IFN-γ resulted in remission of recurrent disease and prolongation of disease-free survival in combination with conventional chemotherapy compared with platinum-containing chemotherapy alone.[12,13] We therefore speculated that IFN-γ expression in ovarian cancer-infiltrating T-lymphocytes might affect tumor biology by directly inhibiting growth as well as by stimulating immune competent cells. These effects should result in better prognosis of cancer patients with elevated IFN-γ production.

Patients and methods

Patients

Tissue samples from patients with invasive ovarian cancer were collected during primary debulking surgery at the Department of Obstetrics and Gynecology in Innsbruck between 1992 and 1999. Tumor slices of 5 mm were stored at −70°C. Two opposite margins were checked by means of histology in all cases and proven to consist of cancer tissue only (>90%). Moreover, normal ovaries removed for reasons other than ovary-related disease were used as normal controls (n = 19). Patient characteristics are given in Table I. Ninety-two percent of patients received adjuvant chemotherapy consisting of either cisplatin and cyclophosphamide (41 patients), carboplatin and paclitaxel (43 patients), or carboplatin alone (7 patients).

All patients were monitored from the outpatient clinic of the Department of Obstetrics and Gynecology, and the median observation period of surviving ovarian cancer patients was 5.8 years.

Table II Nucleotide sequences of the primers and probes

Gene	Oligonucleotide	Sequence
iNOS	Forward-primer	5′-CCA ACA ATG GCA ACA TCA GG -3′
	Reverse-primer	5′- TCG TGC TTG CCA TCA CTC C -3′
	Probe	5′FAM- CGG CCA TCA CCG TGT TCC CC -3′TAM
SOCS1	Forward-primer	5′-TTT TCG CCC TTA GCG TGA AG -3′
	Reverse-primer	5′-CAT CCA GGT GAA AGC GGC -3′
	Probe	5′FAM-CCT CGG GAC CCA CGA GCA TCC-3′ TAM
IRF1	Forward-primer	5′-AAG GAT GCC TGT TTG TTC CG -3′
	Reverse-primer	5′-CAG CGA AAG TTG GCC TTC C -3′
	Probe	5′ FAM- CTG GGC CAT TCA CAC AGG CCG ATA C -3′ TAM

RNA extraction and reverse transcriptase reaction

Total cellular RNA was extracted from the tumor specimens and purified by using the acid guanidium thiocyanate-phenol-chloroform method.[14] Integrity was evaluated by assessing the 18S- and 28S-ribosomal RNA bands in 2% ethidium-bromide-stained agarose gel. RNA concentration was measured by spectrophotometric analysis.

Reverse transcription (RT) of RNA was performed in a final volume of 20 μL containing 1× RT-buffer (50 mmol/L Tris-HCl, pH8.3, 75 mmol/L KCl, 5 mmol/L $MgCl_2$,), 40 units of rRNasin RNase inhibitor (Promega, Madison, Wis), 10 mmol/L dithiothreitol, 200 units of M-MLV RT (Gibco BRL, Gaithersburg, Md), 5 μmol/L random hexamers (Applied Biosystems, Foster City, Calif), and 800 ng of total RNA. The samples were first incubated at 65°C for 5 minutes and then quickly chilled on ice. After addition of the M-MLV enzyme, the samples were incubated at 25°C for 10 minutes and 37°C for 50 minutes, followed by heating at 70°C for 15 minutes to inactivate the RT enzyme.

Primers and probes

Gene Expression Assay Mixes for CD3E, IFN-γ and HER-2 were purchased from Applied Biosystems (Applied Biosystems Assay ID: Hs00167894_m1 [CD3E], Hs00174143_m1 [IFN-γ], Hs00170433_m1 [HER-2]). Primers and probes for the TATA box-binding protein (TBP; a component of the DNA-binding protein complex TFIID as endogenous RNA control) were used according to Bièche et al.[15] With the assistance of the computer program Primer Express (Applied Biosystems) primers and probes for inducible nitric oxide synthase (iNOS), which were kindly provided by Prof Dr Ernst Werner (Innsbruck University, Austria), suppressor of cytokine signalling-1 (SOCS1) and interferon regulatory factor-1 (IRF1) were determined. The nucleotide sequences of these oligonucleotides are shown in Table II.

Real-time polymerase chain reaction amplification

Polymerase chain reactions (PCRs) for CD3E, IFN-γ, and HER-2 were performed with the use of an ABI Prism 7900 Detection System (Applied Biosystems) with a total volume of 25 μL reaction mixture containing 5 μL of each appropriately diluted RT samples (standard curve points and patient samples), 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× Gene Expression Assay Mix or for TBP 900 nmol/L of each primer and 250 nmol/L of the probe, respectively. The thermal cycling conditions comprised initial incubation at 50°C for 2 minutes, a denaturing step at 95°C for 10 minutes, and 40 cycles at 95°C for 15 seconds and 65°C for 1 minute. Each experiment included a standard curve with 5 complementary DNA (cDNA) concentrations, a patient sample used as control sample to determine the reproducibility of each assay, 35 patients and a no template control. The standard curves were generated with serially diluted solutions of standard cDNA derived from the SKOV-6 ovarian carcinoma cell line for HER-2, Jurkat lymphoma cell line for CD3, and a RNA-pool from several patients for IFN-γ messenger RNA (mRNA) expression analysis.

PCR reactions for iNOS, SOCS1, and IRF1 were performed with an ABI Prism 7700 Detection System (Applied Biosystems) with a total volume of 25 μL reaction mixture containing 1 μL of each appropriately diluted RT samples for TBP and 4 μL for INOS, SOCS1, and IRF1, 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 200 nmol/L of each primer and 100 nmol/L of the probe. A typical real-time PCR amplification plot has been shown in Figure 1.

Determination of transcription level

The amount of the target and endogenous reference was determined from the standard curve. For value normalization the target amount was divided by the endogenous reference amount.

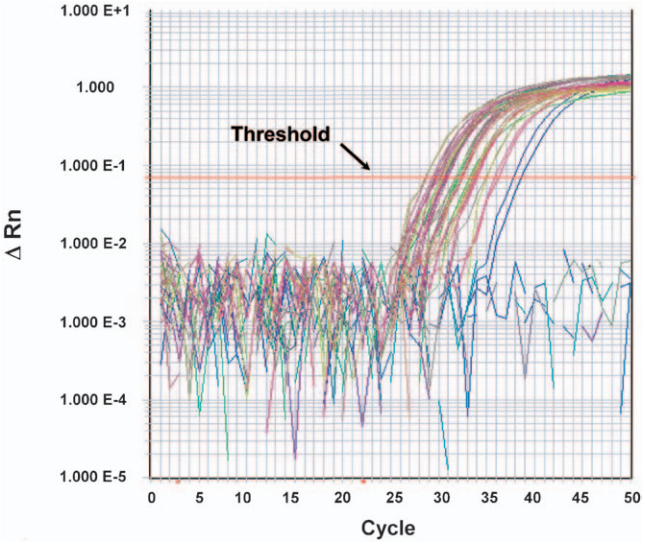

Figure 1 Typical real-time PCR amplification plot: Each experiment included a standard curve, a control sample to verify the reproducibility of each assay, 35 patients and a no template control.

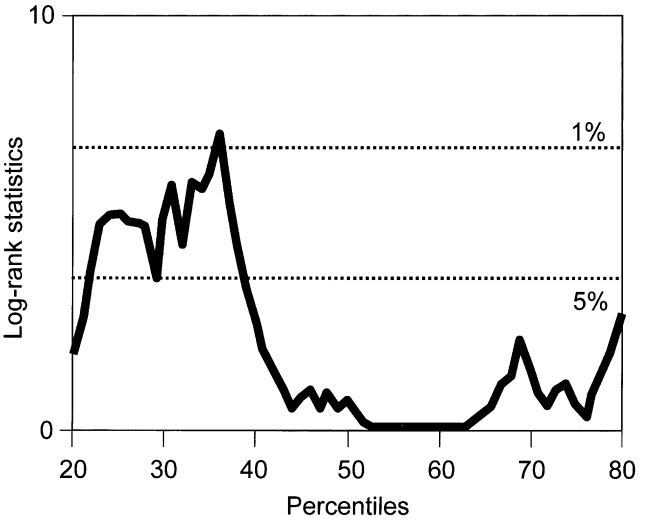

Figure 2 Estimation of an optimal cutoff point for IFN-γ expression level. Patients were stratified into 2 groups according to their IFN-γ expression level, and cutoff points were arbitrarily set between the 20th and 80th percentile. Survival curves were calculated for these cutoffs, and *P* values of Peto statistics are depicted. The optimal cutoff point for IFN-γ expression with the highest level of significance was the 36th percentile. Values above the 5% line are significant at a 5% alpha level, values above the 1% line are significant at the 1% alpha level.

Table III Results of real-time quantitative PCR

	Ovarian cancer (n = 99)	Normal (n = 19)	*P* value*
IFN-γ	0.5548 (0.1791-2.2995)	0.3586 (0.2271-1.1230)	.942
CD-3	0.0106 (0.0041-0.0295)	0.0109 (0.0063-0.0205)	.904
HER-2	2.6741 (1.8487-3.6678)	0.9920 (0.8045-1.1543)	< .001
SOCS-1	0.4795 (0.2773-0.7576)	0.9457 (0.6111-1.7526)	< .001
IRF-1	1.4927 (0.7889-2.1703)	0.8455 (0.7012-1.3640)	.034
iNOS	0.2761 (0.1032-0.4908)	0.8140 (0.4774-1.6168)	< .001

Results are given as median and interquartile range.
* Differences between the 2 groups were evaluated by the Mann-Whitney *U* test.

The gene expression level for iNOS, SOCS1, and IRF1 was determined with the Comparative C_T method according to User Bulletin 2 (Applied Biosystems).

Statistical analysis

Differences in expression levels between normal and malignant tissues were evaluated with the Mann-Whitney *U* test. Data were expressed as median and interquartile range. Because IFN-γ expression showed neither a clear negative value nor a biphasic distribution, the cutoff point had to be determined. For this purpose, data were divided into 2 groups at each percentile from 20 to 80, and differences in overall survival were analyzed with the log-rank test (Figure 2). Groups characterized by high and low IFN-γ expression were tested for differences with the Mann-Whitney *U* test for quantitative data and the χ^2 test for categorical data.

Spearman's correlation coefficient was calculated to show the correlation in the expression of IFN- γ, CD-3, HER-2, and STAT-induced genes (SOCS-1, IRF-1, and iNOS).

Survival curves were estimated with the use of the Kaplan-Meier method, and differences between the groups were evaluated in a univariate analysis by means of the log-rank test.

The Cox proportional hazards model was used to assess the predictive value of IFN-γ expression, with adjustment for potential confounding variables. The final model included performance status, grading, residual disease, and IFN-γ expression. The relative

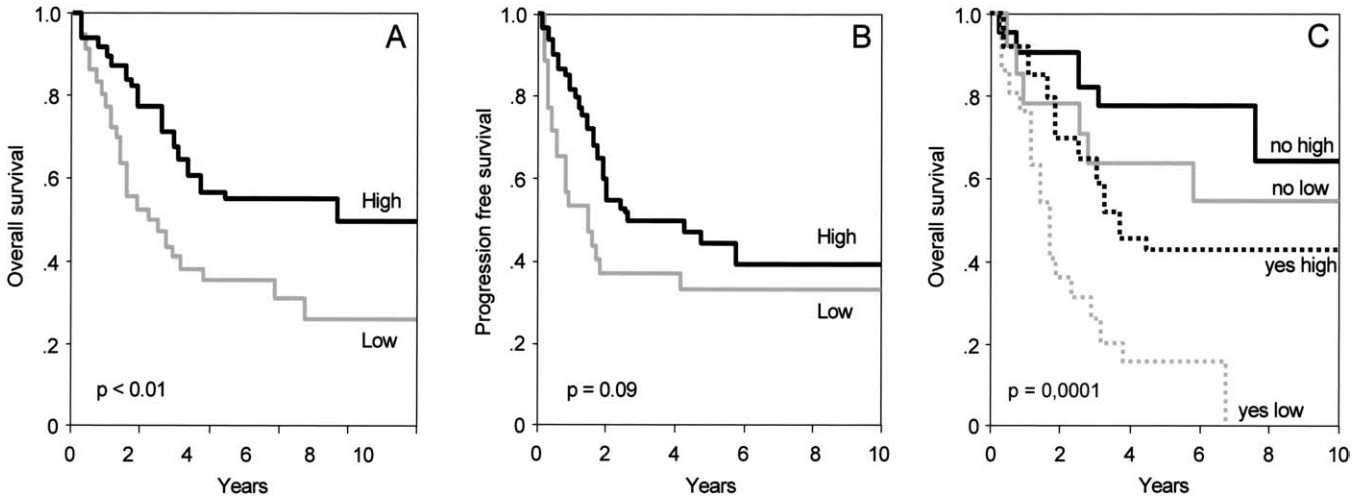

Figure 3 Survival analysis for patients with ovarian cancer according to expression of IFN-γ. **A** and **B** show Kaplan-Meier curves for PFS and OS, respectively, for patients with low and high IFN-γ–expressing tumors. **C** shows Kaplan-Meier curves for OS stratified according to the presence of residual disease after front-line debulking surgery (*yes*/*no*) and according to the expression of IFN-γ (*low*/*high*).

Table IV Multivariate Cox proportional-hazards analysis of PFS and OS

		PFS		OS	
Variable	No. of patients	Hazard ratio (95% CI)	*P* value	Hazard ratio (95% CI)	*P* value
INF-γ-expression					
Low	36	1.00	.005	1.00	< .001
High	63	0.41 (0.22-0.76)		0.37 (0.20-0.67)	
Residual disease					
No tumor left	37	1.00	.004	1.00	< .001
Tumor left	62	2.63 (1.37-5.06)		3.03 (1.54-5.97)	
Grade					
1 + 2	59	1.00	.044	1.00	.397
3	40	1.85 (1.02-3.37)		1.30 (0.71-2.37)	
Performance status					
PS 0	68	1.00		1.00	
PS 1	17	1.51 (0.31-1.43)	.296	2.13 (1.04-4.34)	.039
> PS 1	14	1.61 (0.63-4.1)	.318	1.49 (0.62-3.57)	.379

risk for recurrence or death is expressed as a hazard ratio with 95% CI. Statistical significance was defined as $P < .05$. SPSS for Windows 11.0 software (SPSS, Inc, Chicago, Ill) was used for all analyses.

Results

By means of RT-PCR we were able to detect expression of IFN-γ, CD-3, HER-2, as well as expression of signal transducers and activators of transcription (STAT)-induced genes such as SOCS-1, IRF-1, and iNOS (Table III). IFN-γ and CD-3 expression did not differ significantly in normal and malignant tissue. SOCS-1 and iNOS expression was higher in normal tissue, whereas HER-2 and IRF-1 RNA levels were significantly higher in malignant tumors than in normal tissues.

Expression of IFN-γ in patients with malignant ovarian tumors was strongly correlated to CD-3 ($\rho = 0.638$, $P < .001$) as well as to STAT-induced genes such as SOCS-1 ($\rho = 0.437$, $P < .01$), and IRF-1 ($\rho = 0.394$, $P < .01$), but not iNOS ($\rho = -0.117$, ns). Because IFN-γ has been shown to reduce expression of the oncogene HER-2, we were interested in whether we are able to observe a negative correlation.[9] Analysis of the 99 cases showed a negative Spearman-ρ of −0.209 ($P < .05$) for HER-2 and IFN-γ.

Because IFN-γ expression showed neither a clear negative value nor biphasic distribution, the cutoff point had to be determined. Our hypothesis was that patients with high IFN-γ expression have a better prognosis than those with low expression. With this assumption, survival curves applying cutoff points between the 20th and 80th percentiles were calculated. As a result, the highest

significance level was found at a cutoff corresponding to the 36th percentile (Figure 2). In all further analyses, the 36th percentile was thus used as the cutoff point. IFN-γ expression correlated with no other prognostic factors, except tumor grade (Table I). Surprisingly, high IFN-γ expression was associated with those tumors regarded as having poor prognosis. About 80% of patients with low IFN-γ expression had grade 1 or 2 tumors, whereas the patient group with high IFN-γ expression showed almost 50% of tumors to be grade 3 ($P = .016$). The other important factors analyzed were equally distributed between both IFN-γ groups, including the most important prognostic parameter, namely, residual disease.

To validate our data set survival analysis of classical prognostic factors was performed. Progression-free (PFS) and overall survival (OS) of ovarian cancer patients were significantly influenced by residual disease ($P = .0026$ and $P = .0012$, respectively) and FIGO stage ($P = .0031$ and $P = .079$, respectively), but not by tumor grade (ns for PFS and OS), performance status (ns for PFS and OS), or age (ns for PFS and OS) (data not shown).

Patients with high levels of IFN-γ expression showed significantly longer progression-free and overall survival (Figure 3, *A* and *B*). Median time to progression was 10 and 29 months for patients with low and high IFN-γ expression, respectively ($P = .039$). Median overall survival was 29 and 44 months for patients with low and high IFN-γ expression, respectively ($P < .032$). Because tumor left at primary surgery is 1 of the most important prognostic factors, we stratified patients according to residual disease (yes/no) and IFN-γ level (low/high, Figure 3, *C*). Patients with residual disease and low IFN-γ levels had the poorest prognosis, whereas those with high IFN-γ expression and complete debulking had the best survival rate.

Application of multivariate Cox regression showed IFN-γ expression to be an independent prognostic factor for PFS as well as OS (Table IV). Patients with a high IFN-γ expression had an approximately 60% reduced risk for recurrence or death. Residual disease was also an independent prognostic factor for both PFS and OS. Grading predicted recurrence only, whereas good performance status was associated with improved survival.

Comment

Our study indicates that elevated interferon-γ expression correlates with good clinical outcome in patients with ovarian cancer. Median PFS and OS was increased 2.7 and 1.6 times, respectively, in patients with high IFN-γ expression. The RNA encoding for this cytokine correlated with the message-characterizing cells known to produce IFN-γ, namely, CD-3 positive T-lymphocytes.[16] This is very interesting because Zhang et al[6] recently showed that the presence of intratumoral T cells is associated with prolonged PFS and OS. However, by measuring the CD-3 message we could not confirm this prognostic value. Nevertheless, immunohistochemical determination of active cells might be superior to measurement of mRNA only. For a cytokine, on the contrary, PCR is more sensitive than immunohistochemistry and allows quantification. Indeed, data suggest that IFN-γ might be the most important T-cell product mediating antitumoral action. It is therefore important to elucidate the mechanism of action to explain the beneficial effect of exposure of ovarian cancer to high concentrations of IFN-γ. Expression of several classes of genes is known to be regulated by IFN-γ. Class II genes of the major histocompatibility complex are normally underrepresented in malignant tumors, and IFN-γ is the most important inducer.[7] The action has been shown for ovarian cancer cells in vitro as well as in patients treated intraperitoneally with this cytokine.[17]

In addition, we were able to find a correlation between IFN-γ expression and those genes known to be induced by this cytokine, such as SOCS-1 and IRF-1. SOCS-1 has been shown to be a tumor-suppressor gene in hepatocellular carcinomas and an essential mediator of the effects of IFN-γ on MHC class II expression.[18,19] IRF-1 also acts as a tumor suppressor and is required for upregulation of the tumor suppressor gene *H-REV107-1* in ovarian carcinoma cells.[10,20,21] The failure to observe correlation of iNOS expression, another IFN-γ–inducible gene, might be explained by the fact that expression of iNOS is not only dependent on IFN-γ but also on activation of other signalling pathways, eg, nuclear factor κB (NFκB).[22, 23]

Transcription of both SOCS-1 and IRF-1 is dependent on STAT1, the central intracellular mediator of the action of interferons.[24,25] STAT1 has been demonstrated to be an essential component of IFN-γ–mediated tumor surveillance and a necessary component of proapoptotic and antiproliferative intracellular signalling pathways.[26] In accordance with this biologic function, STAT1 activation in mammary carcinoma correlated with good prognosis.[27] Whether STAT1 has a similar effect in promoting good clinical outcome in patients with ovarian cancer remains to be established.

The oncogene HER-2 belongs to the family of epidermal growth factor receptors, and its amplification has been shown to be associated with poor prognosis in breast and ovarian cancer. It is therefore very interesting that IFN-γ expression inversely correlates with HER-2 expression. We recently demonstrated downregulation of the HER-2 message in several ovarian cancer cell lines using IFN-γ.[9] Our finding in tumor specimens is therefore in agreement with the recent in vitro study. Suppression of the oncogene HER-2 by biologic agents might be an interesting therapeutic approach. In breast cancer patients, the monoclonal antibody trastuzumab

known to block HER-2 is able to prolong survival.[28] IFN-γ produced endogenously or administered therapeutically might evoke a similar response in ovarian cancer patients. Because only about 10% of ovarian tumors overexpress HER-2, this IFN-γ–mediated effect might not play a major role, although normal levels of growth factor receptors could also suffice for relevant signal transduction. IFN-γ was also able to reduce oncogene message in vitro in ovarian carcinoma cells with normal HER-2 expression.[9]

Another mechanism by which IFN-γ might inhibit the proliferation of ovarian cancer cells is the regulation of tumor suppressor genes. One interesting candidate is *H-REV107-1*, a class II tumor suppressor gene strongly reduced in about 50% of human ovarian carcinomas. Treatment of ovarian cancer cells with IFN-γ resulted in a substantial upregulation of *H-REV107-1* and IRF-1.[10]

IFN-γ treatment alone has been shown to induce remission in recurrent ovarian cancer.[12] Moreover a combination of IFN-γ and conventional platinum-containing chemotherapy proved to increase PFS.[13] There is therefore no doubt that IFN-γ is active in primary and recurrent ovarian cancer. Bringing together these studies with our results, one could speculate that patients with low IFN-γ expression might benefit from cytokine treatment. It is therefore necessary to further analyze IFN-γ expression in ovarian cancer patients treated with this cytokine. Recently, an international randomized multicenter phase III ovarian cancer trial was initiated to compare carboplatin plus paclitaxel with the same regimen plus IFN-γ (GRACES sponsored by Intermune Inc). More than 600 patients have been recruited and first results are expected for the end of 2004.

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