c-FLIP_L expression defines two ovarian cancer patient subsets and is a prognostic factor of adverse outcome

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Abstract

The impairment of apoptotic pathways represents an efficient mechanism to promote chemoresistance in cancer cells. We previously showed that in epithelial ovarian cancer (EOC) cells, long isoform of cellular FLICE-inhibitory protein (c-FLIP_L) accounts for apoptosis resistance in a context of functional p53 and resistance could be overcome by c-FLIP_L downmodulation. Here, we studied the association between c-FLIP_L and p53 expressions and their prognostic impact in EOC patients. Tumor tissue from 207 patients diagnosed with primary EOC was analyzed by immunohistochemistry (IHC) for c-FLIP_L and p53 expressions, and multiple correspondence analysis (MCA) was used to evaluate the multivariable pattern of association among patients' clinical–pathological characteristics and biological determinants. IHC revealed c-FLIP_L expression and p53 nuclear accumulation inversely related ($P=0.0001$; odds ratio=0.29, confidence interval (CI)=0.15–0.055). MCA indicated that p53 accumulation was associated to clinical–pathological variables, while c-FLIP_L expression contributed to the overall association pattern independently from other's clinical characteristics and complementary to p53. Kaplan–Meier curves showed a reduced survival time according to c-FLIP_L expression in concert with p53 accumulation (median overall survival (OS): 35 months) compared with lack of expression of both markers (median OS: 110 months; log-rank test, P value=0.024). The multivariable Cox regression model, adjusted for known prognostic factors, identified c-FLIP_L expression, but not p53 nuclear accumulation, as an independent prognostic factor for adverse outcome (hazard ratio=1.82, 95% CI=1.17–2.82; $P=0.008$). Altogether these data support the independent contribution of c-FLIP_L in refining the prognostic information obtained from standard clinical–pathological indicators, confirming its pivotal role in promoting cell survival.

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Introduction

Epithelial ovarian carcinoma (EOC) remains the leading cause of death for gynecologic cancer in women in western countries. Because more than two-third of patients are diagnosed with advanced disease,

surgical debulking is not sufficient to eradicate the tumor, which often spreads into the peritoneal cavity. In the attempt to eradicate residual disease, patients receive a platinum-based therapy. After an initial response to front-line chemotherapy, the patients

frequently relapse, developing resistance to further chemotherapeutic treatment (Ozols *et al.* 2004). The overall prognosis is poor, with the 5-year survival ~40% (Jemal *et al.* 2008).

Resistance to chemotherapy is frequently related to the failure of tumor cells to undergo programmed cell death. Thus, the role of apoptotic or pro-survival pathways in ovarian carcinogenesis and cancer progression is a current focus of intensive studies. Apoptotic program is typically executed through an intrinsic, mitochondrial-dependent and an extrinsic, receptor-dependent pathway (Jin & el Deiry 2005); nevertheless, tumor cells can evade apoptosis through several mechanisms that affect both pathways (Fulda & Debatin 2006). The tumor suppressor gene p53 plays a leading role in determining the efficacy of the intrinsic apoptotic signaling in response to several chemotherapeutic agents, and its inactivation can induce apoptosis resistance (Vogelstein *et al.* 2000). The extrinsic pathway can be inhibited directly at the receptor level by molecules such as the cellular FLICE-inhibitory protein (c-FLIP), which interfere with efficient death-inducing signaling complex formation by inhibiting caspase-8 recruitment and processing (Irmeler *et al.* 1997, Scaffidi *et al.* 1999). An association between c-FLIP expression and resistance to apoptosis mediated by CD95 as well as by TRAIL has been reported for several cancer types (Abedini *et al.* 2004, Rippo *et al.* 2004, Horak *et al.* 2005, Longley *et al.* 2006, Rogers *et al.* 2007), and c-FLIP expression was already reported to be an unfavorable prognostic indicator in some oncotypes other than ovarian carcinoma (Chen *et al.* 2005, Valnet-Rabier *et al.* 2005, Ullenhag *et al.* 2007). In EOC cell lines, we have recently identified the long isoform of c-FLIP (c-FLIP_L) as one of the molecules directly involved in the impairment of apoptotic signaling in the context of functional p53; in the same study, we also found an inverse relationship between c-FLIP_L overexpression and p53 nuclear accumulation in a small number of EOC specimens (Mezzanzanica *et al.* 2004). Additionally, Tsang's group reported the involvement of c-FLIP_L in regulating EOC cisplatin sensitivity in concert with p53 and the ubiquitin ligase Itch (Abedini *et al.* 2004, 2008), further suggesting a role for c-FLIP_L as a survival factor in EOC cell lines.

Although several experimental evidences suggested a major role of c-FLIP_L in regulating apoptotic mechanisms in EOC, little is known about its prognostic relevance in this disease. Here, we analyzed c-FLIP_L expression alone and in combination with p53 nuclear accumulation in more than 200 primary EOC specimens to determine whether

c-FLIP_L behaves as survival prognostic marker that may help in identifying patients at higher risk of death of disease.

Patients and methods

Patients, tissue specimens, and pathologic data

The study was performed on available archival formalin-fixed, paraffin-embedded material collected at surgery before any chemotherapeutic treatment, from 207 patients with primary EOC who underwent surgical resection at the Fondazione IRCCS Istituto Nazionale dei Tumori (INT) between 1990 and 2001 and at the S. Chiara Hospital in Trento between 1992 and 1999. All histological sections and paraffin blocks were obtained from the Departments of Pathology of both Institutes. Pathologists (S P and M B) with specialized expertise in gynecological pathology reviewed all pathological data. All clinical data and follow-up information were available from the Units of Gynecologic Oncology of both Institutes as current follow-up procedures. The use of tissue blocks and patients' record was approved by the Institutional Review Boards.

Table 1 summarizes the patients' clinicopathological characteristics. Tumor staging was in accord with International Federation of Gynecology and Obstetrics (FIGO) criteria. Primary treatment for all patients was surgery and, based on the extent of residual disease after primary surgery, the patient population was divided into three groups: no evident disease (NED); minimal residual disease (mRD, residual tumor smaller than 1 cm); and gross residual disease (GRD, residual tumor larger than 1 cm; Berman 2003). After surgery, 195 patients received front-line treatment with standard platinum-based therapeutic schedules (P: platinum without taxanes; PT: platinum and paclitaxel; PTT: platinum, paclitaxel, and topotecan) according to the time of accrual and Institutional involvement in International Trials; two patients were treated with other chemotherapeutic agents, seven patients (all stage I) received no chemotherapy, and three patients had information missing.

Immunohistochemistry

Immunohistochemistry (IHC) assays were all performed on the whole-case series at the INT, Milan.

c-FLIP_L and p53 expressions were examined by IHC on formalin-fixed, paraffin-embedded EOC sections or tissue microarray, using the UltraVision LP detection system HRP polymer (Lab Vision Corporation, Fremont, CA, USA) according to the manufacturer's

Table 1 Patients' clinical characteristics

Characteristics	Patients (n=207)	
	N	%
Age (years)		
(mean, median 57; range 23–84)		
≤55	93	45
>55	114	55
Tumor histotype		
Serous	137	66
Undifferentiated	21	10
Clear cell	14	7
Endometrioid	24	12
Mucinous	7	3
Others + mixed	4	2
Tumor stage (FIGO)		
I	24	12
II	15	7
III	127	61
IV	39	19
Not available	2	1
Tumor grade		
1, well differentiated	11	5
2, moderately differentiated	68	33
3, poorly differentiated	101	49
Undifferentiated	21	10
Not available	6	3
Amount of residual disease		
NED	54	26
<1 cm, mRD	32	15
>1 cm, GRD	105	51
Not available	16	8
Front-line treatment		
None	7	3
Platinum without taxanes	108	52
Platinum/paclitaxel	71	34
Platinum/paclitaxel/topotecan	16	8
Other or not available	5	2

FIGO, International Federation of Gynecological and Obstetrics staging system; NED, not evident disease; mRD, minimal residual disease; GRD, gross residual disease.

instructions and as previously described (Aldovini *et al.* 2006, Mezzanzanica *et al.* 2008). After xylene deparaffinization and alcohol rehydration, sections were subjected to antigen retrieval in 10 mM, pH 6.0, citrate buffer at 95 °C for 6 min in an autoclave. Goat anti-c-FLIP_L (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and mouse anti-p53 mAb DO7 (Novocastra Laboratories, Newcastle upon Tyne, UK) were used as primary antibodies. Slides incubated with 'primary antibody enhancer' alone provided negative controls (Aldovini *et al.* 2006, Mezzanzanica *et al.* 2008).

Staining of each biopsy sample was evaluated as described (Aldovini *et al.* 2006) based on staining intensity (0, undetectable; 1, faint; 2, moderate; 3, intense) and estimated percentage of reacting cells (0, none; 1, 1–10%; 2, 11–25%; 3, 26–50%; 4, >50%). A final IHC

score was obtained as the sum of the intensity and the percentage scores. For tissue microarray analysis (130 cases), the four cores of each case were scored separately, and the value of the mean score was taken as representative of the case (the four cores of each case were always comparable). Only cases with two or more assessable cores were included in the analyses (Aldovini *et al.* 2006). Tumors were considered positive for p53 accumulation or c-FLIP_L expression if they had a score ≥5 and 3 respectively.

Slides were evaluated by two independent observers blinded to patient characteristics and outcome. All cases with discrepant evaluations were discussed during observation with a double-headed microscope and a consensus was reached.

Statistical methods

Patients were grouped based on similar clinicopathological characteristics (Tables 1 and 2) and age was used as categorical variable. ϕ contingency coefficient (Liebetrau 1983) was used to evaluate the association of c-FLIP_L and p53 with the other clinicopathological characteristics (Table 2). ϕ contingency coefficient ranges between 0 and 1, with 0 signifying no association and 1 signifying perfect association. An exact test to evaluate whether ϕ is significantly different from 0 was reported. The odds ratio (OR), with exact confidence interval (CI), was used to evaluate the association between c-FLIP_L and p53. $P < 0.05$ was considered significant.

The multivariable pattern of association among patients' clinicopathological characteristics and biological determinations was investigated in the 174 (84%) cases informative for all the considered variables using multiple correspondence analysis (MCA; Coradini *et al.* 2000, Ambrogì *et al.* 2006).

MCA can be applied both to categorical and continuous variables. Using MCA, it is possible to visualize association between markers and clinical characteristics on a two-dimensional plot. The use of a two-dimensional plot, easy to interpret, is possible at the expense of losing some information on the multidimensional pattern of associations. To quantify the information retained in the two-dimensional plot, the percentage of information explained (i.e. the percentage of total variability explained by the two axes of the plot) is used following Benzécri (Lebart *et al.* 1995).

The distance between points is based on a χ^2 metric, and therefore the numerical values on the axes could not have a straightforward interpretation in MCA. Points approximately in the same region of the plot and

Table 2 Patients' clinical and pathological characteristics according to long isoform of cellular FLICE-inhibitory protein (c-FLIP_L) expression and p53 nuclear accumulation as assessed by immunohistochemistry

Clinical parameters	c-FLIP _L expression					φ (P^*)	p53 nuclear accumulation					φ (P^*)
	Negative ($n=127$)		Positive ($n=72$)		Missing ($n=8$)		Absent ($n=100$)		Present ($n=100$)		Missing ($n=7$)	
	<i>N</i>	%	<i>N</i>	%			<i>N</i>	%	<i>N</i>	%		
Total ($n=207$)												
Age (years)						0.002						0.02
≤55	58	64	33	36	2	(1)	47	51	45	49	1	(0.887)
>55	69	64	39	36	6		53	49	55	51	6	
Tumor histotype						0.28						0.26
Serous	85	65	46	35	6	(0.0056)	66	49	69	51	2	(0.017)
Undifferentiated	15	79	4	21	2		7	41	10	59	4	
Clear cell	3	21	11	79			12	86	2	14		
Endometrioid	18	75	6	25			8	33	16	67		
Mucinous	3	43	4	57			5	83	1	17	1	
Others	3	75	1	25			2	50	2	50		
Tumor stage (FIGO)						0.08						0.27
I+II	24	63	14	37	1	(0.58)	30	77	9	23		(0.0006)
III	79	66	41	34	7		51	42	71	58	5	
IV	22	56	17	44			19	51	18	49	2	
Missing	2								2			
Tumor grade						0.11						0.25
1	6	55	5	45		(0.53)	10	91	1	9		(0.006)
2	42	64	24	36	2		37	56	29	44	2	
3	63	65	34	35	4		41	41	59	59	1	
Undifferentiated	15	79	4	21	2		7	41	10	59	4	
Missing	1		5				5		1			
Amount of residual disease						0.14						0.18
NED	36	69	16	31	2	(0.15)	33	63	19	37	2	(0.06)
<1 cm mRD	21	70	9	3	2		15	48	16	52	1	
>1 cm GRD	57	55	46	45	2		44	43	58	57	3	
Missing	13		1		2		8		7		1	

NED, not evidence of disease; mRD, minimal residual disease; GRD, gross residual disease; φ , association coefficient. * P values, exact test for the φ association coefficient evaluated on c-FLIP_L expression or p53 nuclear accumulation and available clinical and pathological parameters.

approximately in the same direction starting from the origin correspond to associated marker categories and clinical characteristics. The two tumor markers under study (c-FLIP_L and p53) and the five clinical characteristics (FIGO stage, grading, histotype, age, and surgical debulking) were used to create the MCA plot (active information). The position of the categories of the active variables and the knowledge of which categories have most contributed to the construction of the MCA plot are used to interpret the result obtained.

Front-line therapy was only plotted on the existing MCA plane without modifying it (passive information), for a subsequent study of its relationships with the clinic and pathologic characterization of the tumors. The passive variable may not be associated with the active variables used for the construction of the MCA plot. In this case, the categories of the variable should not be considered for the interpretation of the result.

MCA was performed using SPAD (version 3.5 CISIA-CELESTA, Montreuil, France), while exact tests for φ coefficient were computed using StatXact (version 3.1, Cytel Software Corporation, Cambridge, MA, USA).

Prognostic relationships

Overall survival (OS) was defined as the time (in months) between the date of surgery and the date of death or until the date of last contact for censored events when the patient was still alive. Median follow-up time was 87 months. Three patients were missing at follow-up, 127 patients had died and all, but two observed deaths were cancer related. Progression-free survival (PFS) time was calculated as the time in months from the time of intervention until the first evidence (clinical, instrumental, or biological) of disease progression.

The effect of biological and clinicopathological characteristics on OS and PFS was investigated first by univariate analysis through the inspection of Kaplan–Meier curves. A Cox univariate model was used to estimate the hazard ratio (HR) for each prognostic variable. Multivariable analysis using a Cox regression model was used to evaluate the prognostic impact of c-FLIP_L and p53 in the context of concomitant effects of other known prognostic factors. Front-line therapy was used as a stratification factor accounting for its possible non-proportional effect. To take into account a potential heterogeneity related to the different specialization of the two Institutions (an oncological center versus a general hospital), we firstly performed all the statistical analysis stratifying for center. Since the analysis stratified by center was in agreement with the unstratified analysis, only the latter was reported. For both univariate and multivariable analyses, stage and surgical debulking were coded as indicator variables, (stage III versus stage I+II, stage IV versus stage III; mRD versus NED, GRD versus mRD). Age was modeled with a restricted cubic spline (Herndon & Harrell 1995) with three knots to evaluate nonlinear effects. Also a two-level classification was used for age (>55 vs ≤55; Havrilesky *et al.* 2003, Tanner *et al.* 2006, Winter *et al.* 2007), grading (grading 3 + undifferentiated versus grading 1 + 2), and histotype (serous versus non-serous). Four levels were defined for front-line therapy: no chemotherapy or other therapy; platinum; platinum/paclitaxel; platinum/paclitaxel/topotecan. Survival analyses were carried out using R statistical language (<http://www.R-project.org>).

Results

c-FLIP_L and p53 immunostaining

c-FLIP_L expression was detected in 72 out of 199 valuable cases (36%), with a higher frequency in clear cells and mucinous histotype (79 and 57% respectively) and with no other correlation with

clinicopathological characteristics (Table 2). p53 nuclear accumulation was detected in 100 out of 200 valuable cases (50%) and was associated with advanced tumor stage ($\varphi=0.27$, $P<0.001$) and high grade ($\varphi=0.25$, $P=0.006$). p53 accumulation was less frequently observed in clear cell and mucinous tumors than in other histological types (Table 2).

c-FLIP_L expression versus p53 nuclear accumulation was assessable on 195 cases. Consistent with our previous finding of an inverse relationship between c-FLIP_L expression and p53 nuclear accumulation in a small sample size of EOC specimens (Mezzanzanica *et al.* 2004), p53 nuclear accumulation was observed in 62% of cases negative for c-FLIP_L expression (77 out of 125) and in only 31% of c-FLIP_L-positive cases (22 out of 70; $\varphi=0.29$, $P=0.0001$). OR was 0.29 (CI exact=0.15–0.55). Results of immunohistochemical staining are summarized in Table 3.

Multiple correspondence analysis

The information explained by the MCA plot, obtained as described in materials and methods, amounted to 97%. The MCA plot positions of clinical–pathological characteristics (active information) and therapy (passive information) were jointly plotted in Fig. 1, to highlight their mutual association. The horizontal (first) axis separates on the right the categories of stage I+II, grade 1, no serous histotype, p53 negative, and surgical debulking NED. On the left are plotted stage III, grade 3, serous histotype, presence of p53 nuclear accumulation (p53 pos), and surgical debulking GRD. The first axis thus represents the expected association among well-known clinical–pathological features of the pathology. The vertical (second) axis separates undifferentiated tumors, no serous histotype, p53 positive and c-FLIP_L negative on the bottom, while p53 negative, c-FLIP_L positive, and grade 2 are on the top.

p53 seems to have an intermediate behavior between clinical features and c-FLIP_L; in particular, p53 positive appears to be associated, sharing the same direction from the origin, with mRD and age > 55,

Table 3 Long isoform of cellular FLICE-inhibitory protein (c-FLIP_L) expression versus p53 nuclear accumulation in epithelial ovarian cancer as assessed by immunohistochemical staining

c-FLIP _L expression		p53 nuclear accumulation			φ (P value)*
		Absent	Present	Not available	
Negative	125	48	77	2	0.29
Positive	70	48	22	2	
Not available	8	4	1	3	0.0001

*Exact P value for the φ coefficient.

Figure 1 Multiple correspondence analysis. MCA plot showing the projections of the two biological markers (c-FLIP_L and p53) and patients' age, tumor stage, grading, histology, and surgical debulking. Front-line therapy (FT) was used as passive information. Factor 1, first axis; factor 2, second axis.

whereas p53 negative with age ≤ 55 and grading 2. Therefore, the second axis is mainly associated with c-FLIP_L status, suggesting a greater independent contribution of c-FLIP_L to the overall association pattern. An additional contribution as inverse association of c-FLIP_L and p53 markers is evident, being c-FLIP_L-positive and p53-negative categories and c-FLIP_L negative and p53 positive projected on the same graph side. Therapy (passive information) projected on the plot accordingly to the clinical features as expected.

Prognostic significance of c-FLIP_L and p53 expressions

Univariate survival analysis (Table 4) showed that, as expected for EOC, known clinical prognostic factors such as higher FIGO stage, serous histotype, and suboptimal surgical debulking were all associated with worse prognosis (shorter OS). These clinical variables were also associated with early relapse. Tumor grade showed a slight impact on both PFS and OS. p53 nuclear accumulation, but not c-FLIP_L expression was associated with both worse prognosis and early relapse (see Table 4 for details).

Figure 2 shows OS (panel A) and PFS (panel B) curves estimated by the Kaplan–Meier method, stratified for c-FLIP_L expression and p53 nuclear accumulation; IHC staining representative of the four possible combination is reported in panel C. The concurrent alteration in c-FLIP_L expression and p53

nuclear accumulation when compared with the absence of both proteins led to a considerable reduction in median OS (35 vs 110 months) and median PFS (15 vs 26 months; log-rank test, $P=0.024$ and $P=0.017$ respectively), see Fig. 2 for details.

Use of a bivariable Cox model with c-FLIP_L and p53 as covariates indicated a stronger prognostic impact of the biological markers when compared with univariate analysis for both clinical end points. For c-FLIP_L expression, HRs became 1.50 (95% CI: 1.01–2.21; $P=0.04$) and 1.45 (95% CI: 1.01–2.06; $P=0.042$) for OS and PFS respectively. Considering p53 nuclear accumulation, HRs became 1.72 (95% CI: 1.17–2.53; $P=0.0056$) and 1.71 (95% CI: 1.20–2.42; $P=0.0027$) for OS and PFS respectively.

We next applied the multivariable Cox regression model with c-FLIP_L expression and p53 nuclear accumulation adjusting for all known clinical and pathological prognostic factors. There was no evidence for the inclusion of non-linear effects of age. As model results were similar (as far as the HR of c-FLIP_L) for age modeled as a linear function and for age dichotomized, only the latter was reported. The estimated HR and their CIs are reported in Table 5. In this model, c-FLIP_L expression maintained its prognostic impact for OS. FIGO stage and surgical debulking were of prognostic relevance for both OS and PFS, whereas p53 nuclear accumulation, grading, and histotype were not. In our study population, c-FLIP_L expression was an independent prognostic

Table 4 Univariate analysis of the prognostic impact of clinical and biological variables on overall and progression-free survival

	Overall survival			Progression-free survival				
	P*	HR	(95% CI)	Median OS (months)	P*	HR	(95% CI)	Median PFS (months)
Age at diagnosis								
> 55 vs ≤55	0.037	1.46	1.02–2.09	44 vs 58	0.06	1.36	0.99–1.88	20 vs 23
Stage								
III vs I–II	<0.0001	5.83	2.69–12.63	45 vs n.y.r.	<0.0001	6.18	3.22–11.88	20 vs n.y.r.
IV vs III	0.031	1.57	1.04–2.37	28 vs 45	0.0028	1.8	1.22–2.64	13 vs 20
Histotype								
Serous versus others	0.0067	1.75	1.17–2.61	45 vs 110	0.0026	1.76	1.22–2.55	20 vs 25
Grade								
3+undiff versus 1+2	0.08	1.40	0.96–2.03	46 vs 62	0.027	1.47	1.04–2.06	20 vs 25
Surgical debulking								
mRD versus NED	<0.0001	4.31	2.12–8.78	53 vs n.y.r	<0.0001	4.15	2.20–7.83	21 vs n.y.r.
GRD versus mRD	0.062	1.60	0.98–2.61	40 vs 53	0.0008	2.20	1.39–3.48	16 vs 21
FLIP expression*								
Positive versus negative	0.19	1.28	0.89–1.86	40 vs 58	0.23	1.23	0.88–1.72	18 vs 22
p53 nuclear accumulation								
Present versus absent	0.02	1.52	1.06–2.18	48 vs 63	0.0092	1.54	1.11–2.14	20 vs 25

*P value determined using log-rank test; HR, hazard ratio; CI, confidence interval; mRD, minimal residual disease; n.y.r, not yet reached; NED, not evident disease; GRD, gross residual disease.

Figure 2 Kaplan–Meier estimate of (A) overall survival and (B) progression-free survival following stratification for c-FLIP_L expression and p53 nuclear accumulation. *P* values estimated using the log-rank test. (C) Representative IHC staining of c-FLIP_L expression and p53 nuclear accumulation on formalin-fixed, paraffin-embedded EOC tissue sections. Original magnification ×200.

Table 5 Multivariable analysis (Cox regression) of overall and progression-free survival for clinical and biological variables. Models are stratified according to front-line therapy

	Overall survival			Progression-free survival		
	<i>P</i> *	HR	(95% CI)	<i>P</i> *	HR	(95% CI)
Age at diagnosis						
>55 vs ≤55	0.052	1.51	0.99–2.29	0.11	1.36	0.94–1.97
Stage						
III vs I–II	0.018	3.77	1.26–11.33	0.002	4.78	1.80–12.68
IV vs III	0.051	1.64	0.99–2.70	0.061	1.54	0.98–2.41
Histotype						
Serous versus others	0.53	0.85	0.51–1.41	0.21	0.74	0.47–1.18
Grade						
3+undiff versus 1+2	0.8	0.95	0.6–1.48	0.4	0.85	0.57–1.25
Surgical debulking						
mRD versus NED	0.034	2.57	1.07–6.13	0.02	2.65	1.21–5.83
GRD versus mRD	0.041	1.83	1.03–3.26	0.0002	2.84	1.65–4.9
FLIP expression*						
Positive versus negative	0.008	1.82	1.17–2.82	0.15	1.34	0.90–1.97
p53 nuclear accumulation						
Present versus absent	0.49	1.17	0.75–1.83	0.52	1.14	0.76–1.72

**P* value determined using log-rank test; HR, hazard ratio; CI, confidence interval; mRD, minimal residual disease; NED, not evident disease; GRD, gross residual disease.

factor for adverse outcome (HR=1.82, 95% CI =1.17–2.82; *P*=0.008), consistent with the pattern of association indicated by the MCA.

Discussion

The impairment of both intrinsic and extrinsic apoptotic pathways represents an efficient mechanism to promote chemoresistance (Fulda & Debatin 2006). Although several mechanisms of resistance to apoptosis have been defined *in vitro*, their relevance in patients frequently remains unclear. In the present study, expression of the anti-apoptotic molecule c-FLIP_L was related to survival of EOC patients, being associated in the multivariable Cox proportional-hazard regression model with poor prognosis, independently of the established clinical prognostic factors.

Our previous findings, indicating the relevance of c-FLIP_L expression in the ability of EOC cells to evade apoptosis *in vitro*, also suggested that c-FLIP_L might ultimately favor tumor cells survival when p53 mutation has not yet occurred (Mezzanzanica *et al.* 2004). Here, we demonstrated the previously suggested inverse relationship between c-FLIP_L expression and p53 nuclear accumulation in EOC and, except for a higher frequency in clear cells and mucinous histotypes, we found that, at variance of p53 nuclear accumulation, c-FLIP_L expression was not associated with EOC clinicopathological characteristics.

MCA analysis provided evidence of the different contribution given by the two markers in information explaining. Whereas relevance of p53 nuclear accumulation appeared to be more related to unfavorable clinicopathological variables, c-FLIP_L expression clearly defined two groups of patients independently from others, clinical characteristics and complementary to p53. The contribution of c-FLIP_L expression in identifying patients that do not yet display p53 nuclear accumulation supports a role for this molecule at the tumor onset, while p53 overexpression is apparently more related to later stages of the disease. Consistent with our finding, other authors (Horak *et al.* 2005, Ouellet *et al.* 2007), by studying deregulation of TRAIL cascade in EOC patients, identified c-FLIP_L mainly expressed in early-stage ovarian cancer further suggesting that this survival factor is expressed by tumor cells when other mechanisms of apoptosis resistance, like loss of p53 function, are not yet present.

Multivariable regression analysis correctly takes into account the association structure among the biological markers and the clinical/pathological features. Indeed, in agreement with MCA findings, c-FLIP_L expression became of relevance, while the prognostic impact of p53 was downsized when the effects of the main, well-established clinical and pathological factors were also considered.

Kurman & Shih (2008) have recently proposed a new model of ovarian carcinogenesis which, by taking into account the molecular, histopathologic, and

clinical evidences, stratifies EOC in type I and type II tumors. Dysfunctional p53 has been recognized as one of the main features of type II EOC (Kurman & Shih 2008). Consistently, in our study, the association of p53 nuclear accumulation with known unfavorable clinicopathological factors was evident in both MCA and multivariable Cox regression analyses. Our observations describing c-FLIP_L expression as decreased from well-differentiated to undifferentiated tumors, inversely related with p53 nuclear accumulation and more frequent in mucinous and clear cells histotypes, suggest that c-FLIP_L associates to type I EOC and, since its independent contribution, it might be included in the hallmarks of this tumor subset.

A general consensus regarding the prognostic or predictive impact of p53 alteration in EOC has not yet been reached. Despite the clear prognostic impact of p53 accumulation on both overall and progression-free survival in univariate analysis, we found no evidence that p53 nuclear accumulation behaves as an independent prognostic marker. Accordingly, Havrilesky et al. (2003) found that p53 overexpression in primary advanced-stage EOC was not associated with risk of death, regardless of adjustments for clinical characteristics. Although they found TP53 gene status associated with patients' outcome, a more recent study has determined that the p53 overexpression rather than mutation correlated with shortened OS (Bartel et al. 2008). In this context, stratification for c-FLIP_L expression and p53 nuclear accumulation indicated that the concurrent presence of both alterations was associated with shorter OS and early relapse. Our data are in substantial agreement with recent findings showing a downmodulation of c-FLIP_L expression in platinum-treated EOC cells by p53-dependent proteasomal degradation (Abedini et al. 2008). p53 nuclear accumulation, mostly associated with protein functional alteration, precluding an appropriate control of c-FLIP_L ubiquitination and proteasomal degradation, could represent an additional mechanism of apoptosis resistance mediated by c-FLIP_L expression.

Altogether these data, confirming the pivotal role of c-FLIP_L in promoting cell survival, further support the independent contribution of c-FLIP_L in refining the prognostic information obtained from other standard clinicopathological indicators.

Our study, directly implicating c-FLIP_L as an independent adverse prognostic factor, suggests that it might significantly improve the selection of a subgroup of patients at higher risk of death of disease who could benefit from more frequent follow-up or alternative therapeutic modalities. Furthermore, c-FLIP_L may be proposed as a possible alternative

therapeutic target (Wajant 2003, Geserick et al. 2008), since its inhibition might be relevant not only in receptor-dependent apoptosis but also in sensitizing cancer cells to conventional chemotherapy (Siervo-Sassi et al. 2003, Bagnoli et al. 2007).

Declaration of interest

Authors declare no potential conflicts of interest.

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