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# Gene expression analysis identifies two groups of ovarian high-grade serous carcinomas with different prognosis

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**Gene expression profiling is an important tool to evaluate genetic heterogeneity in carcinomas and is useful to develop expression-based classifications for many types of cancer, as well as markers of disease outcome. In this study, we have investigated the expression profile of 22 genes involved in the PI3K–AKT pathway in 26 high-grade ovarian carcinomas (19 serous and 7 clear cell carcinomas). Unsupervised hierarchical clustering divided high-grade ovarian carcinomas into three groups. Although all clear cell carcinomas clustered in one group, high-grade serous carcinomas were segregated into two separate groups with different prognosis ($P=0.05$). High expression of *CASP3, XIAP* (X-linked inhibitor of apoptosis) *, NFKB1*, *FAS*, and *GSK3B* mRNAs identified high-grade serous carcinomas with better prognosis. In multivariate analysis, these cluster groups were of prognostic significance independent of age, tumor size, and tumor stage ($P=0.008$). To validate the mRNA expression data, we studied the immunohistochemical expression of caspase-3 and XIAP on a tissue microarray. Immunoreaction for caspase-3 was concordant with the results obtained by mRNA expression analysis (Spearman $r=0.762$, $P=0.000$). Caspase-3 was exclusively expressed by the macrophages. Furthermore, co-expression of caspase-3 and XIAP identified high-grade serous carcinomas with different prognosis ($P=0.03$). Our results suggest that there are different biological subtypes of high-grade serous carcinomas.**
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**Keywords:** clustering analysis; high-grade serous carcinomas; outcome; PI3K–AKT pathway

Malignant surface epithelial tumors (carcinomas) are the most lethal gynaecological malignancies and the most frequent forms of ovarian cancer accounting for 90% of all malignant ovarian tumors.[1,2] Although they respond initially to platinum/taxane chemotherapy, most patients will subsequently experience recurrence.[3,4] Despite numerous investigations, new therapeutic approaches to reduce mortality have been unsuccessful and there is a need for a better understanding of the origin and pathogenesis of ovarian carcinomas.

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The phosphatidylinositol 3-kinase (PI3K)–AKT signaling pathway regulates the expression of several downstream target genes that inhibit apoptosis and promote cell proliferation.[5,6] The effect of such dysregulation on the clinical outcome of human tumors has recently become a subject of intense investigation. Activation of this pathway has been associated with aggressive phenotype and poor prognosis in brain tumors (glioblastoma and neuroblastoma), as well as various carcinomas including ovarian carcinomas.[7–13]

In an attempt to improve our knowledge on the pathogenesis of ovarian carcinomas, we have examined the expression profiling of 22 genes involved in the PI3K–AKT pathway in 26 high-grade ovarian carcinomas (19 serous and 7 clear cell carcinomas) by Taqman Low-Density Arrays. We analyzed the gene expression pattern by hierarchical clustering analysis and correlated the results with the clinicopathological features of the tumors including

outcome. From this gene set, we subsequently selected two markers for which commercial antibodies were available (caspase-3 and XIAP (X-linked inhibitor of apoptosis)) and investigated their immunohistochemical expression in 18 high-grade serous carcinomas.

## Materials and methods

### Selecting Tissue Samples

Samples from 26 high-grade ovarian carcinomas, including 19 serous and 7 clear cell carcinomas, were retrieved from the Tumor Bank and the Surgical Pathology files of Hospital de la Santa Creu i Sant Pau, Barcelona, Spain. All cases were reviewed and classified using the 2003 World Health Organization (WHO) criteria. All patients received neoadjuvant chemotherapy with taxane and cisplatinum or carboplatinum. We considered resistant tumors those from patients who had recurrent disease in less than 6 months after treatment. Cases were anonymized and the study was approved by the Institutional Ethics Committee.

### RNA Extraction and cDNA Synthesis

Total RNA from tumors and corresponding non-tumor tissues was extracted from fresh frozen biopsies using Trizol reagent (Invitrogen, Carlsbad, CA, USA) and the RNeasy mini kit (Qiagen, Hilden, Germany) as specified by the manufacturer's instructions. RNA was eluded in 25 $\mu$l of RNase-free water. RNA yield and quality were assessed by agarose electrophoresis and spectrophotometry and then stored at −80 °C. RNA was digested with DNase I (Invitrogen). A measure of 1 $\mu$g of total RNA was used for cDNA synthesis according to the protocol provided with the HighCapacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). Recombinant RNasin Ribonuclease Inhibitor (Applied Biosystems) was added to prevent RNase-mediated degradation. The cDNA was stored at −20 °C.

### Gene Expression Analyses

Gene expression analyses were performed at mRNA level by Taqman Low-Density Arrays. Pre-designed TaqMan probe and primer sets for target genes were chosen from an on-line catalog (Applied Biosystems). Once selected, the sets were factory loaded into the 384 wells of Taqman Low-Density Array cards. Each card was configured into eight identical 24 gene sets in duplicate. A total of 22 genes were chosen based on literature reviews of the PI3K–AKT, apoptosis, and cell-cycle molecular pathways and their involvement in carcinogenesis (Figure 1).[5,6,14] Each set of genes also contained two housekeeping genes, *GAPDH* and *ABL*. We mixed 5 $\mu$l of single-stranded cDNA (equivalent to 100 ng of total RNA) with 45 $\mu$l of nucleases-free water and 50 $\mu$l of TaqMan Universal PCR Master Mix. After gentle mixing and centrifugation, 100 $\mu$l of mixture was transferred into a loading port on a card. The card was centrifuged twice for 1 min at 1100 r.p.m. to distribute the samples from the loading port into each well. Then was sealed and placed in the Micro Fluidic Card Sample Block of an Applied Biosystems 7900HT PCR system (Applied Biosystems).

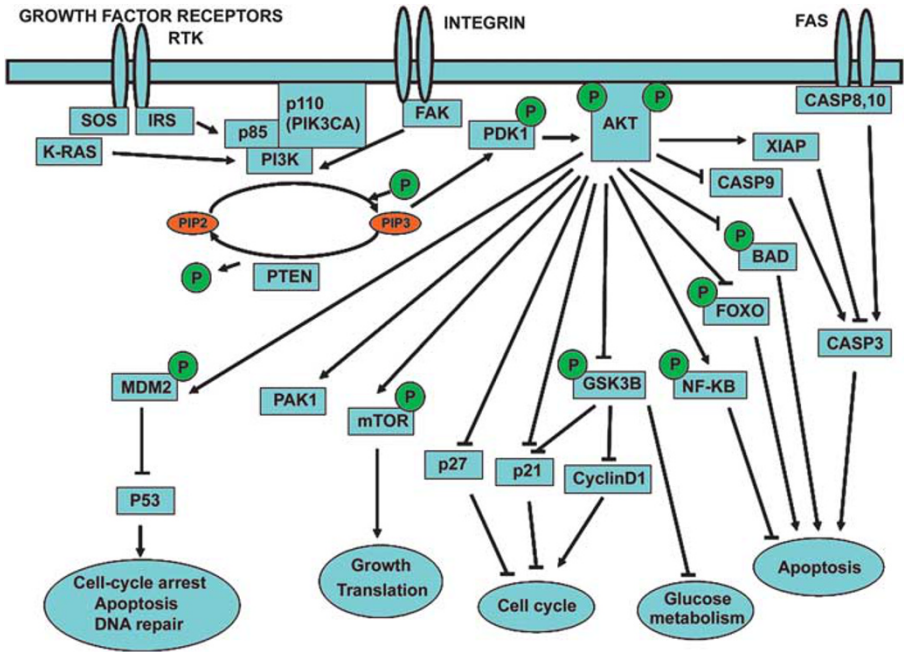


**Figure 1** Diagram of phosphatidylinositol 3-kinase (PI3K)–AKT signaling pathway.

**Table 1** Gene expression assays used for configure the Taqman Low-Density Array card

| *Gene symbols* | *Gene names* | *Ref seq* | *Assay ID* | *Amplicon lenght* |
|---|---|---|---|---|
| *XIAP (BIRC4)* | X-linked inhibitor of apoptosis | NM_001167.2 | Hs00236913_m1 | 116 |
| *AKT1* | v-Akt murine thymoma viral oncogene homolog 1 | NM_005163 | Hs00178289_m1 | 66 |
| *TWIST1* | Twist homolog 1 (Drosophila) | NM_000474.3 | Hs00361186_m1 | 115 |
| *BAD* | BCL2-associated agonist of cell death | NM_004322.2 | Hs00188930_m1 | 69 |
| *CDKN1A (p21)* | Cyclin-dependent kinase inhibitor 1A (p21, Cip1)) | NM_000389.2 | Hs00355782_m1 | 66 |
| *ABL1* | v-abl Abelson murine leukemia viral oncogene homolog 1 | NM_005157.3 | Hs00245443_m1 | Endogenous control |
| *CDH1* | Cadherin 1, type 1, E-cadherin | NM_004360.3 | Hs00170423_m1 | 117 |
| *TP53* | Tumor protein p53 | NM_000546 | Hs00153340_m1 | 81 |
| *CASP3* | Caspase 3, apoptosis-related cysteine peptidase | NM_004346.2 | Hs00263337_m1 | 111 |
| *PAK1* | p21/Cdc42/Rac1-activated kinase 1 | NM_002576.4 | Hs00176815_m1 | 93 |
| *GAPDH* | Glyceraldehyde-3-phosphate dehydrogenase | NM_002046.3 | Hs99999905_m1 | Endogenous control |
| *FAS* | TNF receptor superfamily, member 6 | NM_000043.3 | Hs00531110_m1 | 97 |
| *AKT2* | v-Akt murine thymoma viral oncogene homolog 2 | NM_001626.3 | Hs00609846_m1 | 129 |
| *PTK2 (FAK)* | PTK2 protein tyrosine kinase 2 | NM_005607.3 | Hs00178587_m1 | 68 |
| *PTEN* | Phosphatase and tensin homolog | NM_000314.4 | Hs00829813_s1 | 154 |
| *CCND1* | Cyclin D1 | NM_053056.2 | Hs00277039_m1 | 94 |
| *NFKB1* | Nuclear factor kappa light polypeptide gene enhancer B-cells 1 | NM_003998.2 | Hs00765730_m1 | 66 |
| *GSK3B* | Glycogen synthase kinase 3 beta | NM_002093.2 | Hs00275656_m1 | 73 |

Only genes with reproducible amplification curves of both duplicates are shown.

The thermal cycling conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. Expression levels were measured in duplicate. Only the genes with reproducible amplification curves of both duplicates were analyzed and presented (Table 1). Taqman Low-Density Array cards were analyzed with RQ Manager Software for automated data analysis. Gene expression values RQ were calculated based on the ΔΔCt method. Delta cycle threshold (Ct) values, defined as the point at which the fluorescence rises above the background fluorescence, were calculated with SDS 2.3 software (Applied Biosystems). Commercial frozen RNA from normal ovary was used (Stratagene, Agilent technologies, Santa Clara, CA, USA) as a calibrator and *ABL-1* housekeeping gene was the reference for normalization. Hierarchical clustering analysis of Taqman Low-Density Arrays data was performed using the *R* program from R Development Core Team (2009) (R Foundation for Statistical Computing, Vienna, Austria; ISBN 3-900051-07-0). Program can be downloaded at http://www.R-project.org. Clustered data were displayed with tumors on the vertical axis and genes on the horizontal axis. For validation, we made 22 comparisons between the cluster groups, one for each gene.

## Immunohistochemical Analysis

Caspase-3 and XIAP expression was evaluated by immunohistochemistry on tissue arrays constructed with a tissue arrayer device (Beecher Instruments, Sun Prairie, WI, USA). Slides were cut at 4 μm. Deparaffination and antigen retrieval were carried out in a PT Link module (Dako, Glostrup, Denmark) and immunohistochemistry was carried out in a Dako Autostainer (Dako). The primary antibodies used were cleaved caspase-3 (rabbit monoclonal 5A1E, 1/100, Cell Signalling Technology 9664, Danvers, MA, USA) and XIAP (mouse monoclonal 48/hILP/XIAP, 1/25, BD Biosciences 610762, San Jose, CA, USA). Antigen retrieval solution was Dako high pH. Visualization system used was EnVision + Flex (Dako). Detection was carried out with DAB/HRP and hematoxylin as a counterstain. Adequate immunoreactive tissue sample was used as positive control. Negative control was obtained by omission of the primary antibodies. The stained sections were semiquantitatively scored by two pathologists (IE, ED). Caspase-3 was evaluated counting the number of cells per core (1 mm) showing positive immunostaining. Caspase-3 was considered positive if more than 50 positive cells/mm were present. This cut-off was chosen because it was close to the median. XIAP immunostaining was scored as follows: 1 indicates the absence of any staining; 2 indicates any weak staining whether diffusely or focally present in the tumor; 3 indicates strong staining whether diffusely or focally present in the tumor.

## Statistical Analysis

The Kaplan–Meier method was used to estimate disease-free survival (DFS) distributions. DFS was calculated from the time of diagnosis to the date of the first recurrence. Multivariate Cox regression analysis was used to study the relationship between survival and the different variables. The following variables were introduced into the analysis: age, tumor size, tumor stage, and high-grade serous carcinoma groups identified by gene expression analysis. To create Cox regression models with the subset of features that showed the strongest association with survival, we used forward step-wise variable selection using likelihood ratio. *P* values

**Table 2** Clinicopathological features of 19 patients with high-grade serous carcinoma and 7 with clear cell carcinoma

| | *High-grade serous carcinoma* | *Clear cell carcinoma* | P-*value* |
|---|---|---|---|
| Total cases | 19 | 7 | |
| Age (years) | 60 (30–83) | 51 (27–64) | 0.1 |
| *Stages* | | | 0.04 |
| I | 2/19 (11%) | 4/7 (57%) | |
| II | 0/19 (0%) | 0/7 (0%) | |
| III | 12/19 (63%) | 2/7 (29%) | |
| IV | 5/19 (26%) | 1/7 (14%) | |
| Tumor size (cm) | 11 (3–22) | 16 (9–30) | 0.08 |
| *Outcome* | | | 0.66 |
| NED | 8/18 (44.5%) | 3/5 (60%) | |
| AWD | 2/18 (11%) | 1/5 (20%) | |
| DOD | 8/18 (44.5%) | 1/5 (20%) | |
| LFU | 1 | 2 | |
| *Survival* | | | |
| Mean follow-up (years) | 3.6 (0.8–7.4) | 4.6 (0.7–13.6) | |
| 5DFS Rate | 40% | 40% | 0.5 |
| *Responsive to chemotherapy* | | | 0.48 |
| Early stage (I) | 2/2 (100%) | 3/3 (100%) | |
| Advanced stage (II–IV) | 7/16 (44%) | 1/3 (33%) | |

Abbreviations: NED: no evidence of disease; AWD: alive with disease; DOD: dead of disease; LFU: Loss of follow-up; 5DFS: 5-year disease-free survival in percentage.

$\leq 0.05$ were considered significant. Statistical calculations were performed using SPSS software 18.0 (SPSS, Chicago, IL, USA).

## Results

### Clinicopathological Features

The clinicopathological features of the 19 patients with high-grade serous carcinoma and 7 patients with clear cell carcinoma are summarized in Table 2. Briefly, the average age of 19 patients with high-grade serous carcinoma was 60 (range, 30–83 years) and that for 7 patients with clear cell carcinoma was 51 years (range, 27–64 years). Although 57% of the clear cell carcinomas (4/7) were stage I, the corresponding rate for stage I high-grade serous carcinomas was only 11% (2/19). In all, 89% of high-grade serous carcinomas had extended beyond the pelvis (stages III and IV). Concordantly, at the end of the follow-up interval, almost 50% of the patients with high-grade serous carcinoma had died of tumor (8/18; 44%). In contrast, the corresponding rate for the patients with clear cell carcinoma was 1/5 (20%). In advanced stage tumors, 33% (1/3) of the patients with clear cell carcinoma and 44% (7/16) with high-grade serous carcinoma responded to chemotherapy.

### Gene Expression Analysis

By using unsupervised hierarchical clustering, tumors were grouped together according to the histological subtype (Figure 2a). All clear cell carcinomas clustered on group 1. In contrast, high-grade serous carcinomas were separated into two groups (group 2 and group 3).

We subsequently analyzed the expression data by a non-parametric test (Kruskall–Wallis test) to identify and rank order the genes that distinguish the two groups of high-grade serous carcinoma. First, we considered high-grade serous carcinoma cases as a group distinct from clear cell carcinomas (group 1), and then we analyzed genes that separated high-grade serous carcinomas (groups 2 and 3). The highest-ranking genes identified in these analyses are show in Table 3.

### Association of High-Grade Serous Carcinoma Clusters with Clinicopathological Parameters

A relationship between the two clustering subgroups of high-grade serous carcinoma and the prognostic clinicopathological parameters was investigated (Table 4). In advanced stage tumors, response to chemotherapy was better in group 3 than in group 2 high-grade serous carcinoma but it did not reach statistical significance (6/10 (60%) *vs* 1/6 (17%), $P=0.13$). Kaplan–Meier DFS analysis showed a poorer outcome in group 2 than in group 3 ($P=0.05$) (Figure 2b). In the multivariate analysis of various parameters including age, tumor size, stage, and high-grade serous carcinoma groups (group 2 *vs* 3), distribution either into group 2 or 3 was the feature most predictive of outcome ($P=0.008$).

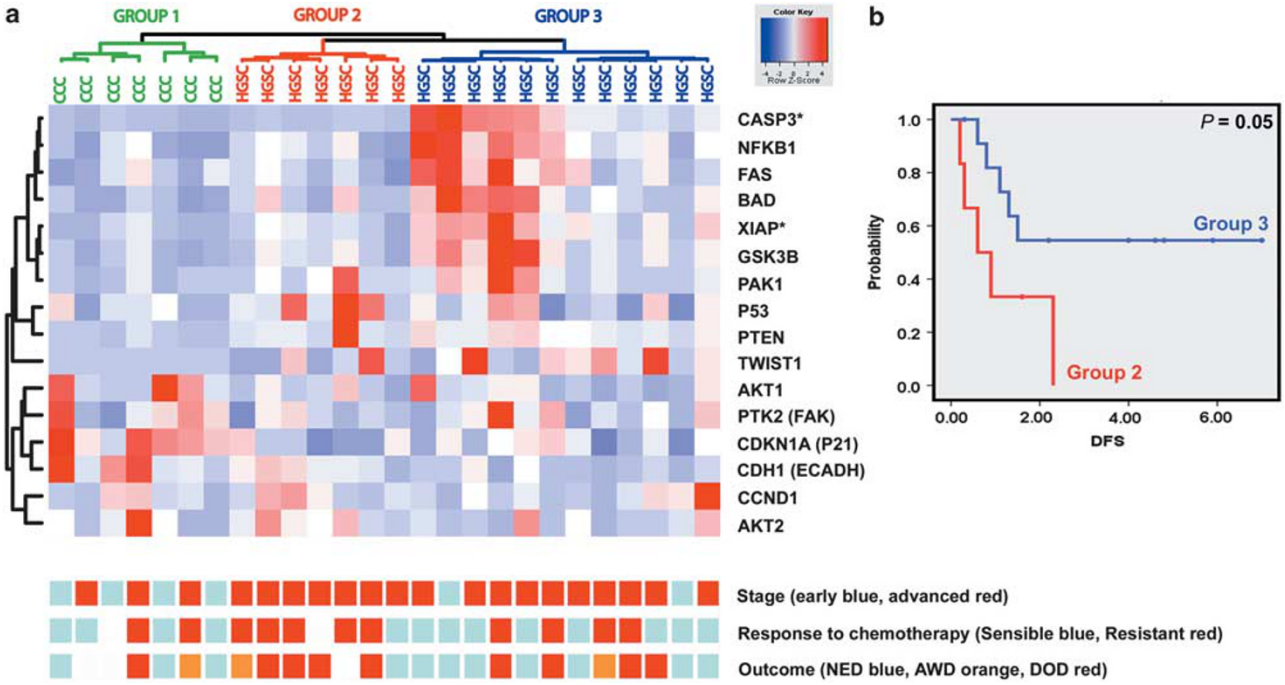


**Figure 2** (**a**) Expression profiling of 16 phosphatidylinositol 3-kinase (PI3K)–AKT pathway genes in 26 high-grade ovarian carcinomas. Enclosed in the clustering image, clinicopathological parameters such as stage, response to chemotherapy, and outcome are graphically represented for each case. CCC, clear cell carcinoma; HGSC, high-grade serous carcinoma. Asterisks identify the two markers selected for immunohistochemistry. (**b**) Kapplan–Meier analysis (disease-free survival) for the two high-grade serous carcinoma groups (groups 2 and 3) identified by gene expression analysis.

**Table 3** The highest-ranking genes identified in the Kruskall–Wallis test

| *Genes* | *Description* | *Fold change* | P-*value* |
|---|---|---|---|
| *(a) Highest differential expression in high-grade serous carcinomas* | | | |
| *PTEN* | Phosphatase and tensin homolog | 3.7 | 0.000 |
| *CASP3* | Caspase 3, apoptosis-related cysteine peptidase | 3.3 | 0.001 |
| *BAD* | BCL2-associated agonist of cell death | 2.7 | 0.003 |
| *PAK1* | p21 protein (Cdc42/Rac)-activated kinase 1 | 2.7 | 0.003 |
| *XIAP* | X-linked inhibitor of apoptosis | 2.4 | 0.005 |
| *GSK3B* | Glycogen synthase kinase 3 beta | 2.4 | 0.006 |
| *NFKB1* | Nuclear factor kappa-B DNA binding subunit | 2.3 | 0.008 |
| *(b) Highest differential expression in group 2 of high-grade serous carcinomas* | | | |
| *CDH1* | E-cadherin | 1.8 | 0.028 |
| *(c) Highest differential expression in group 3 of high-grade serous carcinomas* | | | |
| *CASP3* | Caspase 3, apoptosis-related cysteine peptidase | 3.0 | 0.000 |
| *XIAP* | X-linked inhibitor of apoptosis | 2.0 | 0.022 |
| *NFKB1* | Nuclear factor kappa-B DNA binding subunit | 2.0 | 0.022 |
| *FAS* | TNF receptor superfamily, member 6 | 2.0 | 0.022 |
| *GSK3B* | Glycogen synthase kinase 3 beta | 1.8 | 0.043 |

## Validation of Gene Array Findings

Using a tissue microarray, we tried to confirm by immunohistochemistry the expression profiles of two genes selected from the Kruskall–Wallis test on 18 of the 19 high-grade serous carcinomas. We tested caspase-3 and XIAP.

The apoptotic protein caspase-3 is highly expressed in macrophages, natural killer cells, and lymphocytes.[15] Immunohistochemical expression of caspase-3 protein was concordant with the results obtained by gene expression analysis (Spearman $r = 0.762$, $P = 0.000$). The immunostaining pattern was granular and cytoplasmic (Figure 3a). Most caspase-3-positive cells showed typical macrophage morphology with large cytoplasm and round or oval nuclei. Caspase-3 was not expressed by the epithelial tumor cells. Of 18 high-grade serous carcinoma cases, 8 (44%) immunoreacted for caspase-3.

**Table 4** Clinicopathological features of groups of high-grade serous carcinoma identified by gene expression analysis

| | *Group 2* | *Group 3* | P-*value* |
|---|---|---|---|
| Total cases | 7 | 12 | |
| Age (years) | 55 (30-67) | 64 (42-83) | 0.10 |
| *Stages* | | | 0.49 |
| I | 0 | 2 | |
| II | 0 | 0 | |
| III | 4 | 8 | |
| IV | 3 | 2 | |
| Tumor size (cm) | 11 (3-20) | 11 (3-22) | 0.95 |
| *Outcome* | | | 0.17 |
| NED | 1/6 (17%) | 7/12 (58%) | |
| AWD | 1/6 (17%) | 1/12 (8%) | |
| DOD | 4/6 (67%) | 4/12 (33%) | |
| LFU | 1 | — | |
| *Survival* | | | |
| Mean follow-up (years) | 3 (1.3-4.9) | 3.9 (0.8–7.4) | |
| 5DFS Rate | 0% | 55% | 0.05 |
| *Responsive to chemotherapy* | | | 0.13 |
| Early stage (I) | — | 2/2 (100%) | |
| Advanced stage (II–IV) | 1/6 (17%) | 6/10 (60%) | |

Abbreviations: NED: no evidence of disease; AWD: alive with disease; DOD: dead of disease; LFU: Loss of follow-up; 5DFS: 5-year disease-free survival in percentage.

XIAP interacts and inhibits processed caspase-3 and caspase-9 among other apoptotic markers.[16] It was expressed by epithelial tumor cells and the immunostaining pattern was membranous and cytoplasmic (Figure 3a). XIAP was expressed in nine (9/16; 56%) high-grade serous carcinoma cases (score 2: 5/9(56%), score 3: 4/9 (44%)).

Kapplan–Meier analysis for caspase-3 protein expression showed a significant association with favorable prognosis ($P=0.04$). No association with survival was found with expression of XIAP. Co-expression of caspase-3 and XIAP identified a distinctive group of tumors (7/16, 44%) with better 5-years DFS than those that did not co-express the two markers (60% *vs* 0%; $P=0.03$) (Figure 3b).

## Discussion

Ovarian carcinoma is the most common malignant tumor of the ovary. However, in spite of numerous investigations, the origin and pathogenesis of these tumors are poorly understood. PI3K–AKT pathway is frequently involved in the development of ovarian carcinomas.[10–12] Therefore, we hypothesized that expression profiling of genes involved in this pathway might help to better understand the pathogenesis of these tumors. Unsupervised hierarchical clustering separated clear cell carcinomas from high-grade serous carcinomas and revealed two groups of high-grade serous carcinoma with different prognosis. The gene-array analysis data were confirmed by immunohistochemistry using caspase-3 and XIAP antibodies in 18 high-grade serous carcinomas.

Epithelial ovarian cancer is not a single disease but rather a heterogeneous group of tumors that can be classified based on distinctive morphological and molecular genetic features. Different histological subtypes of ovarian carcinoma differ with respect to epidemiological and genetic risk factors, precursor lesions, patterns of spread, molecular events during oncogenesis, response to chemotherapy, and outcome. In our study, clear cell and high-grade serous carcinomas had a very distinct expression profile, even if there was overlap, which supports their different nature. High-grade serous carcinomas were associated with high expression levels of *PTEN, CASP3, BAD, PAK1, XIAP, GSK3B,* and *NFKB1*. In contrast, clear cell carcinoma showed low expression of these genes.

*PTEN* is a tumor suppressor gene located on chromosome 10q23.3. Inactivation of tumor suppressor genes secondary to allelic lose (LOH) is a common event in clear cell carcinomas. In fact, loss of *PTEN* expression has been noted in 40% of early-stage clear cell carcinomas, suggesting that *PTEN* inactivation may be an early event in the development of these tumors.[17] Furthermore, functional inactivation of *PTEN* has been also identified in endometriosis, the precursor lesion of clear cell carcinomas. In contrast, most undifferentiated and high-grade serous carcinomas show high expression of *PTEN* and higher level of chromosomal instability.[18,19] In fact, several studies have revealed frequent gene-copy number gains of *PAK1* at 11q13.5-q14 in high-grade serous carcinomas.[20] Moreover, we recently found that the proportion of high-grade serous carcinomas immunoreactive for PAK1 was higher than that of clear cell carcinomas.[21] More importantly, PAK1 activation has been implicated to contribute to tumor development.

*XIAP* is upregulated in various cancers and has an important role in tumor formation and invasion/metastases in both animal models and cancer patients.[22] *XIAP* has been shown to be one of the important regulators in cisplatin-induced apoptosis in ovarian cancer cells and its downregulation sensitizes cells to cisplatine.[23] A recent study showed that *XIAP* gene siRNA inhibited the proliferation of ovarian carcinoma cells and caused cells to be more sensitive to cisplatine suggesting that XIAP is a potential target for therapeutic anticancer drugs.[24]

BAD, GSK3B, and transcription factor NFKB1 are downstream substrates of AKT kinase. These molecules directly or indirectly regulate apoptosis and are involved in the chemotherapy resistance in cancer cells. Downregulation of BAD, GSK3B, and NFKB1 overcome resistance of human ovarian cancer cells to growth inhibition by cisplatin.[25]

The most recent studies suggest that the cell of origin of some or even most epithelial ovarian

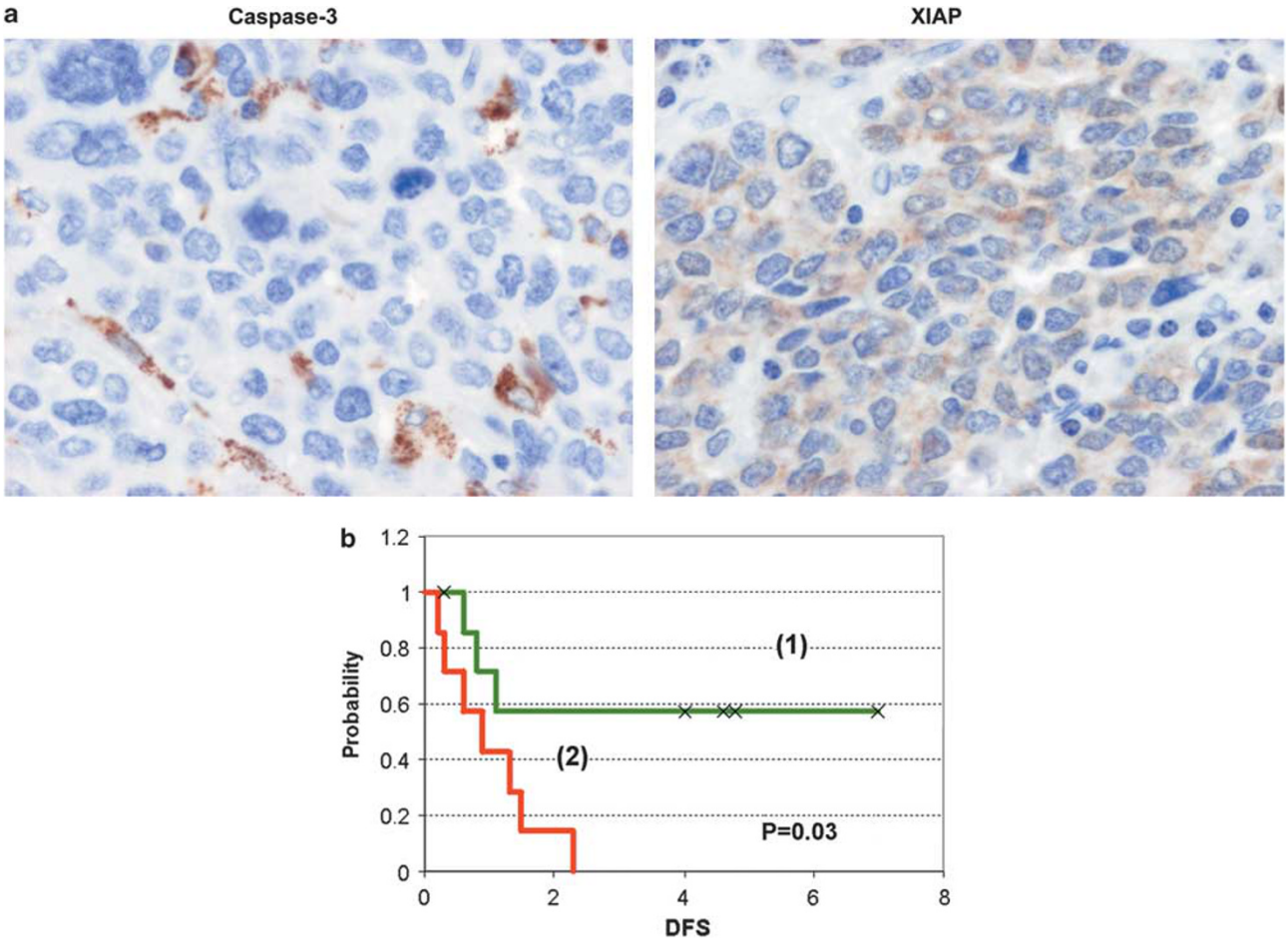


**Figure 3** (**a**) Caspase-3 and XIAP (X-linked inhibitor of apoptosis) immunoreactions of a representative case. (**b**) Kaplan–Meier analysis (disease-free survival); 1: high-grade serous carcinomas with co-expression of caspase-3 and XIAP; 2: other high-grade serous carcinomas.

cancer is not an ovarian cell. It has been stated that high-grade serous carcinomas may arise from the fallopian tube in a significant number of cases.[26–31] These tumors are designated, together with undifferentiated carcinomas and malignant mixed mesodermal tumors (carcinosarcomas), as 'type II' tumors.[32] In contrast to 'type I' tumors, these high-grade malignancies are highly aggressive, are found in advance stage at the time of diagnosis, and have high chromosomal instability. However, our findings indicate the existence of two different groups of high-grade serous carcinoma suggesting that this neoplasm encompasses a highly heterogeneous group of tumors and that, perhaps, limiting the carcinogenetic pathways to just 'type I or type II' tumors is artificial.

This study shows that clear-cut differences between the two high-grade serous carcinoma groups are based mainly on the expression of *CASP3*. Caspase-3 is considered to be the central protein in triggering apoptosis and has also been reported to be involved in drug resistance in human cancer cell lines.[15,33,34] Few data are available regarding the prognostic relevance of caspase-3 in patients with ovarian carcinoma. Materna *et al*[35] found that patients with postchemotherapy biopsies expressing active caspase-3 in less than 50% of tumor cells showed poorer prognosis than those with higher caspase-3 expression. Recently, Kleinberg *et al*[36] showed active caspase-3 is a prognostic factor in metastatic serous ovarian carcinoma. They found that high levels of cleaved caspase-3 were associated with improved survival. In high-grade serous carcinoma patients studied in the current report, high expression of cleaved caspase-3 was associated with good prognosis and showed a significant trend toward better response to chemotherapy.

An interesting observation that emerged from this study was the expression of active caspase-3 by the inflammatory cells from the stroma, mainly macrophages and lymphocytes. There is an increasing body of evidence that tumor microenvironment have an important role in tumor development and progression.[37] Lymphocytes and macrophages infiltrate ovarian tumors and are found in ascites in addition to a wide range of cytokines and chemokines.[38] Moreover, the presence of immune inflammatory cells has been reported to be associated with

prognosis in several types of carcinoma, including ovarian carcinoma.[39–41] Macrophage colony-stimulator 1 (CSF1) secreted by ovarian cancer cells and lymphocytes is a potent chemoattractant for macrophages. Activated macrophages produce interleukine-6, an interleukin associated with chemoresistace in several carcinomas, including ovarian carcinomas.[42,43] A recent study showed that chemoresistance caused by interleukine-6 is associated with activation of the PI3K–AKT pathway and downregulation of proteolytic activation of caspase-3 (see ref. 44).

In summary, hierarchical clustering analysis of genes involved in the PI3K–AKT pathway identified groups of high-grade serous carcinoma with different prognosis. Expression of active caspase-3 in the tumor stroma (lymphocytes and macrophages) was associated with good prognosis and response to chemotherapy. These findings offer insight into the pathogenesis of high-grade serous carcinoma and suggest that the measurement of active caspase-3 in the inflammatory cells, if confirmed on an independent and larger series of cases, could be useful in clinical practice to identify patients with the highest risk of poor outcome. Identification of these patients may aid clinicians in determining which patients require more aggressive treatment.

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## Disclosure/conflict of interest

The authors declare no conflict of interest.

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