The Zinc Finger Gene ZIC2 Has Features of an Oncogene and Its Overexpression Correlates Strongly with the Clinical Course of Epithelial Ovarian Cancer

Sergio Marchini1, Elizabeth Poynor3,4, Richard R. Barakat4, Luca Clivio1, Michela Cinquini1, Robert Fruscio2,5, Luca Porcu1, Cecilia Bussani7, Maurizio D’Incalci1,2, Eugenio Erba1, Michela Romano1, Giorgio Cattoretti6, Dionyssios Katsaros2,9, Andrew Koff10, and Lucio Luzzatto3,8

Abstract

Purpose: Epithelial ovarian tumors (EOT) are among the most lethal of malignancies in women. We have previously identified ZIC2 as expressed at a higher level in samples of a malignant form (MAL) of EOT than in samples of a form with low malignant potential (LMP). We have now investigated the role of ZIC2 in driving tumor growth and its association with clinical outcomes.

Experimental Design: ZIC2 expression levels were analyzed in two independent tumor tissue collections of LMP and MAL. In vitro experiments aimed to test the role of ZIC2 as a transforming gene. Cox models were used to correlate ZIC2 expression with clinical endpoints.

Results: ZIC2 expression was about 40-fold in terms of mRNA and about 17-fold in terms of protein in MAL (n = 193) versus LMP (n = 39) tumors. ZIC2 mRNA levels were high in MAL cell lines but undetectable in LMP cell lines. Overexpression of ZIC2 was localized to the nucleus. ZIC2 overexpression increases the growth rate and foci formation of NIH3T3 cells and stimulates anchorage-independent colony formation; downregulation of ZIC2 decreases the growth rate of MAL cell lines. Zinc finger domains 1 and 2 are required for transforming activity. In stage I MAL, ZIC2 expression was significantly associated with overall survival in both univariate (P = 0.046) and multivariate model (P = 0.049).

Conclusions: ZIC2, a transcription factor related to the sonic hedgehog pathway, is a strong discriminant between MAL and LMP tumors: it may be a major determinant of outcome of EOTs. Clin Cancer Res; 18(16); 4313–24. ©2012 AACR.

Introduction

Ovarian cancer is the gynecologic malignancy with the highest mortality (1); it comprises several types of tumors with different pathologic and clinical features (2, 3). Not surprisingly, karyotypic and molecular lesions reported in these tumors are also heterogeneous (4, 5) and to some extent they correlate with histologic types and grading (4, 6, 7). The majority of malignant ovarian tumors are of epithelial origin (epithelial ovarian tumor; EOT), and the majority of these are aggressive, with a tendency to metastasize in the peritoneal cavity and elsewhere: for brevity, we will refer to them as MAL. On the other hand, it has been recognized for a long time that some 10% to 20% of EOTs are confined to the ovaries at the time of diagnosis, grow more slowly, and have little tendency to metastasize (8): these have been designated as borderline tumors, or tumors with low malignant potential (LMP). In recent years, it has been abundantly confirmed that LMP tumors have a much better prognosis than MAL tumors (9).

At the time this work was initiated, GEP was used extensively in comparing a variety of tumors with "normal counterpart" tissue (10); however, many of the differences observed were related essentially to differences in growth rate. In the case of ovarian cancer, we thought that a more promising approach than comparing MAL with normal ovarian epithelium would be comparing MAL with LMP because this comparison might bring to the fore selectively those differences that confer to MAL tumors the invasive
ZIC encode zinc finger transcription factors. The homology to the drosophila genes which belongs to a family of 5 genes originally defined by MAL versus LMP. One of the highest-ranking was ZIC2, revealed 61 genes with significantly higher expression in a doctoral thesis (11), a quantitative profile study in mice and humans, heterozygous deletions or other mutations in the establishment of left–right asymmetry and in the development of the central nervous system (12). In both mice and humans, heterogeneous deletions or other mutations of the ZIC2 gene result in severe brain malformation (13). We focused on ZIC2 because other transcription factors important in development are known to play a major role in embryonic development, particularly in the establishment of left–right asymmetry and in the development of the central nervous system (12). In both mice and humans, heterozygous deletions or other mutations of the ZIC2 gene result in severe brain malformation (13). We focused on ZIC2 because other transcription factors important in development are known to play a major role in oncosogenesis. In the normal adult, ZIC2 mRNA has been found only in brain and testis, but it is now known that ZIC2 is also expressed in a high proportion of several types of tumors (14, 15), including endometrial cancer (16).

In this article, we report that the overexpression of ZIC2 in MAL compared with LMP tumors has been confirmed independently in 2 large sets of archival material: in fact, the mean ratio in expression level is greater than 30-fold. At the same time, we show by in vitro studies that ZIC2, when overexpressed, behaves as a transforming oncogene; and that the level of overexpression of ZIC2 correlates with clinical outcome in patients with MAL.

Translational Relevance
In the manuscript, we investigate the molecular basis for the remarkable difference in clinical course between the invasive form of epithelial ovarian cancer (malignant; MAL) and that of the so-called low malignant potential (LMP) or borderline tumors. By gene expression profiling (GEP), we have discovered a markedly increased expression of the transcription factor ZIC2 in MAL versus LMP; and this has been validated quantitatively and statistically in two separate sets of archival material (totaling 232 tumors): the mean ZIC2 mRNA value is about 40 times higher in MAL tumors. Overexpression of ZIC2 was also documented at the protein level, and whenever ZIC2 is overexpressed, it is selectively localized to the nucleus. We have further shown by transformation assays and by siRNA experiments that overexpressed ZIC2 has features of an oncogene and that this depends on the integrity of two of the zinc finger domains. We have then gone back full circle to our original work and have found that in patients with MAL tumors, ZIC2 expression levels correlate significantly with early mortality in both univariate and multivariate models. ZIC2 is of special interest because it is expressed in early embryonic development and it is related to the GLI genes in the sonic hedgehog pathway: from our work, it now appears that ZIC2 is also a key factor in the biology of ovarian cancer.

Materials and Methods
Cell lines
The HOSE cell lines (gift from J. Boyd), the LMP cell line ML46 (gift from L. Dubeau), and all the other ovarian cancer cell lines analyzed (Ovcar-8, Igrov, Skov-3, A2780, Ovcar-432, Ovcar-420, Ovcar-433, Ovcar-5, Caov-2, and Ovcar-3) were routinely cultured in dedicated fresh medium with fetal calf serum (FCS) at 37°C in a 5% CO2 incubator. The SBOT3.1 cells (gift from Dr. Auerperg) were grown as reported (17).

Tumor sample collection
A total of 232 samples of EOT, staged according to the International Federation of Gynaecological and Obstetrics criteria (FIGO; ref. 18), were obtained from 2 separate tumor tissue collections:
i. A total of 170 biopsies were part of 1,300 samples stored in a frozen tissue bank collection between September 1992 and March 2005 and available at Department of Oncology, “Mario Negri” Institute, Milano, Italy. Tumor tissue was collected from patients undergoing surgery for EOC at the Obstetrics and Gynecology Department, San Gerardo Hospital (Monza, Italy), as described (19).

Samples were collected in the operating theatre from patients undergoing cytoreductive surgery, frozen within 15 minutes in liquid nitrogen, and stored at −80°C. The tumor content of the specimens was assessed by hematoxylin and eosin stain in the Pathology Units. Only specimens containing more than 70% of tumor were used. Patients’ clinical and anatomopathologic information were registered. Histology, grade, and stage of each tumor are listed in Supplementary Table SI. The collection and use of tumor samples was approved by the local scientific ethical committee, and written informed consent was obtained from the patients.

Real-time quantitative reverse transcriptase PCR and data analysis
Total RNA was purified using RNeasy Plus Mini Kit according to manufacturer’s instructions (Qiagen). Absolute copy numbers were determined by quantitative real-time reverse transcriptase PCR (qRT-PCR; ABI-7900) with SYBR Green protocol (Qiagen) using methods and protocols previously standardized (20). Primer pair sequences are reported in Supplementary Table SII. Statistical analysis was carried out using the R 2.1.0 software as described (20). For gene expression experiments, statistical comparisons between median values were conducted using Wilcoxon t test. Differences were considered statistically significant with a 2-sided P < 0.05. Data are presented as median with
interquartile range (IQR, 25%–75% quartile). All tests and data plots were done using GraphPad Prism Version 5.01 (GraphPad Software).

**Western blot analysis**

Total proteins were isolated through standard methods, separated through 10% SDS-PAGE and electrotransferred to nitrocellulose filters. For cytoplasmic and nuclear fractions, whole cells were prepared in buffer A [10 mmol/L HEPES, pH 7.9, 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L phenylmethylsulfonylfluoride (PMSF)] containing protease inhibitors (Roche). Nuclear extracts were obtained using buffer C [20 mmol/L HEPES, pH 7.9, 0.4 mol/L NaCl, 1 mmol/L EDTA, 1 mmol/L EGTA, 1 mmol/L DTT, 0.5 mmol/L PMSF].

Immunoblotting was carried out with ZIC2 monoclonal antibodies (Zymed and Abcam); glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Abcam), laminin (B-2, Santa Cruz Biotechnology), or β-actin were used as loading controls (sc-130656, Santa Cruz). H2B (N-20, Santa Cruz Biotechnology) was used for loading control of nuclear fraction only. Antibody binding was revealed by peroxidase-labeled secondary antibodies and visualized using enhanced chemiluminescence (Amersham).

**ZIC2 constructs**

The human full-length ZIC2 cDNA sequence was provided by A. Gure (Ludwig Institute, New York Presbyterian Medical Center, NY) and cloned into the pWZL/hygro/mycin retroviral vector or into the lentiviral pLENTI5/6 vector (Invitrogen). The Phoenix packaging cell line was transfected with the pWZLZIC2 construct and vector alone. The 293 packaging cell line (Invitrogen) was transfected with the pWZLZIC2 construct and vector. The 293 packaging cell line (Invitrogen) was transfection with pLENTI-ZIC2 construct and control construct pLENTI + LACZ (Invitrogen). Lipofectamine 2000 (Invitrogen) was used for all transfection experiments. After 48 hours, supernatants of packaging cell lines were collected and applied onto NIH3T3 cell monolayers, along with 5 mg/mL polybrene (Sigma-Aldrich). Pools of stably transfected cells were isolated through selection with the appropriate antibiotic, and ZIC2 protein overexpression was confirmed with ZIC2 immunoblots. Cells were plated in triplicate in 6-well plates at a density of 2.5 × 10^5 cells per 35-mm plate, along with pools of 1 × 10^3 cells stably transfected with pLENTI-LACZ, pLENTI-ZIC2, or pBABE rasV12. Cells were grown for 21 days at 37°C in a 5% CO₂ incubator. Foci were visualized by Giemsa staining. For assays of anchorage-independent growth, 35-mm plastic dishes were first coated with an underlayer of Dulbecco’s Modified Eagle’s Media (DMEM) + 10% FCS containing 0.5% agar. Cells from pools of the stably transfected cell lines pLENTI-LACZ, pLENTI-ZIC2, or pBABE rasV12 were suspended in DMEM + 10% FCS containing 0.35% agar and plated on the bottom layer of agar at a density of either 1 × 10^5 cells or 1 × 10^4 cells per 35-mm dish. After growth for 14 days at 37°C in 5% CO₂, colonies were visualized either by bright-field microscopy or after staining with crystal violet.

**Statistical analysis**

**Transformation assay**

For foci assays, mouse embryo fibroblasts (MEF) were mixed at a density of 1 × 10^5 cells per 35-mm plate, along with pools of 1 × 10^3 cells stably transfected with pLENTI-LACZ, pLENTI-ZIC2, or pBABE rasV12. Cells were grown for 21 days at 37°C in a 5% CO₂ incubator. Foci were visualized by Giemsa staining. For assays of anchorage-independent growth, 35-mm plastic dishes were first coated with an underlayer of Dulbecco’s Modified Eagle’s Media (DMEM) + 10% FCS containing 0.5% agar. Cells from pools of the stably transfected cell lines pLENTI-LACZ, pLENTI-ZIC2, or pBABE rasV12 were suspended in DMEM + 10% FCS containing 0.35% agar and plated on the bottom layer of agar at a density of either 1 × 10^5 cells or 1 × 10^4 cells per 35-mm dish. After growth for 14 days at 37°C in 5% CO₂, colonies were visualized either by bright-field microscopy or after staining with crystal violet.

**Statistical analysis**

Tumor tissue collection A and B were analyzed separately. Categorical variables were statistically described using absolute and relative frequencies; continuous variables were statistically described using median as measure of central tendency and range or IQR as measure of dispersion. We assessed the statistical association between clinical and histopathologic characteristics and ZIC2 expression with nonparametric tests building univariate linear regression model and reporting t-test value and P value (after checking for normality); whether more than one clinical features resulted statistically associated with gene expression multivariable model were built. Correlations between ZIC2 value and known prognostic variables (substage, grading, histology) were described with relative and absolute frequencies and analyzed with the χ² test for association or trend (21).

Prognostic was evaluated using the following time-to-event endpoints: overall survival (OS) was defined as the time from diagnosis to death from any cause; progression-free survival (PFS) was defined as the time from diagnosis to tumor progression or death from any cause; survivor function were estimated using the Kaplan–Meier method; the log-rank test was used to assess the statistical differences in survival among compared groups; the Conatal and O’Quigley method was used to categorize patients into high- or low-risk groups for OS and PFS endpoints based on the ZIC2 expression level and to assess the statistical significance of the cutoff point identified (21); and the Cox proportional hazards model was used to estimate HR. All tests were 2-sided, and a P < 0.05 was considered statistically significant. Statistical analysis was conducted using SAS software (version 9.1; SAS Institute, Inc.).
Results

**ZIC2 is overexpressed in MAL versus LMP ovarian tumors and cell lines**

GEP was originally carried out on Research Genetics GeneFilters by hybridizing tumor cDNA to 31,203 genes and expression sequence tags. From pairwise comparison of 3 MAL and 3 LMP tumors, it was shown that gene expression profiles were significantly more similar within the MAL set and within the LMP set than when the 2 sets were compared (10, 22). Among 61 genes with higher expression in MAL versus LMP, ZIC2 stood out on grounds of its biologic role, and its overexpression in MAL was confirmed by testing 10 additional samples (22).

We next proceeded to validate these preliminary data on large tumor tissue collections, and this was done on 2 separate sets of tumors (Fig. 1): collection A consisted of 170 tumors and collection B consisted of 62 tumors (23). The clinical and histopathologic features of these 2 sets of tumors are reported in Supplementary Table SI.

Levels of ZIC2 mRNA measured by qRT-PCR were markedly different in LMP and in MAL samples (Fig. 2A and B and Supplementary Table SIII). Indeed, the median value of ZIC2 mRNA was 36.5 times higher in MAL tumors than in LMP tumors in set A (P = 0.001) and 41.7 times higher in set B (P = 0.008). In set A, we were able to stratify patients according to their FIGO stage; we found that ZIC2 overexpression was 13.8 higher in stage III tumors than in stage I tumors (P < 0.001; Fig. 2C and Supplementary Table SIII). Western blot analysis, conducted in a subset of 32 MAL and 18 LMP tumors, revealed that ZIC2 protein levels were again much higher (about 17-fold) in MAL than in LMP tumors (Fig. 2E and F and Supplementary Table SIII), mirroring the data that we have found at the mRNA level.

In 6 MAL and 2 LMP tumors, we carried out immunoblot analysis on nuclear and cytoplasmic fractions separately: we found that whenever ZIC2 is expressed, the localization of the protein is strictly nuclear (see Fig. 2G).

In cell lines from normal ovarian epithelium (HOSE 1, HOSE 2), ZIC2 mRNA was undetectable (Fig. 2D). In contrast, ZIC2 is expressed in a panel of 10 cell lines that have been established from MAL ovarian cancers: the level of expression is greatly variable over a span of several logs, with Skov-3 having the highest level and A2780 the lowest level; Igrov, Ovar-8, Ovar-5, and Caov-2 were intermediate (Fig. 2D). Interestingly, in the cell lines SBOT3.1 and ML46, to our knowledge, the only 2 cell lines established from LMP tumors, ZIC2 mRNA was undetectable (Fig. 2D).

These data mirror those obtained in our tumor collections, and they support the notion that ZIC2 may be highly relevant to the biology of EOT, as its pattern of expression appears to be preserved in the transition from primary tumors to cell lines. We do not yet know the mechanism underlying ZIC2 overexpression in MAL tumors and in cell lines derived from these tumors. In 10 tumor samples, we have tested that there was no correlation between ZIC2 mRNA levels and the methylation of C residues within the ZIC2 promoter region (data not shown).

Overall, these data fully confirm, in 2 independent tumor tissue collections, the initial evidence that ZIC2 overexpression discriminates strongly between MAL and LMP tumors.

**Overexpression of the ZIC2 protein causes increased proliferation of NIH3T3 cells**

To define whether ZIC2 overexpression in MAL tumors is an agent of malignant transformation, we proceeded to test this directly in a standard assay system. NIH3T3 mouse fibroblasts were transduced with lentiviral vectors containing the human ZIC2 cDNA; as control, we used a vector containing the LACZ gene. After 2 to 3 passages, ZIC2-transduced cells overexpressed ZIC2 and had a higher proliferation rate than control cells (Supplementary Fig. S1A); after 6 days, the number of cells was more than double that in the control culture, and the fraction of
Higher in bromodeoxyuridine (BrdUrd)-positive S-phase cells was ZIC2 had intermediate expression (Ovcar-8 and Caov-2). Inhibition of ZIC2 decreases the growth of ovarian cancer cell lines

NIH3T3 are an assay system quite remote from EOT. To test more directly the impact of ZIC2 on the growth of ovarian cancer cells, we selected 2 of the cell lines in which ZIC2 had intermediate expression (Ovcar-8 and Caov-2). Three duplex 21-mer synthetic oligonucleotides (siRNA) directed against the untranslated portion of the ZIC2 mRNA were transfected into these cells; a scrambled siRNA was used as control. On the basis of immunoblot analysis, at 48 hours posttransfection, all 3 siRNA molecules (siRNAa and siRNAc more than siRNAb) effectively decreased ZIC2 protein levels in Ovcar-8 cells; correspondingly, their proliferation was markedly impaired, particularly with siRNA C (Supplementary Fig. S1D). Similar results were obtained with the Caov-2 cell line (Supplementary Fig. S1E). We infer
that in both of these 2 different tumor cell lines, ZIC2 is a major determinant of the rate of growth.

Overexpression of ZIC2 induces transformed foci and anchorage-independent growth

Having determined that ZIC2-transduced cells have increased growth, whereas ZIC2 inhibition inhibits cell growth, we proceeded to test directly whether ZIC2 is a transforming gene. MEFs stably transfected with a ZIC2 vector or with a LACZ control vector were tested for their ability to form foci in monolayer cultures. After 2 to 3 passages, there was a 6-fold increase in foci from ZIC2-transduced cells. A similar number of foci was obtained from rasV12-transduced cells (RAS) used as a positive control, although ZIC2 foci were generally smaller than RAS foci (Fig. 3A and B). The transforming activity of ZIC2 was further tested in NIH3T3 cells in terms of the ability of ZIC2 to induce anchorage-independent growth in soft agar (Fig. 3C). The number of colonies in soft agar was approximately 3-fold higher for ZIC2-transduced cells than for our negative control (LACZ); and it was about half compared with the number of colonies obtained with RAS-transfected cells (Fig. 3C). Again, ZIC2 colonies were smaller than RAS colonies. The same result was obtained by plating cells at different densities (data not shown). Overall, the data show that overexpression of ZIC2 produces cellular transformation in vitro.

Zinc fingers 1 and 2 are required for the transforming activity of ZIC2

The zinc fingers of ZIC2 bind DNA and are involved in the ZIC/GLI interaction (24); therefore, one might surmise they are important for the transforming activity of the protein. Some of the ZIC2 mutations causing holoprosencephaly (HPE) have been identified in zinc finger domains (or disrupting them as a result of frameshifts), whereas others are in the region of ZIC2-encoding polyalanine tracts (see OMIM 603073). Therefore, we proceeded to prepare constructs with an expanded polyalanine tract (M-1) and constructs in which one or more individual zinc finger elements had been deleted or the nuclear localization signal had been disrupted (M2-M7; Supplementary Fig. S2A). After cloning these mutant constructs into pLENTIV5/6 and transduction into NIH3T3 cells (Supplementary Fig. S2B), both foci and colonies in soft agar were counted. All constructs retained transforming activity except M7, in which both zinc fingers 1 and 2 had been deleted (Supplementary Fig. S2C and S2D).

Correlation of ZIC2 expression levels with parameters of clinical outcome

Because the clinical course of LMP is characteristically favorable compared with that of MAL, it is not surprising that we found a marked difference in both OS and PFS between these 2 groups of tumors (Supplementary Fig. S3). A further question is whether within the set of MAL tumors, ZIC2 expression levels correlated with the clinical course of the disease. When we plot ZIC2 levels against time to death or time to progression, several points emerge (Fig. 4). First, ZIC2 mRNA levels are widely spread in stage I, whereas stage III patients have very high ZIC2 levels in nearly all cases. Second, as the proportion of the serous histotype was much higher in the stage I group than in the stage III group (Supplementary Table S1), we wondered whether the above difference was due to this fact, clearly it is not. However, there is a statistically significant association of ZIC2 expression with clinical histotype: within stage I, between clear cells and mucinous ($P = 0.032$; Supplementary Table SIV); in stage III between serous and endometroid ($P = 0.048$; Supplementary Table SIV). Third, with respect to both OS and PFS there is a clear trend: the patients with the highest levels of ZIC2 are clustered where survival time and time to
progression are lowest, and this is true for both stage I and stage III patients.

Next, we analyzed the data in a way that lends itself to rigorous statistical analysis. We considered in a Cox model using univariate analysis, $ZIC2$ mRNA absolute copy number, grading, substage, histotype, chemotherapy, and age at diagnosis with respect to clinical variables OS and PFS. By using $ZIC2$ level as a continuous variable (see Materials and Methods), we found that stage I patients who expressed high levels of $ZIC2$ had a significant lower OS than did patients with low $ZIC2$ expression levels [$HR, 1.062; 95\%$ confidence interval (CI), 1.001–1.127; $P = 0.046$; Table 1, stage I; for PFS the difference was not statistically significant]; and in stage III patients, the association was not significant for either OS or PFS (Table 1, stage III). All other baseline characteristics analyzed, along with chemotherapy, correlated with OS endpoint (i.e., stage I patients with clear cells histotype and grade III had a worst OS than patients with other characteristics; Table 1, stage I). We next sought a statistically significant cutoff point that would maximally discriminate prognosis (Table 1, $ZIC2$ expression levels). Kaplan-Meier curves (Fig. 5) built for stage I patients show clearly that patients with $ZIC2$ expression levels higher than 55 had a shorter OS and PFS than patients with levels lower than 55: these differences were

---

Figure 4. $ZIC2$ expression correlates with measures of clinically important endpoints. Each symbol represents an individual patient: squares are for serous histotype, triangles for all other histotypes. Full symbols are for those patients who experienced fatal outcome or relapse; empty symbols are those who were alive and censored (or relapse-free and censored) at the times indicated. $ZIC2$ mRNA levels are $10^5$ multiplied.

---

Oncogenic Role of $ZIC2$ in Epithelial Ovarian Cancer

www.aacrjournals.org Clin Cancer Res; 18(16) August 15, 2012

Published OnlineFirst June 25, 2012; DOI: 10.1158/1078-0432.CCR-12-0037
statistically significant ($P = 0.014$ for OS, $P = 0.01$ for PFS; Table 1, ZIC2 expression levels). Specifically, this analysis shows a 5-year OS rate of 96% versus 75% for patients with ZIC2 expression levels higher and lower than 55, respectively (Fig. 5). A multivariate Cox proportional hazards model was fitted to test the effect of ZIC2 expression levels on survival, taking into account the effect of the previously analyzed demographic characteristics and clinical features. In stage I cohort of patients from tumor tissue collection A, ZIC2 maintained its significance as an independent prognostic factor for OS but not for PFS ($P = 0.049$; Table 1, stage I).
Discussion

GEP reveals ZIC2 overexpression as a discriminant between MAL versus LMP ovarian tumors

GEP has been used extensively to investigate differences between tumor cells and their putative cell of origin (25, 26). In general, these differences are so marked that they prove difficult to pinpoint which ones may be important in oncogenesis. In the case of EOT, however, we can take advantage of a rather unique situation, whereby the MAL and LMP tumors, although similar in histopathology and in other respects, have remarkably different clinical behavior, as their respective designations indicate (27). When our work started (10), there were no data on GEP studies in EOTs; since then, 3 studies have specifically addressed the comparison of MAL and LMP tumors (28, 29). Gilks and colleagues (29) reported that several genes expressed in MAL tumors and regarded as important in ovarian carcinogenesis were, somewhat paradoxically, even more highly expressed in LMP tumors. Meinhild-Heerlein and colleagues (30) observed that overall, the GEP of LMP tumors was significantly different from that of MAL tumors of high histologic grading (II or III); but virtually undistinguishable from that of grade I MAL tumors. Marchini and colleagues (19) confirmed the latter finding. At the same time, it was shown that, within MAL tumors, the different histologic types clustered separately from each other. In this study, we found that within the MAL group and within the LMP group, gene expression profiles were more consistent than between the 2 groups and genes with markedly different expression were relatively few (22). The overexpression of ZIC2 in MAL versus LMP was originally uncovered through an analysis of just 3 pairs of tumors but, remarkably, it was fully validated in 2 separate much larger sets of tumors (232 in total) in a different laboratory, and the extent of overexpression proved impressive (see Fig. 2A–C). One might assume that increased ZIC2 mRNA will likely entail an increase in ZIC2 protein and this is indeed the case (see Fig. 2F). This result was further strengthened by our findings on cell lines. Although these do not necessarily retain all the characteristics of primary tumors we observed that, strikingly, the 2 cell lines originating from LMP tumors were the only ones (along with the ovarian epithelium nontumor cell lines), in which ZIC2 mRNA was altogether undetected, despite of the high sensitivity of the RT-PCR technique used. These findings are consistent with the notion that ZIC2 overexpression may be a crucial determinant of the malignancy of ovarian cancer.

The ZIC gene family

ZIC2 is now known to be overexpressed in several cases of brain tumors (14, 15, 31) and in other tumors (16), and it has been noted recently in a GEP study of oral cancer (32). Interestingly, antibodies against the ZIC2 protein have been shown in 5 of 17 patients with small cell lung cancer. Members of the ZIC family of zinc finger transcription factors play a major role in early embryonic development. Although the 5 ZIC genes are highly homologous, they must fulfill different roles, as spontaneous mutations in humans and targeted inactivation of individual ZIC genes in mice

![Figure 5. Correlations between ZIC2 expression and measures of clinical outcome are statistically validated. Kaplan–Meier curves of OS and PFS for all MAL tumors in collection A (left, stage I; right, stage III), Samples were divided into 2 clusters on the basis of ZIC2 expression levels measured by qRT-PCR, as detailed in the Materials and Methods. P values are from log-rank test.](Image)
give distinct pathologies. For instance, ZIC1 inactivation causes abnormalities of the cerebellum and of the axial skeleton (33). ZIC2 mutations cause HPE, a congenital malformation of the forebrain due to a transient defect in the organizer region during a stage of development as early as gastrulation: indeed, ZIC2 is expressed already in pluripotent cells in the blastocyst (34). ZIC3 mutations affect left–right asymmetry and can cause congenital heart abnormalities (35). ZIC proteins have significant sequence homology to GLI proteins, transcription factors that are crucial downstream effectors of the sonic hedgehog (SHH) pathway. There is evidence that this pathway may be upregulated in ovarian cancer compared with the ovarian epithelium (36). The relationship between the ZIC set and the GLI set of proteins is remarkable because the DNA elements to which they bind are almost identical (37). In addition, GLI1, GLI2, and GLI3 interact with ZIC1, ZIC2, and ZIC3; specifically, zinc fingers 3 to 5 of ZIC1 have been shown to bind to zinc fingers 3 to 5 of GLI1 (38). At the functional level, ZIC proteins can act either as coactivators or as repressors of GLI-mediated transactivation and this modulating action depends on cell types (37). Such cell-specific differences may be mediated by interactions with DNA-PK, Ku70/80, and PARP proteins (39). Mutations in genes involved in early embryonic development have been well characterized in the case of leukemia, for instance, the HOX and RUNT genes (40). In a very broad sense, it is not surprising that transcription factors with features of master genes, when expressed out of context, can cause uncontrolled cell growth.

**Overexpressed ZIC2 behaves as an oncogene**

By using classic transformation assays—the formation of foci and growth of colonies in soft agar—we found that ZIC2 overexpression is oncogenic-like; conversely, ZIC2 inhibition by siRNAs inhibits proliferation of ovarian cancer cell lines. Thus, expression studies and functional studies converge in suggesting that ZIC2 may play a major role in determining the aggressive behavior of ovarian cancer. That the mechanism of action of ZIC2 in causing invasive growth is mediated by its transcriptional function is corroborated by our finding that transforming activity is abolished by deletion of zinc fingers 1 and 2. However, at the moment, we do not know what causes ZIC2 overexpression. Because it has been shown that in neural progenitor cells, p53 inhibits the transcriptional activity of GLI1 and GLI2 and conversely loss of p53 activates GLI1 (41), we wondered whether a similar circuit might be relevant to ZIC2 overexpression in EOTs. Of 45 stage III tumors that were tested, 27 (60%) had p53 mutations (20): there was no significant difference in the distribution of ZIC2 levels between these tumors and the 18 in which no p53 mutation was found. From these data, it would appear that the main mechanism of ZIC2 overexpression may be p53-independent; and this has been observed also with respect to regulation of Nanog by GLI1 and GLI2 (42) and with respect to regulation of GLI in melanoma cells (Stecca, personal communication). In zebrafish, ZIC2 is regulated by FOXD5 (43). Considering the close relationship between ZIC and GLI genes, it is tempting to surmise that ZIC2 may act through the SHH pathway because much evidence suggests that in some adult tissues, this pathway controls growth and tissue patterning by regulating the stem cell compartment (24). Recent work by Chan and colleagues strongly supports this notion: They have shown that in cervical cancer cells, overexpressed ZIC2 causes nuclear retention of GLI1 and thus enhances SHH signaling (44).

That the SHH pathway is disturbed in a variety of tumors [particularly in basal cell carcinoma (BCC); ref. 45, but also in melanoma (46) and in pancreatic cancer (47)], is well established. Indeed, somatic mutations of PATCH1, the gene encoding the SHH receptor, and of Smoothened, a protein normally controlled by PATCH1, are highly prevalent in BCC; whereas, inherited PATCH1 mutations are responsible for the nevoid BCCs or Gorlin syndrome (45); and in view of the fact that medulloblastoma is part of the Gorlin syndrome spectrum, it is interesting that ZIC2 may be highly expressed in this tumor (31). Although the distal part of the SHH pathway is not yet elucidated in detail, there is overlap between the transcriptional targets of the GLI family and of the ZIC family.

The evidence that ZIC2 is a determinant of malignancy in ovarian cancer is not just from *in vitro* studies of cell lines but from data on clinical outcomes. We have found that by all major parameters (OS and PFS,) the clinical course of patients correlates with the extent of ZIP2 overexpression. Interestingly, a similar observation has been recently reported with respect to oral squamous cell carcinoma (32).

**Conclusion**

From this work, ZIC2 expression emerges as a strong discriminator between MAL and LMP tumors. In addition, there is a significant correlation between the extent of ZIC2 overexpression and the clinical course of patients with MAL tumors. Overall, these data support the notion that ZIC2 plays a major role in the highly malignant behavior of EOTs. Several avenues are being currently explored to target the SHH pathway for therapeutic purposes (24, 48), and one small molecule, vismodegib, is at an advanced preclinical stage of development (49). There is now a rationale for including EOT among the tumors that might respond to such molecules.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

**Authors’ Contributions**

**Concept and design:** S. Marchini, E. Poynor, M. D’Incalci, L. Luzzatto  
**Development of methodology:** S. Marchini, E. Poynor, L. Luzzatto  
**Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.):** E. Poynor, R. Fruscio, E. Erba, G. Cattoretti, D. Katsaros, A. Koff, L. Luzzatto  
**Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis):** E. Poynor, R. Clivio, M. Cinquini, R. Fruscio, L. Porcu, M. Romano, A. Koff, L. Luzzatto  
**Writing, review, and/or revision of the manuscript:** S. Marchini, R.R. Barakat, L. Clivio, M. Cinquini, R. Fruscio, L. Porcu, M. D’Incalci, M. Romano, G. Cattoretti, A. Koff, L. Luzzatto
Oncogenic Role of ZIC2 in Epithelial Ovarian Cancer

Grant Support

Financial support was received from NIH, from the ‘Nerina and Mario Mattioli’ Foundation, and from the Italian Association for Cancer Research (IG11673). Cariplo Foundation (Grant Number 2010-0744) and ACTO Foundation.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 10, 2012; revised May 15, 2012; accepted June 8, 2012; published OnlineFirst June 25, 2012.

References


Acknowledgments

The authors thank Drs. N. Auerperg, Salamanca, J. Boyd, and L. Dubeau for providing us with cell lines; Dr. Juan Rosai for authoritative views on ovarian cancer; Drs. Barbara Stecca and Valter Torri for helpful comments on the manuscript; and Dr. Diane Tabarini and Ellen Bordignon for technical and administrative support.
The Zinc Finger Gene ZIC2 Has Features of an Oncogene and Its Overexpression Correlates Strongly with the Clinical Course of Epithelial Ovarian Cancer


Updated version
Access the most recent version of this article at:
doi:10.1158/1078-0432.CCR-12-0037

Supplementary Material
Access the most recent supplemental material at:
http://clincancerres.aacrjournals.org/content/suppl/2012/06/25/1078-0432.CCR-12-0037.DC1

Cited articles
This article cites 45 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/18/16/4313.full.html#ref-list-1

Citing articles
This article has been cited by 1 HighWire-hosted articles. Access the articles at:
/content/18/16/4313.full.html#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.