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The zinc finger gene *ZIC2* has features of an oncogene and its over-expression correlates strongly with the clinical course of epithelial ovarian cancer

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Abstract

Purpose—Epithelial ovarian tumors (EOTs) are amongst 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.

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Experimental Design—*ZIC2* expression levels were analysed 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. Over-expression of *ZIC2* was localized to the nucleus. *ZIC2* over-expression increases the growth rate and foci formation of NIH 3T3 cells, and stimulates anchorage-independent colony formation; down-regulation 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 EOT.

Keywords

Epithelial ovarian tumors; low malignant potential; *ZIC2*; oncogenes; sonic hedgehog

Introduction

Ovarian cancer is the gynecological malignancy with the highest mortality (1): it comprises several types of tumors with different pathological 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 histological types and grading (4, 6, 7). The majority of malignant ovarian tumors are of epithelial origin (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 10–20% 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, gene expression profiling 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 character that LMP tumors lack. In a study that was recorded in a doctoral thesis (11) a quantitative profile study revealed 61 genes with significantly higher expression in MAL versus LMP. One of the highest-ranking was *ZIC2*, which belongs to a family of five genes originally defined by their homology to the drosophila genes *odd-paired*, that encode zinc-finger transcription factors. The *ZIC* genes 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 oncogenesis. 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 paper we report that the over-expression of *ZIC2* in MAL compared to LMP tumors has been confirmed independently in two 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 over-expressed, behaves as a transforming oncogene; and that the level of over-expression of *ZIC2* correlates with clinical outcome in patients with MAL.

Materials and Methods

Cell lines

The HOSE cell lines (gift of J. Boyd), the LMP cell line ML46 (gift of 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, Ovcar-3), were routinely cultured in dedicated fresh medium with fetal calf serum at 37°C in a 5% CO₂ incubator. The SBOT 3.1 cells (gift of Dr. Auersperg) 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) (18) as obtained from two separate tumor tissue collections:

- A. A total of 170 biopsies were part of 1300 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 Dept., San Gerardo Hospital (Monza, Italy), as described (19).
- B. A total of 62 biopsies were part of 600 samples stored in a frozen tissue bank collection between January 1992 and December 2005 and available at Department of Gynaecology-Oncology, “S. Anna”, University of Torino.

Samples were collected in the operating theatre from patients undergoing cytoreductive surgery, frozen within 15 min in liquid nitrogen and stored at –80°C. The tumor content of the specimens was assessed by H&E stain in the pathology Units. Only specimens containing >70% of tumor were used. Patients’ clinical and anatomic-pathological information were registered. Histology, grade, and stage of each tumor are listed in Supplementary Table I. The collection and use of tumor samples was approved by the local scientific ethical committees and written informed consent was obtained from the patients.

Real-time qRT-PCR and data analysis

Total RNA was purified using RNAeasy™ Plus Mini Kit according to manufacturer's instructions (Qiagen, Milan, Italy). Absolute copy numbers were determined by quantitative real-time RT-PCR (qRT-PCR) (ABI-7900, Applied Biosystems) with Syber Green protocol (Qiagen, Milan Italy) using methods and protocols previously standardized (20). Primer pair sequences are reported in Supplementary Table II. 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 performed using Wilcoxon *t* test. Differences were considered statistically significant with a two sided $p < 0.05$. Data are presented as median with inter-quartile range (IQ range, 25%–75% quartile). All tests and data plots were done using GraphPad Prism Version 5.01 (GraphPad Software, La Jolla, CA).

Western blot analysis

Total proteins were isolated through standard methods, separated through 10% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to nitrocellulose filters. For cytoplasmic and nuclear fractions, whole cells were prepared in buffer A (10 mM Hepes pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF) containing protease inhibitors (Roche). Nuclear extracts were obtained using Buffer C (20 mM Hepes pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM DTT, 0.5 mM PMSF).

Immuno-blotting was carried out with ZIC2 monoclonal antibodies (Zymed, South San Francisco, CA); GAPDH (Abcam, Cambridge FL), laminin (B-2, Santa Cruz Biothecnology, Palo Alto CA,) or beta actin were used as loading controls (sc-130656, Santa Cruz). H2B (N-20, Santa Cruz Biothecnology) was used for loading control of nuclear fraction only. Antibody binding was revealed by peroxidase labeled secondary antibodies and visualized using enhanced chemiluminescence (Amersham, Piscataway, NJ).

ZIC2 constructs

The human full length ZIC2 cDNA sequence was provided by A. Gure (Ludwig Institute, New York Presbyterian Medical Center) and cloned into the pWZL/hygromycin retroviral vector or into the lentiviral pLENTN5/6 vector (Invitrogen, Carlsbad, California). The Phoenix packaging cell line was transfected with the pWZLZIC2 construct and vector alone. The 293 packaging cell line (Invitrogen) was transfected with pLENTIZIC2 construct and the control construct pLENTI+LACZ (Invitrogen). Lipofectamine 2000 (Invitrogen) was utilized 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, St Louis, MO). Pools of stably transfected cells were isolated through selection with the appropriate antibiotic and ZIC2 protein over-expression was confirmed with ZIC2 immunoblots. Cells were plated in triplicate in 6 well plates and viable cells were counted every 48 hours by trypan blue exclusion. Average counts were recorded in order to assay the DNA synthesis NIH 3T3 cells were pulsed with FITC-BrdU (Clontech, Mountain View, CA) for 1 hour and harvested. Biparametric BrdU/DNA flow cytometric analysis was performed on at least 20.000 cells by using FACS Calibur instrument (Becton Dickinson) and analyzed by Cell Quest software.

ZIC2 siRNA

Three siRNA oligonucleotides (Supplementary Table II), targeted to the *ZIC2* mRNA (Qiagen) were utilized along with control scrambled oligonucleotide. Ovarc 8 and Caov2 cells were transfected with these four oligonucleotides utilizing Oligofectamine (Qiagen). Cells were plated in triplicate in 6 well plates at a density of 2.5×10^5 cells, transfected with 1.6 micrograms of scrambled RNA or a siRNA, and counted with trypan blue exclusion at 24-hour intervals. The average of 3 wells was recorded.

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 either: 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 DMEM + 10% FCS containing 0.5% agar. Cells from pools of the stably transfected cell lines pLENTI-LACZ, pLENTI-ZIC2, 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 1×10^4 cells or 1×10^3 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 interquartile range (IQR) as measure of dispersion. We assessed the statistical association between clinical and histopathological characteristics and *ZIC2* expression with non parametric 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 (sub-stage, grading, histology) were described with relative and absolute frequencies and analyzed with the χ^2 test for association or trend. (21).

Prognosis 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 Contal 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 cutpoint identified (21); the Cox proportional hazards model was used to estimate Hazard Ratio (HR). All tests were two-sided and a p-value < 0.05 was considered statistically significant. Statistical analysis was performed using SAS software (version 9.1; SAS Institute, Inc, Cary NC).

Results

ZIC2 is over-expressed in MAL versus LMP ovarian tumors and cell lines

Gene expression profiling was originally carried out on Research Genetics GeneFilters by hybridizing tumor cDNA to 31,203 genes and expression sequence tags. From pairwise comparison of three MAL and three 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 two sets were compared (10, 22). Among 61 genes with higher expression in MAL versus LMP, *ZIC2* stood out on grounds of its biological role, and its over-expression 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 two separate sets of tumors (Fig. 1): collection A consisted of 170 tumors and collection B consisted of 62 tumors (23). The clinical and histopathological features of these two sets of tumors are reported in Supplementary Table I.

Levels of *ZIC2* mRNA measured by qRT-PCR were markedly different in LMP and in MAL samples (Fig. 2 and Supplementary Table III). 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* over-expression was 13.8 higher in stage III tumors than in stage I tumors ($p < 0.001$, Fig. 2 and Supplementary Table III). Western Blot analysis, performed 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, F; and Supplementary table III), mirroring what we have found at the mRNA level. In 6 MAL and 2 LMP tumors we carried out immuno-blot 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. 2C and D). In contrast, *ZIC2* is expressed in all of 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. 2C and D). Interestingly in the cell lines SBOT3.1 and ML46, to our knowledge the only two cell lines established from LMP tumors, *ZIC2* mRNA was undetectable (Fig. 2). 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, since its pattern of expression appears to be preserved in the transition from primary tumors to cell lines. We don't yet know the mechanism underlying *ZIC2* over-expression in MAL tumors and in cell lines derived from these tumors. In 10 tumor samples we have tested 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 two independent tumor tissue collections, the initial evidence that *ZIC2* over-expression discriminates strongly between MAL and LMP tumors.

Over-expression of the ZIC2 protein causes increased proliferation of NIH3T3 cells

In order to define whether ZIC2 over-expression 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 over-expressed ZIC2 and had a higher proliferation rate than control cells (Supplementary Figure 1A): after 6 days the number of cells was more than double that in the control culture and the fraction of BrdU positive S phase cells was higher in ZIC2-transduced cells than in control cells (Supplementary Figure 1B).

Inhibition of ZIC2 decreases the growth of ovarian cancer cell lines

NIH3T3 are an assay system quite remote from EOT. In order to test more directly the impact of ZIC2 on the growth of ovarian cancer cells we selected two 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. Based on immuno-blot analysis at 48 hours post-transfection, all three siRNA molecules (siRNAb and siRNAc more than siRNAa) effectively decreased ZIC2 protein levels in Ovcar-8 cells; correspondingly their proliferation was markedly impaired, particularly with siRNA C (Supplementary Figure 1C). Similar results were obtained with the Caov2 cell line (Supplementary Figure 1D). We infer that in both of these two different tumor cell lines ZIC2 is a major determinant of the rate of growth.

Over-expression 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. Mouse embryo fibroblasts (MEF) 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. 3, A 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 compared to our negative control (LACZ); and it was about one-half compared to 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 density (data not shown). Overall, the data show that over-expression 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 poly-alanine 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 NLS had been disrupted (M2-M7: supplementary Figure 2A). After cloning these mutant constructs into pLENTIV5/6 and transduction into NIH3T3 cells (supplementary Figure 2B), both foci and colonies in soft agar were counted. All constructs retained transforming activity except M7, in which both zinc finger 1 and zinc finger 2 had been deleted (Supplementary Figure 2C and 2D).

Correlation of *ZIC2* expression levels with parameters of clinical outcome

Since the clinical course of LMP is characteristically favorable compared to that of MAL, it is not surprising that we found a marked difference in both OS and PFS between these two group of tumors (Supplementary Figure 3). 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, there is a wide spread of *ZIC2* mRNA levels in stage I patients, whereas stage III patients have very high *ZIC2* levels in nearly all cases. Second, since the proportion of the serous histotype was much higher in the stage I group than in the stage III group (Supplementary Table I), 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 IV); in stage III between serous and endometrioid ($p=0.048$; Supplementary Table IV). 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, sub-stage, histotype, chemotherapy and age at diagnosis with respect to clinical variables OS, PFS. By using *ZIC2* level as a continuous variable (see Material 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% CI: 1.001–1.127; $p=0.046$; Table IA; for PFS the difference was not statistically significant); and in stage III patients the association was not significant for either OS or PFS (Table IB). All other baseline characteristics analyzed so far as well as chemotherapy resulted associated to OS endpoint, (i.e. stage I patients with clear cells histotype and grade 3 had a worst OS than patients with other characteristics, Table IA). We next sought a statistically significant cutoff point that would maximally discriminate prognosis (Table IC). 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 statistically significant ($p=0.014$ for OS, $p=0.01$ for PFS, Table IC). Specifically, this analysis shows an OS at 5 years 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 IA).

Discussion

Gene expression profiling reveals *ZIC2* over-expression 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 many that it proves 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, though 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, three studies have specifically addressed the comparison of MAL and LMP tumors (28, 29). Gilks et al (30) 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. Meinhold-Herlein et al. (31) observed that, overall, the GEP of LMP tumors was significantly different from that of MAL tumors of high histological grading (G2 or G3); but virtually undistinguishable from that of grade G1 MAL tumors. Marchini et al (19) confirmed the latter finding: at the same time, it was shown that, within MAL tumors, the different histological 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 two groups; and genes with markedly different expression were relatively few (22). The over-expression 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 two separate much larger sets of tumors (232 in total) in a different laboratory, and the extent of over-expression 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 two cell lines originating from LMP tumors were the only ones (along with the ovarian epithelium non-tumor cell lines), in which *ZIC2* mRNA was altogether undetectable, in spite of the high sensitivity of the RT-PCR technique we used. These findings are consistent with the notion that *ZIC2* over-expression may be a crucial determinant of the malignancy of ovarian cancer.

The *ZIC* gene family

ZIC2 is now known to be over-expressed in several cases of brain tumors (14, 15, 32) and in other tumors (16); and it has been noted recently in a GEP study of oral cancer (33). Interestingly, antibodies against the *ZIC2* protein have been demonstrated in five out 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 five *ZIC* genes are

highly homologous, they must fulfill different roles, since spontaneous mutations in humans and targeted inactivation of individual ZIC genes in mice give distinct pathologies. For instance, *ZIC1* inactivation causes abnormalities of the cerebellum and of the axial skeleton (34). *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 (35). *ZIC3* mutations affect left-right asymmetry and can cause congenital heart abnormalities (36). 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 up-regulated in ovarian cancer compared to the ovarian epithelium (37). The relationship between the ZIC set and the GLI set of proteins is remarkable because the DNA element to which they bind are almost identical (38). In addition, GLI1, GLI2 and GLI3 interact with ZIC1, ZIC2 and ZIC3; specifically, zinc fingers 3–5 of ZIC1 have been shown to bind to zinc fingers 3–5 of GLI1 (39). At the functional level, ZIC proteins can act either as co-activators or as repressors of GLI-mediated trans-activation, and this modulating action depends on cell types (38). Such cell-specific differences may be mediated by interactions with DNA-PK, KU70/80 and PARP proteins (40). Mutations in genes involved in early embryonic development have been well characterized in the case of leukemia: for instance, the *HOX* and *RUNT* genes (41). 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.

Over-expressed ZIC2 behaves as an oncogene

By using classic transformation assays – formation of foci and growth of colonies in soft agar – we found that *ZIC2* over-expression 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 don't know what causes *ZIC2* over-expression. Since it has been shown that in neural progenitor cells p53 inhibits the transcriptional activity of GLI1-GLI2, and conversely loss of p53 activates GLI1 (42), we wondered whether a similar circuit might be relevant to *ZIC2* over-expression in EOTs. Out 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* over-expression may be p53-independent; and this has been observed also with respect to regulation of Nanog by GLI1-GLI2 (43), and with respect to regulation of GLI in melanoma cells (Stecca, personal communication). In zebrafish, *ZIC2* is regulated by *FOXD5* (44). 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 et al. strongly supports this notion: they have shown that in cervical

cancer cells over-expressed *ZIC2* causes nuclear retention of *GLI1* and thus enhances *SHH* signaling (45).

That the *SHH* pathway is disturbed in a variety of tumors [particularly in basal cell carcinoma (BCC) (46), but also in melanoma (47) and in pancreatic cancer (48)], is well established. Indeed, somatic mutations of *PATCH1*, the gene encoding the *SHH* receptor, and of *Smoothed*, a protein normally controlled by *PATCH1*, are highly prevalent in BCC; whereas inherited *PATCH1* mutations are responsible for the nevoid basal cell carcinoma, or Gorlin syndrome (46); 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 (32). 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, PFS,) the clinical course of patients correlates with the extent of *ZIC2* over-expression. Interestingly, a similar observation has been recently reported with respect to oral squamous cell carcinoma (33).

Conclusion

From this work *ZIC2* expression emerges as a strong discriminant between MAL and LMP tumors. In addition, there is a significant correlation between the extent of *ZIC2* over-expression 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 EOT. Several avenues are being currently explored to target the *SHH* pathway for therapeutic purposes (24, 49), and one small molecule, vismodegib, is at an advanced pre-clinical stage of development (50). There is now a rationale for including EOT among the tumors that might respond to such molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

BCC	basal cell carcinoma
BrdU	bromodeoxyuridine
Cyc-A	cyclophilin A

EOT	epithelial ovarian tumor
FIGO	International Federation of Gynecology and Obstetrics
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEP	gene expression profile
HPE	holoprosencephaly
HRs	hazard ratios
LMP	low malignant potential
MEF	mouse embryo fibroblasts
MAL	malignant form
NLS	nuclear localization signal
MSKCC	Memorial Sloan Kettering Cancer Center
OS	overall survival
PFS	Progression-free survival
qRT-PCR	quantitative real-time RT-PCR
SDS-PAGE	SDS polyacrylamide gel electrophoresis
siRNA	small interfering RNA

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Statement of Significance

In this manuscript we investigate the molecular basis for the remarkable difference in clinical course between the invasive form of epithelial ovarian cancer (MAL) and that of the so-called low malignant potential (LMP), or borderline tumors. By gene expression profiling 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. Over-expression of ZIC2 was also documented at the protein level, and whenever ZIC2 is over-expressed it is electively localized to the nucleus. We have further shown by transformation assays and by siRNA experiments that over-expressed 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 clinical material, and we 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.

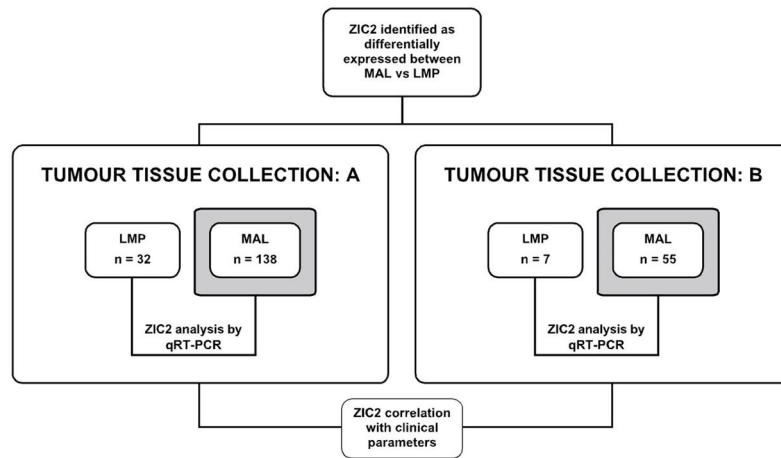


Figure 1. Schematic workflow

The difference in *ZIC2* expression levels between MAL and LMP EOT, originally discovered by GEP analysis on a small sample of tumors (see text), was validated in a separate lab in two distinct tumor tissue collections: **A** and **B**. *ZIC2* expression was measured by qRT-PCR; the figures were then correlated with pathology and with clinical variables, including OS and PFS.

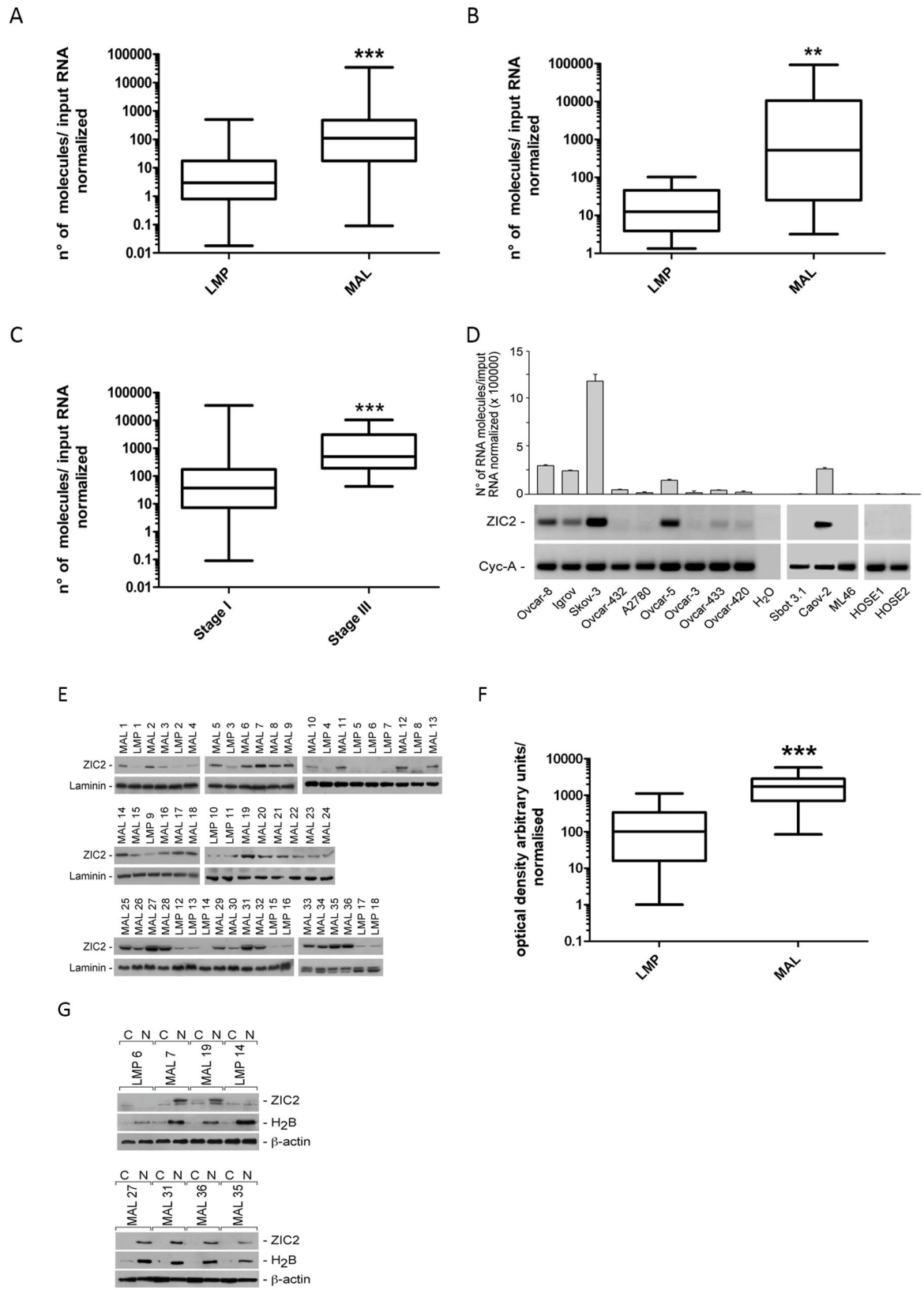


Figure 2. Analysis of the quantitative expression of ZIC2 in primary tumors and in cell lines

A, B: Box plot diagrams showing the expression levels of ZIC-2 measured by qRT-PCR in tumors of collection A and in tumors of collection B. Within each box the horizontal line indicates the median. The top edge of the boxes represents the 75th percentile, the bottom edge the 25th percentile (note that the scale on the ordinate is a log scale). The range is shown as a vertical line ending above and below the 75th and 25th percentile values, respectively. **C:** Tumor samples from collection A divided by clinical stages: there is a significant difference between stage I and stage III tumors. **D;** ZIC2 expression levels in 14 cell lines: 10 originating from MAL tumors, 2 from LMP tumors (Sbot3.1 and ML46) and two from normal ovarian epithelial cells (HOSE 1 and HOSE 2). Below the bar diagram, the original agarose gel electrophoresis picture is shown. Cyclophilin A (Cyc-A) was used as a housekeeping gene internal control. **E:** ZIC2 protein analysis by Western Blot on whole cell extracts (40 µg of protein) of a subset of 18 LMP and 32 MAL tumors. **F:** Box plot diagrams showing the expression levels of ZIC-2 protein measured by densitometry on Western blots analysis in the samples of panel E. In panels A, B, C, F asterisks define the level of significance according to the Mann-Whitney *t* test: ** for < 0.01; *** for < 0.001. **G:** The intracellular distribution of ZIC2 was determined by immuno-blot analysis of equivalent amounts of nuclear (N) and cytoplasmic (C) fractions (see Materials and Methods). The enrichment in nuclear fraction was confirmed by immuno-blotting with an antiserum against the nuclear protein histone H2B; β-actin was used instead as a loading control since it is present in both the nucleus and the cytoplasm.

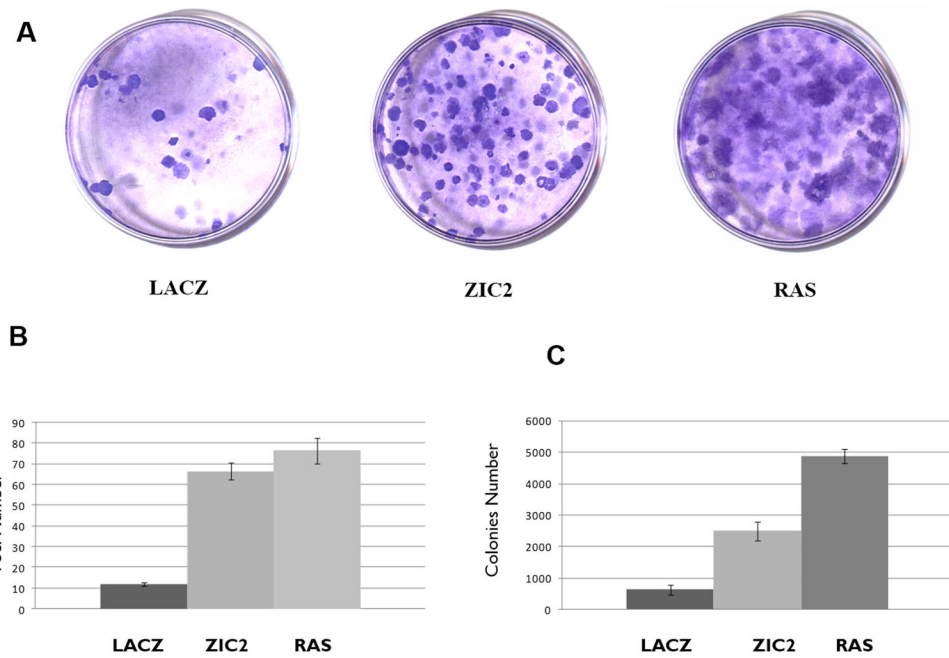


Figure 3. Transforming activity of *ZIC2* assayed by foci formation in mouse embryonic fibroblasts (MEF)

A. Representative plates after Giemsa staining of MEF stably transfected with pLenti+LacZ (LACZ) or pLENTI+ZIC2 (ZIC2) plasmid; pBABE+RAS (RAS) was used as positive control. **B.** Quantitative data from experiments as above carried out in duplicate. **C.** Number of colonies formed by NIH3T3 cells stably transfected with pLenti+LacZ (LACZ), pLENTI+ZIC2 (ZIC2), or pBABE+RAS (RAS). Numbers are the average of triplicate plates and the experiment has been carried out in duplicate. Bars are the \pm 1 standard deviation.

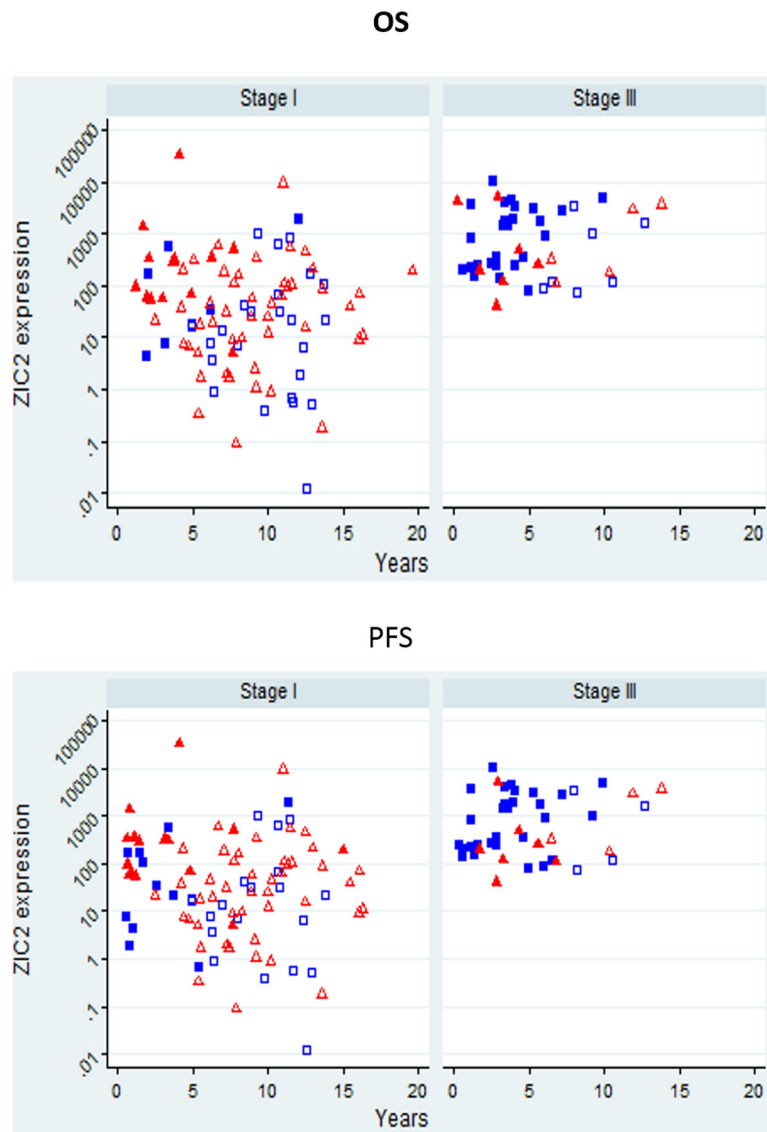
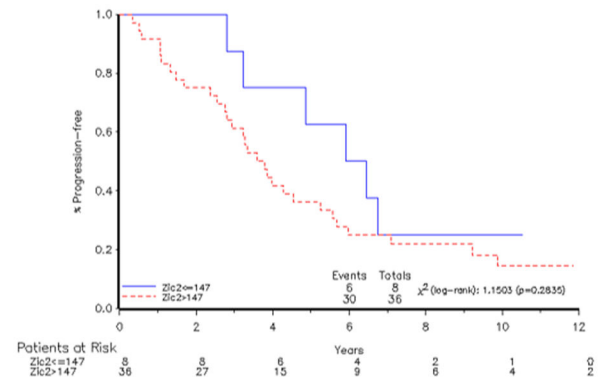
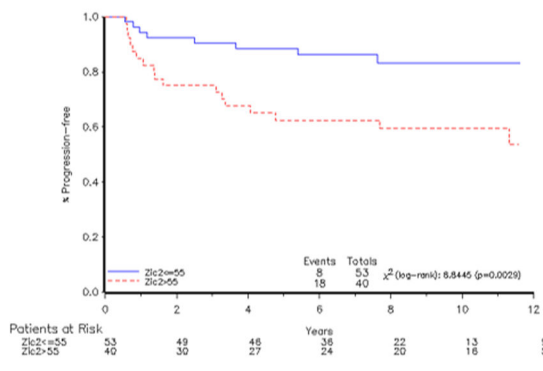
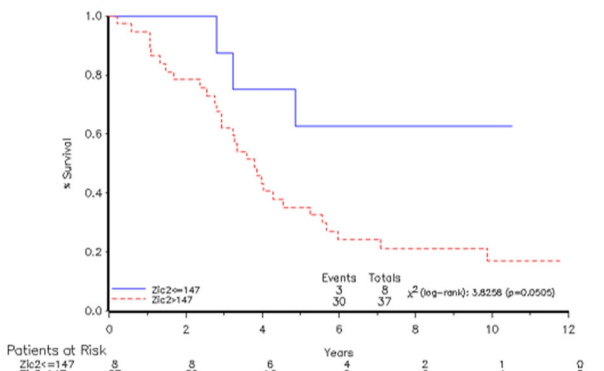
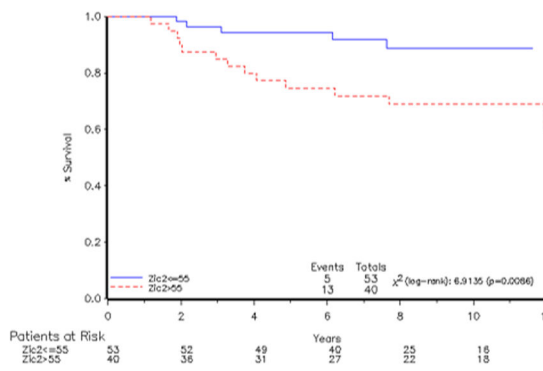


Figure 4. *ZIC2* expression correlates with measures of clinically important end-points
 Each symbol represents an individual patient: squares are for serous histotype, triangles for all other histotypes. Full symbols are for those patients who experienced fatal outcome or relapse; empty symbols are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

PFS



OS



Stage I

Stage III

Figure 5. Correlations between ZIC2 expression and measures of clinical outcome are statistically validated

Kaplan–Meier curves of OS, PFS for all MAL tumors in collection A (left, stage I; right, stage III). Samples were divided into two clusters based on ZIC2 expression levels measured by qRT-PCR, as detailed in the Methods section. *p* values are log rank test.

Table I

HR estimates for ZIC2 expression levels

Cox proportional hazards regression analysis of OS, PFS in univariate and multivariate analysis in tumor tissue collection A. Analysis performed in continuum, in stage I patients (A), or in stage III patients (B). C. ZIC2 expression levels were dichotomized (see Materials and Methods), with a cutoff set at 55 for stage I tumors, and at 147 for stage III tumors. *p* values are log rank test. CI, confidential interval HR is estimated for each 10⁻² ZIC2 units increase. Cut-point values are 10⁻³ multiplied.

A		Tumor Tissue Collection A										
		Stage I										
Endpoint	Variable	Value	Univariate analysis				Multivariate Analysis*					
			HR	95% CI	χ^2	P	HR	95% CI	χ^2	P		
OS	ZIC2*	-	1.062	1.001-1.127	3.969	0.046	1.065	1.001-1.133	3.884	0.049		
	Age (years)	-	1.061	1.016-1.109	7.132	0.008	1.060	1.007-1.116	4.887	0.027		
	Grading	1,2	1	-	4.882	0.027	1	-	1.403	0.236		
		3	3.025	1.133-8.073			2.074	0.620-6.935				
	Histotype	Clear cell	1	-	5.079	0.024	1	-	0.442	0.506		
		Not clear cell	0.323	0.121-0.863			0.672	0.208-2.171				
	Chemotherapy	No	1	-	4.459	0.035	1	-	3.021	0.082		
		Yes	8.797	1.169-66.193			6.114	0.794-47.098				
	ZIC2*	-	1.043	0.984-1.106	1.978	0.160	1.036	0.976-1.099	1.322	0.250		
	Age (years)	-	1.045	1.010-1.081	6.559	0.010	1.046	1.005-1.088	4.837	0.028		
PFS	Grading	1,2	1	-	5.152	0.023	1	-	1.653	0.199		
		3	2.506	1.134-5.541			1.850	0.724-4.726				
	Histotype	Clear cell	1	-	1.742	0.187	1	-	0.113	0.737		
		Not clear cell	0.539	0.215-1.350			1.201	0.412-3.501				
Chemotherapy	No	1	-	6.983	0.008	1	-	5.711	0.017			
	Yes	14.894	2.009-110.409			11.711	1.557-88.116					

Tumor Tissue Collection A											
Stage III											
Endpoint	Variable	Value	Univariate analysis			Multivariate Analysis*					
			HR	95% CI	χ^2	p	HR	95% CI	χ^2	p	
OS	ZIC2*	-	1.071	0.896-1.280	0.565	0.452	1.014	0.823-1.250	0.017	0.897	
	Age (years)	-	1.061	1.019-1.105	8.208	0.004	1.062	1.017-1.109	7.459	0.006	
	Grading	1,2	1	-	1.430	0.232	1	-	0.453	0.501	
		3	1.896	0.664-5.413	1.452	0.232	1.452	0.491-4.296	0.453	0.501	
PFS	Histotype	Serous	1	-	1.087	0.297	1	-	0.057	0.812	
		Not serous	0.640	0.277-1.481	1.115	0.297	1.115	0.455-2.729	0.057	0.812	
	ZIC2*	-	0.974	0.806-1.178	0.073	0.787	0.899	0.721-1.120	0.900	0.343	
	Age (years)	-	1.043	1.003-1.084	4.437	0.035	1.042	1.001-1.086	3.865	0.049	
PFS	Grading	1,2	1	-	2.230	0.135	1	-	1.273	0.259	
		3	2.213	0.780-6.276	1.865	0.135	1.865	0.632-5.508	1.273	0.259	
	Histotype	Serous	1	-	2.192	0.139	1	-	0.389	0.533	
		Not serous	0.535	0.233-1.225	0.754	0.139	0.754	0.310-1.832	0.389	0.533	

Tumor Tissue Collection A												
C												
Endpoint	"Stage I"						"Stage III"					
	Cut-point	HR	95% CI	Chi ²	p	Cut-point #	HR	95% CI	Chi ²	p		
OS	55	3.642	1.298-10.223	6.028	0.014	147	3.084	0.939-10.129	3.447	0.063		
PFS	55	3.277	1.334-8.046	6.706	0.010	147	1.612	0.668-3.887	1.129	0.288		