

High insulin-like growth factor-2 (IGF-2) gene expression is an independent predictor of poor survival for patients with advanced stage serous epithelial ovarian cancer

Robyn A. Sayer^{a,b}, Johnathan M. Lancaster^{a,b,*}, Jennifer Pittman^c, Jonathon Gray^d, Regina Whitaker^b, Jeffrey R. Marks^e, Andrew Berchuck^b

^aH. Lee Moffitt Cancer Center and Research Institute, 12902 Magnolia Drive, MCC-GYNPROG, Tampa, FL 33612-9497, USA

^bDepartments of Obstetrics and Gynecology/Division of Gynecologic Oncology, Tampa, FL 33612-9497, USA

^cInstitute of Statistics and Decision Sciences, Tampa, FL 33612-9497, USA

^dInstitute of Medical Genetics, University Hospital of Wales, Cardiff, UK

^eSurgery, Duke University Medical Center, Durham, NC 27710, USA

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Abstract

Objective. Epithelial ovarian cancer is the deadliest gynecologic malignancy, yet its molecular etiology remains poorly understood. Evidence is accumulating to support a role for the insulin-like growth factor family in human carcinogenesis, and recently using microarray expression analysis, we demonstrated over-expression of the insulin-like growth factor-2 (IGF-2) gene in advanced stage epithelial ovarian cancers. The purpose of the current study is to further elucidate the role of the IGF-2 gene in ovarian cancer development and progression.

Methods. Relative expression of *IGF-2* was measured in 109 epithelial ovarian cancers and eight normal ovarian surface epithelial (NOSE) samples, using quantitative real-time polymerase chain reaction. Associations with clinicopathological parameters were examined.

Results. Expression of the IGF-2 gene was more than 300-fold higher in ovarian cancers compared with normal ovarian surface epithelium samples ($P < 0.001$). High *IGF-2* expression was associated with advanced stage disease at diagnosis ($P < 0.001$), high-grade cancers ($P < 0.05$) and sub-optimal surgical cytoreduction ($P = 0.08$). In multivariate analysis, relative *IGF-2* expression was an independent predictor of poor survival.

Conclusions. Expression of the IGF-2 gene is significantly higher in ovarian cancers relative to normal ovarian surface epithelium. Further, high IGF-2 gene expression is associated with high grade, advanced stage disease, and is an independent predictor of poor survival in patients with epithelial ovarian cancer. As such, IGF-2 is a molecular marker and potential therapeutic target for the most aggressive epithelial ovarian cancers.

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Introduction

Epithelial ovarian cancer is the most lethal gynecologic malignancy. Currently, no effective screening or early detection techniques exist and approximately 80% of ovarian cancers have metastasized extensively throughout the peritoneal cavity at the time of diagnosis. Despite surgical

cytoreduction and platinum-based chemotherapy, median survival is approximately 3–5 years; however, a minority of women survive much longer [1]. To date, efforts to fully elucidate the molecular etiology of such survival differences have been unsuccessful [2,3]. It is likely that the marked clinical differences in ovarian cancer stage at presentation, response to therapy, and ultimately survival are manifestations of a complex underlying molecular heterogeneity, such that traditional single-gene analysis techniques have proven inadequate. The recent development of microarray

* Corresponding author. Fax: +1 813 745 4801.

E-mail address: lancasjm@moffitt.usf.edu (J.M. Lancaster).

expression analysis technology has transformed the study of carcinogenesis by enabling the simultaneous study of thousands of genes in a single sample. Study of genome-wide expression is beginning to provide a more global understanding of the complex genetic changes that lead to malignant transformation in a variety of cancers.

Our laboratory recently applied microarray technology to identify patterns of gene expression associated with outcome in women with advanced stage epithelial ovarian cancers. A series of 31 advanced stage serous ovarian cancers from patients who survived less than 2 years and more than 7 years were compared with normal ovarian epithelium. Approximately 7070 genes were evaluated using Affymetrix HumanGeneFL arrays. Hierarchical clustering identified patterns of gene expression that distinguished cancer from normal ovarian epithelium, and several members of the insulin-like growth factor family exhibited differential expression between ovarian cancers and normal ovarian surface epithelium samples. Notably, the IGF-2 gene was 13-fold ($P < 0.01$) more highly expressed in 31 advanced ovarian cancers compared to three normal ovarian surface epithelium samples [4].

Previously, expression of the IGF-2 gene has been associated with development of cancers of the breast, colon, and prostate [5–7]. Additionally, IGF-2 expression has been shown to be elevated in ovarian cancers versus normal ovarian tissue. Sawiris et al. [8] recently analyzed a large series of microarray data in order to develop a gene chip specific to the study of ovarian cancer. The analysis identified 516 up-regulated genes with the IGF-2 gene being the most highly over-expressed of this group.

In light of this, and our recent array findings in advanced serous ovarian cancers, we sought to more fully elucidate the role of IGF-2 in ovarian carcinogenesis by applying quantitative real time polymerase chain reaction to measure the expression of the IGF-2 gene in a more comprehensive panel of epithelial ovarian cancers.

Materials and methods

Study population

One hundred and nine fresh frozen epithelial ovarian cancers and eight primary cultures of normal human ovarian surface epithelium samples were obtained with IRB-approved informed consent from patients treated by the Division of Gynecologic Oncology at Duke University Medical Center. Histopathologic subtypes included 83 serous, three endometrioid, seven mucinous, six clear cell, one undifferentiated, and nine mixed type. Ten of the cancers were early stage (I/II), and 99 advanced stage (III/IV). All patients with advanced stage disease were treated with primary surgical cytoreduction (49 optimal, 48 suboptimal, 2 unknown) followed by platinum-based chemotherapy.

Tissue preparation and primer design

Primary cultures of normal human ovarian surface epithelium were obtained by gently scraping ovarian surface epithelium from fresh ovaries. Primary cell cultures were grown in medium 199/MCDB 105 supplemented with 10% bovine serum albumin [9]. Samples were processed using microdissection in order to exclude ovarian stroma. Ovarian tumor tissue was harvested at the time of initial surgery, snap frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Hematoxylin and eosin staining of representative sections of each tumor was performed to determine the percentage of tumor in each specimen. Only samples consisting of more than 70% tumor were used for these studies. Total RNA was extracted using the RNeasy RNA extraction kit (Qiagen Inc., Valencia, CA), and an aliquot of 1 μg was subjected to a reverse transcriptase reaction with the Roche First Strand cDNA kit (Roche, Basel, Switzerland). Complementary DNA (cDNA) was quantified by fluorometry. Based on the published genomic sequence of IGF-2, two gene-specific intron-spanning primers were designed (F5'-CTGTGCTACCCCCGC-CAAGT-3' and R5'-ACGTTTGGCCTCCCTGAACG-3').

Quantitative RT-PCR

Quantitative RT-PCR was performed on the Roche LightCycler system (Roche) using SYBR Green I dye. For each sample, 5 ng of cDNA was subjected to 40 cycles of QRT-PCR in a total volume of 20 μL containing 10 mM of each primer, 10 units of AdvanTaq Plus DNA Polymerase with PCR reaction buffer (Clontech, Palo Alto, CA), dNTP, and SYBR Green I dye according to manufacturer's instructions in the presence of intron-spanning cDNA primers to the house-keeping gene, beta-microglobulin, to confirm adequate cDNA normalization. Serial dilutions of the sample demonstrating highest IGF-2 expression were used to create a concentration curve and relative expression levels calculated for each sample from this curve. The standard curves were included in each run. Sample concentrations fell within the range of the serial dilution curve thus ensuring adequate assay sensitivity (Fig. 1). All samples were run in triplicate and mean values were calculated.

Statistical analysis

Statistical analyses were performed using the R statistical programming environment software [10]. Associations between clinicopathological parameters such as stage, grade, histology (serous and nonserous), and debulking status, and relative IGF-2 expression were analyzed by the chi square test or the Fisher's exact test, when appropriate. Only patients for whom the status of all variables was known were included in the multivariate regression models, therefore, a subset of 98 samples (81 serous and 17 nonserous)

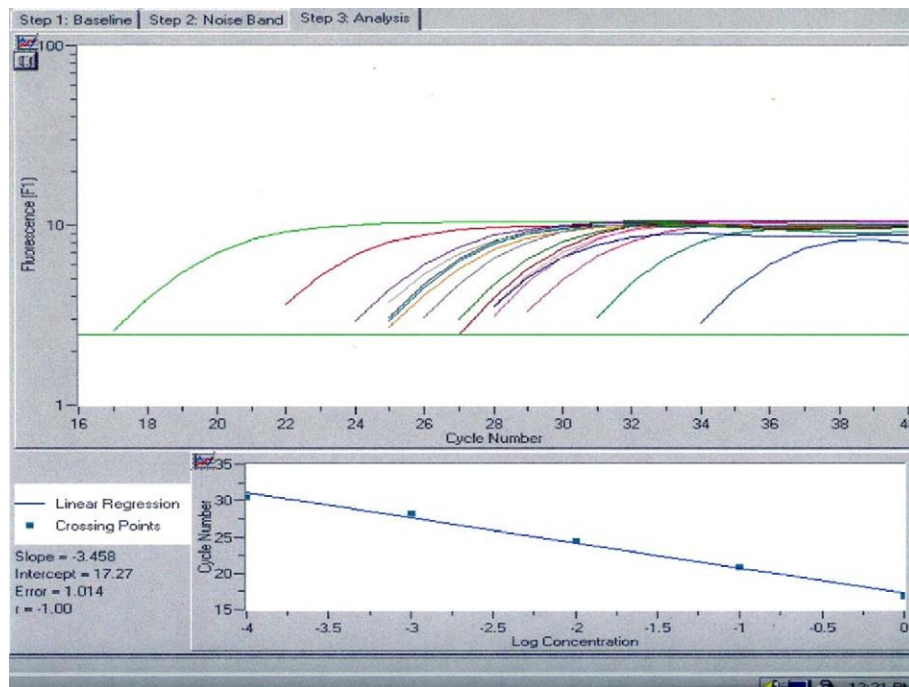


Fig. 1. Epithelial ovarian cancers and normal ovarian surface epithelial samples were subject to quantitative real-time polymerase chain reaction to determine relative expression of the IGF-2 gene. Serial dilutions of the most highly expressed cancer were used to create a concentration curve (bottom panel) and relative expression levels were calculated for each case from the real time increase in fluorescence demonstrated in the top panel.

was analyzed in this way. Information on progression-free interval was not available for all 98 patients, despite review of all available medical records. Progression-free interval was not, therefore, included in the analysis. Multivariate survival analyses were performed by constructing Kaplan–Meier survival curves and differences between curves were evaluated by the log-rank test. Cox proportional hazards models were used to estimate relative risk to determine whether the prognostic value of relative *IGF-2* expression disappeared when other prognostic factors were considered. For further analysis, patients were divided into two groups, high *IGF-2* expression and low *IGF-2* expression, using the mean value of relative *IGF-2* expression for all samples as a cut-off. The differences between the survival curves between *IGF-2* expression groups were tested for statistical significance by the log-rank test.

Results

Quantitative real-time polymerase chain reaction (QRT-PCR) was used to determine relative expression of the *IGF-2* gene in 109 epithelial ovarian cancers and 8 normal ovarian surface epithelial samples. Table 1 depicts the distribution of relative *IGF-2* expression in the ovarian tissue samples in relation to the various clinicopathological variables of the study population.

Mean relative *IGF-2* gene expression in 109 ovarian cancers was approximately 330-fold higher than in 8 normal ovarian surface epithelial samples (0.43 vs. 0.001, $P <$

0.001). Advanced stage cancers demonstrated higher relative *IGF-2* gene expression than early stage cancers. Relative *IGF-2* gene expression from 99 patients with advanced (stage III/IV) disease was 5-fold higher than 10 cancers from patients with early (stages I/II) disease (0.47 vs. 0.09, $P <$ 0.001). In light of the distinction in clinical behavior that has previously been demonstrated between well-differentiated and moderate/poorly differentiated ovarian cancers, relative *IGF-2* expression was compared between these two histopathologic groups [11,12]. Overall, relative expression of the *IGF-2* gene in 97 moderately and poorly differentiated (grade II/III) cancers was 6-fold higher than in six well-differentiated (grade I) cancers (0.48 vs. 0.08, $P <$ 0.001). Within the group of patients with advanced (stages III/IV) disease, relative expression of the *IGF-2* gene in 96 moderately and poorly differentiated (grades II/III) cancers was 3.6-fold higher than in 3 well-differentiated (grade I) cancers (0.5 vs. 0.14, $P = 0.03$). Ovarian cancers that expressed high levels of *IGF-2* were more likely to be incompletely resected at the time of initial surgery, that is, high relative expression of the *IGF-2* gene was associated with suboptimal tumor debulking (residual tumor >1 cm at conclusion of surgery). Debulking status was available for 97/99 (98%) patients with advanced stage disease. Relative *IGF-2* gene expression was 1.8-fold higher in 48 patients with residual tumor >1 cm at conclusion of surgery (suboptimally debulked), compared to 49 patients with residual tumor <1 cm (optimally debulked) at conclusion of surgery (0.62 vs. 0.33, $P = 0.08$). Additionally, a survival detriment was associated with high *IGF-2* gene expression in patients who were subject to optimal and

Table 1
Relationship between relative IGF-2 gene expression and other clinicopathologic variables in 109 ovarian cancers

Variable	n	Mean IGF-2 expression	Difference (fold)	P value
Ovarian cancers	109	0.43	330	<0.001
Normal ovarian epithelium	8	0.001		
Advanced stage				
Survival <45 months	47	0.7	7	<0.001
Survival >45 months	40	0.1		
Survival <24 months	27	0.7	7	<0.01
Survival >60 months	29	0.1		
Stage				
Advanced stage (III/IV)	99	0.47	5	<0.001
Early stage (I/II)	10	0.09		
Grade				
Grade II/III	97	0.48	6	<0.001
Grade I	6	0.08		
Debulking status				
Suboptimal	48	0.62	1.8	0.08
Optimal	49	0.33		
Suboptimally debulked				
Survival <45 months	26	0.89	6.6	0.001
Survival >45 months	17	0.13		
Optimally debulked				
Survival <45 months	18	0.5	4	0.07
Survival >45 months	26	0.12		

Parameters including stage, grade, histology (serous and nonserous), and debulking status, and relative IGF-2 expression were analyzed by the chi square test or the Fisher's exact test, when appropriate. For further analysis, patients were divided into two groups using the median survival of 45 months as a cut-off.

suboptimal debulking at time of initial surgery. In the suboptimally debulked category, 28 cancers from patients who died within 45 months (median survival for all patients in this study) of diagnosis exhibited approximately 6.6-fold higher relative IGF-2 gene expression than cancers from 17 patients who survived more than 45 months (0.89 vs. 0.13, $P = 0.001$, 4 samples censored). Similarly, in patients who underwent optimal debulking, cancers from 18 patients who died within 45 months of diagnosis exhibited approximately 4-fold higher relative IGF-2 gene expression than cancers from 26 patients who survived more than 45 months (0.5 vs. 0.12, $P = 0.07$, 5 samples censored).

Multivariate analyses were performed to assess predictors of survival. Covariates considered included age, grade, stage, histology, and relative IGF-2 expression. A subset of 98 cancers were included in the analysis (10 samples missing complete covariate data were excluded). Considering all cancers, high relative IGF-2 gene expression was associated with poor outcome. Using the median survival as a cut-off, across all cancers the survival curves were significantly different ($P < 0.001$) between those patients with a mean relative IGF-2 gene expression above or below the median (Fig. 2). Using multivariate Cox proportional hazards models with relative IGF-2 gene expression as the only predictor of overall survival, IGF-2 gene expression was highly significant across all cancers ($P < 0.001$) and

also in serous cancers alone ($P < 0.001$); IGF-2 gene expression was not significant among the nonserous cancers ($P = 0.48$) (Fig. 3). Only IGF-2 expression ($P < 0.001$) and stage at diagnosis ($P < 0.001$) were found to be significant independent predictors of outcome overall and also among serous cancers alone.

Discussion

The insulin-like growth factor pathway is a complex system comprised of two growth factors (IGF-1 and IGF-2), two cell surface receptors (IGF-1 R and IGF-2 R), six specific high-affinity binding proteins (IGFBP-1 to IGFBP-6), IGFBP proteases as well as several other IGFBP-interacting molecules, which regulate and potentiate IGF actions.

The IGF ligands, IGF-1 and IGF-2, are single chain molecules of 70 and 67 amino acids, respectively. Both ligands interact with high affinity membrane bound receptors and are thought to work in an autocrine, paracrine, and endocrine manner to mediate cell growth, differentiation, apoptosis, and transformation. Circulating levels are under complex physiological regulation. The majority of circulating IGFs are bound to high-affinity IGF binding proteins (IGFBPs), with >90% bound to a high molecular weight complex comprised of IGF binding protein 3 (IGFBP-3) and a separate protein known as the acid-labile subunit. IGFBPs appear to be responsible for protecting circulating IGFs and delivering them to their specific target sites. IGFBPs can also interact with IGFs in both an inhibitory and enhancing manner [13–15]. Disruption of the delicate balance of these complex processes may result in uncontrolled cell proliferation leading to malignant transformation, and evidence is accumulating that IGFs are important mitogens involved in the development of many types of malignancy [16].

To date, the role of IGFs in ovarian carcinogenesis and progression remains unclear. The most comprehensive study

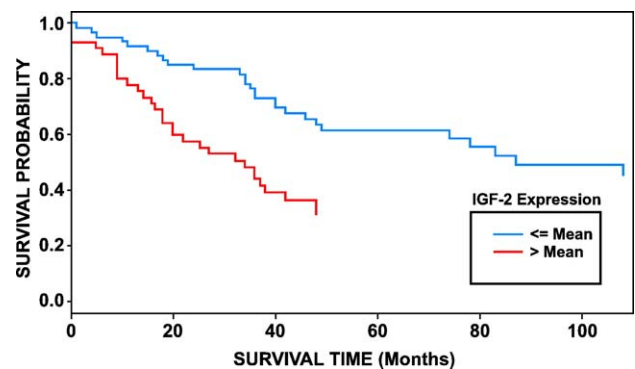


Fig. 2. Kaplan–Meier log-rank survival analysis of survival for patients with advanced (stages III/IV) epithelial ovarian cancer. The cut-off value used to determine high versus low IGF-2 gene expression was selected as the overall mean IGF-2 gene expression value calculated for all 109 ovarian cancer samples. Chi-square = 3.3, $df = 1$, $P = 0.07$.

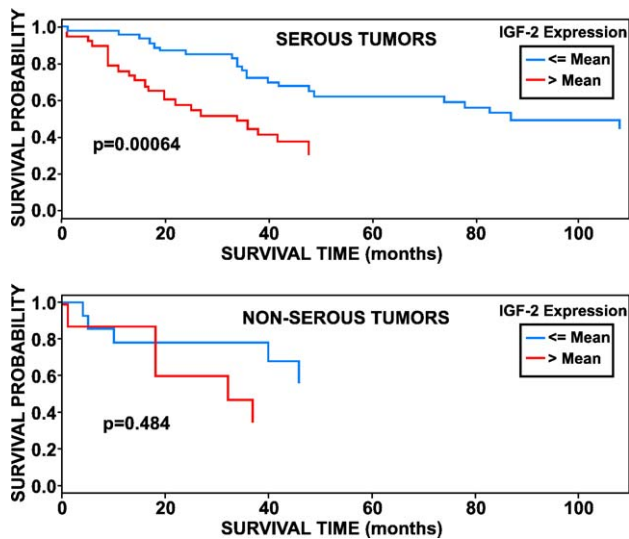


Fig. 3. Logistic regression models were applied to histological subgroups of patients using the median survival of 45 months as a cut-off. *IGF-2* expression was found to be a significant predictor of survival in the subgroup of patients with serous cancers ($P = 0.000638$), but not among nonserous cancers ($P = 0.484$).

of the IGF pathway in ovarian cancer was presented by Conover et al. [17]. They found that primary ovarian epithelial cell lines derived from previously untreated ovarian cancers expressed all major components of the IGF system and were able to demonstrate functional responses to exogenous IGFs.

Using microarray analysis, we recently demonstrated that the *IGF-2* gene exhibits higher expression in advanced stage ovarian cancers compared to normal ovarian epithelium [4]. We now report a quantitative analysis of insulin-like growth factor-2 gene expression levels in a set of 109 epithelial ovarian cancers, using quantitative real-time polymerase chain reaction. In this study that includes both early and advanced stage cancers as well as serous and nonserous histopathologic subtypes, we have demonstrated more than 300-fold higher *IGF-2* gene expression in ovarian cancers compared to normal ovarian surface epithelium. Such findings are consistent with other studies that have demonstrated increased *IGF-2* gene expression in cancers compared to normal tissue [5–7].

Several mechanisms have been proposed to explain increased levels of *IGF-2* including: loss of imprinting (LOI), loss of heterozygosity (LOH) with paternal duplication, excessive transcriptional activation, loss of transcriptional suppression, or alteration in *IGF*-binding proteins [18]. The basis of genomic imprinting involves a complex multistep process that results in parent-of-origin-specific inactivation of alleles of some genes. The *IGF-2* gene is one of the few known imprinted genes that has a major role in fetal development. In most tissues, the paternally derived copy of *IGF-2* is expressed, whereas the maternal copy is silent. Disruption of *IGF-2* imprinting is

a common event in a number of malignancies, including Wilm's tumors, gastric adenocarcinomas, lung cancers, and colorectal cancers [19–22]. When loss of imprinting occurs, biallelic expression of *IGF-2* results, ultimately leading to overexpression of this potent growth factor. The precise role of loss of *IGF-2* imprinting in carcinogenesis and tumor growth is currently unknown.

Loss of *IGF-2* imprinting has previously been implicated in the development of ovarian carcinomas. Chen et al. [23] determined the imprinting status of the *IGF-2* gene in 43 ovarian cancers, seven low malignant potential ovarian tumors, and their matched normal tissues. Loss of imprinting (LOI) was found in 5 of 20 (25%) cancers. Overexpression of the *IGF-2* gene was demonstrated in all five LOI samples. These results suggested that LOI may underlie overexpression of the *IGF-2* gene resulting in development of some ovarian cancers.

Our analysis suggests that high *IGF-2* expression is associated with a more aggressive ovarian cancer phenotype. High *IGF-2* gene expression was associated with higher stage at diagnosis, tumor grade, and in multivariate analysis, shorter overall survival. Additionally, cancers exhibiting high *IGF-2* gene expression demonstrated a trend towards being more likely to be incompletely resected at the time of primary surgery ($P = 0.08$), and within this group of sub-optimally debulked patients, a high *IGF-2* gene expression was associated with shorter survival ($P < 0.01$). These findings are consistent with studies in other cancer types, which have demonstrated a correlation between increased *IGF-2* expression and decreased survival. In colorectal carcinomas, *IGF-2* expression was associated with advanced Duke's stage and poor clinical outcome [24]. In our analysis, advanced stage ovarian cancers have 5-fold higher relative *IGF-2* gene expression than early stage cancers, and moderately and poorly differentiated (grade II/III) cancers have 6-fold higher expression than well-differentiated (grade I) cancers. In a similar fashion, a correlation between high *IGF-2* expression and poor prognostic clinical factors has been demonstrated in other cancers. *IGF-2* expression was recently shown to be correlated with pathologic stage, lymph node metastasis, histologic differentiation, and serum prostate-specific antigen (PSA) levels in 24 prostate carcinomas [25]. Previous studies have shown that *IGF-2* acts as a potent mitogen for a variety of cancer cell lines. Based on work in breast, lung, and colon cancers, a general pattern emerges that *IGF-2* expression increases as the malignant potential of the tumor increases [26,27].

While mRNA levels do not account for regulatory events at the translational and post-translational level, several studies have demonstrated corresponding increases in bioactive *IGF-2* protein levels in cancer cell lines. Nielsen et al. [28] have used radioimmunoassay to demonstrate a correlation of increased levels of *IGF-2* mRNA expression and secretion of bioactive *IGF-2* in human rhabdomyosar-

coma. Additionally, IGF-2 protein levels have been shown to be differentially expressed in gastric cancer cell lines when compared with normal gastric cell cultures [29]. IGF-2 protein levels, and the interactions of IGF-2 with its receptors and binding proteins, have yet to be described in ovarian cancers.

Preliminary studies in breast, prostate, hepatocellular, pancreatic, and ovarian cancer cell lines suggest that agents that block the action of IGF-2 may have potential therapeutic utility [30–33], such that the ability to down-regulated *IGF-2* expression could have significant clinical application in the management of patients with ovarian cancer in the future.

Our results support the hypothesis that insulin-like growth factor-2 plays an important role in ovarian carcinogenesis. Additionally, we have demonstrated that IGF-2 is a potential molecular marker of the most aggressive forms of epithelial ovarian cancer. Further elucidation of the role of the IGF pathway in ovarian cancer development and progression will increase our understanding of the biology of ovarian carcinogenesis, and may yield novel opportunities for cancer prevention and therapy.

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