Short Communication

Role of β -Catenin/T-Cell Factor-Regulated Genes in Ovarian Endometrioid Adenocarcinomas

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In various cancers, inactivating mutations in the adenomatous polyposis coli or Axin tumor suppressor proteins or activating mutations in β -catenin's amino-terminal domain elevate β -catenin levels, resulting in marked effects on T-cell factor (TCF)-regulated transcription. Several candidate β -catenin/TCF-regulated genes in cancer have been proposed. Expression of a few of these genes has been studied in primary human cancers, but most studies have focused on colon cancers and not on other cancer types that harbor mutational defects in adenomatous polyposis coli, AXIN, or β -catenin. Mutations leading to β -catenin deregulation are found in nearly half of ovarian endometrioid adenocarcinomas (OEAs). We report here on the expression of 6 candidate β -catenin/TCF-regulated genes in a panel of 44 primary OEAs, more than a third of which carry demonstrable defects in β -catenin regulation. Using quantitative assays of gene expression, we found significantly elevated expression of the *MMP-7*, *CCND1* (*Cyclin D1*), *CX43* (*Connexin 43*), *PPAR- δ* , and *ITF2* genes in OEAs with deregulated β -catenin. This correlation was not observed for *c-myc*, another putative β -catenin/TCF-regulated gene. Immunohistochemical studies confirmed that overexpression of cyclin D1 and MMP-7 was highly associated with nuclear accumulation of β -catenin and mutational defects of the Wnt/ β -catenin/TCF-signaling pathway. Our findings indicate cyclin D1, MMP-7, connexin 43, PPAR- δ , and ITF-2, likely play important roles in the pathogenesis of those OEAs that manifest defects in β -catenin regulation. (*Am J Pathol* 2002, 160:1229–1238)

In addition to its well-defined role in regulating cell-cell adhesion via interactions with E-cadherin and other classical-type cadherins, β -catenin has a critical role in the highly conserved Wnt signaling pathway.¹ Wnts are secreted glycoproteins with important roles in regulating cell fate specification, proliferation, and differentiation during development and in various adult tissues. Consistent with β -catenin's ostensibly independent functions in cell adhesion and signaling, distinct pools of β -catenin exist in cells, including a cadherin-associated (cell membrane-associated) pool and a cytoplasmic/nuclear pool involved in Wnt signaling. The β -catenin pool that functions in Wnt signaling is regulated in part by a multiprotein complex, consisting of the adenomatous polyposis coli (APC) tumor suppressor, AXIN, and glycogen synthase kinase 3 β (GSK3 β) proteins.^{2,3} In the absence of Wnt signals, β -catenin is bound by APC and either AXIN1 or AXIN2 and then phosphorylated by GSK3 β at one or more serine or threonine residues in its amino(N)-terminal domain. N-terminally phosphorylated β -catenin is recognized and ubiquitinated by a multiprotein complex and degraded by the proteasome. Wnt ligands, after their binding to a Frizzled-LRP transmembrane receptor complex, activate a pathway that inhibits GSK3 β activity, with resultant stabilization of β -catenin. Stabilization of β -catenin leads, in turn, to its enhanced interaction with members of the T-cell factor (TCF) family of transcription factors. β -catenin's interaction with TCF proteins alters their transcriptional activity, resulting in altered expression of genes with TCF-binding sites in their regulatory regions.

Defects in the Wnt signaling pathway seem to play a very significant role in human cancer. Mutations in the *CTNNB1* gene sequences that encode the crucial GSK3 β phosphorylation sites in β -catenin's N-terminal domain have been found in many different cancer types.^{4–14} Inactivating mutations in the *APC*, *AXIN1*, or *AXIN2* tumor

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suppressor genes in colorectal and selected other cancer types seem to inhibit formation of the functional complex necessary for phosphorylation of β -catenin.^{4,15–18} In the case of *APC*, the mutations may also interfere with the function of the APC protein in exporting β -catenin from the nucleus.¹⁹ A presumed critical consequence of Wnt pathway mutations, whether in the *CTNNB1*, *APC*, or *AXIN* genes, is elevation of β -catenin levels in the cytoplasm and nucleus. Deregulation of β -catenin leads to constitutive formation of β -catenin-TCF complexes and altered expression of TCF-regulated target genes. The proteins encoded by these TCF-regulated genes presumably play important roles in effecting neoplastic transformation. Proposed β -catenin/TCF target genes in cancer cells include well-established oncogenes, such as *c-myc* and *CCND1* (*Cyclin D1*), as well as the *MMP-7* (*matrix metalloproteinase 7/matrilysin*), *TCF7*, *LEF1*, *PPAR- δ* (*peroxisome proliferator-activated receptor delta*), *MDR1*, *WISP1*, *ID-2*, *CX43* (*connexin 43*), and *gastrin* genes.^{20–32}

Nearly all of these candidate β -catenin/TCF-regulated genes have been proposed based on data from *in vitro* or animal model studies. Very few studies have evaluated expression of the presumptive target genes in primary human tumors with documented mutational defects in β -catenin regulation. Of the published studies, nearly all have focused on colorectal cancers. For instance, a strong correlation was reported between β -catenin deregulation and cyclin D1 and MMP-7 expression in primary colorectal tumors.^{33,34} Brabletz and colleagues³⁵ also found a tight correlation between nuclear β -catenin accumulation and *c-myc* expression in colorectal adenomas, although neither feature correlated with grade of dysplasia or proliferative activity. In hepatoblastomas, β -catenin mutations were strongly associated with cyclin D1, but not with *c-myc* overexpression.³⁶ The findings obtained thus far suggest that only a subset of the proposed β -catenin/TCF target genes may play a key role in human cancer pathogenesis. An alternative view is that at least some of the candidate β -catenin/TCF target genes have critical but not wide-ranging roles in cancer, with certain candidate genes showing tissue-specific or context-specific roles in cancers with β -catenin deregulation. Therefore, given the many uncertainties about the relationship between defective β -catenin regulation and specific gene expression changes in human cancer, we sought to study expression of candidate β -catenin/TCF target genes in ovarian endometrioid adenocarcinoma (OEA), a tumor type known to have mutations affecting β -catenin regulation in upwards of half the cases.^{37–40} Examining expression of candidate target genes in OEAs with regulated *versus* deregulated β -catenin/TCF signaling should assist in identifying those target genes of greatest relevance in the pathogenesis of OEAs and perhaps other cancers. We report here on the analysis of 6 putative β -catenin/TCF target genes—namely, *c-myc*, *CCND1*, *PPAR- δ* , *ITF2* (immunoglobulin transcription factor 2), *CX43*, and *MMP-7*—in a panel of 44 primary OEAs previously characterized for specific defects in β -catenin regulation.⁴¹

Materials and Methods

Tumor Samples

A total of 44 snap-frozen primary OEAs were analyzed. Five specimens were obtained from the Johns Hopkins Hospital; 2 from the University of Michigan Hospital; and 37 from the Cooperative Human Tissue Network/Gynecologic Oncology Group Tissue Bank. A small portion of formalin-fixed, paraffin-embedded tissue from each specimen was histologically verified as OEA by a gynecological pathologist (KRC), and classified as well differentiated, moderately differentiated, or poorly differentiated, based on the tumor's histological features. Tumor stage (I to IV) was assigned according to the International Federation of Gynecology and Obstetrics system. Analysis of tissues from human subjects was approved by the University of Michigan's Institutional Review Board (IRB-MED no. 2001-0568).

RNA and cDNA Preparation

Primary tumor tissues were manually microdissected before nucleic acid extraction to ensure each tumor sample contained at least 70% neoplastic cells. Hematoxylin- and eosin-stained sections of the frozen tumor tissues were used to guide dissection. Total RNA was extracted from pooled frozen tissue sections with Trizol (Life Technologies, Inc., Rockville, MD), according to the manufacturer's protocol. First-strand cDNA was synthesized from DNase I-treated mRNA samples using random hexamer primers (Pharmacia Biotech, Piscataway, NJ) and Superscript II (Life Technologies, Inc.).

Ribonuclease (RNase) Protection Assay

Before RNase protection analysis, the integrity of primary tumor RNA was monitored by electrophoresis on 1% agarose/MOPS gels. Ethidium-bromide-stained gels were examined and only samples with intact 28S and 18S ribosomal bands were studied further. cDNA fragments used for generation of riboprobes were reverse transcriptase-polymerase chain reaction products subcloned into pGEM-4Z (Promega, Madison, WI). The length of the subcloned cDNA fragments were: *L32* (76 bp), *CX43* (180 bp), *ITF2* (203 bp), *MMP-7* (226 bp), *PPAR- δ* (255 bp), *CCND1* (286 bp), and *c-myc* (320 bp). The different probe lengths allowed their use in multiprobe reactions to conserve RNA from primary tumor samples. The probe for the ribosomal *L32* gene was generously provided by Dr. R. Rochford (University of Michigan School of Public Health, Ann Arbor, MI).⁴² To synthesize anti-sense probe sets, the pGEM-4Z subclones were linearized, and ³²P-labeled riboprobes were synthesized using T7 RNA polymerase and the Riboprobe *In Vitro* Transcription System (Promega). The final reaction contained 100 μ Ci of [α -³²P] UTP (3000 Ci/mmol; Amersham, Arlington Heights, IL); 1 μ l of ATP, CTP, GTP, and UTP mixture (2.5 mmol/L for ATP, CTP, and GTP; 10 μ mol/L for UTP); 2 μ l of dithiothreitol (100 mmol); 1 μ l of RNasin (40 U); 1 μ l of

T7 polymerase (10 U) and equal amounts of linearized probes (50 μ g of DNA for each probe). After 1 hour incubation at 37°C, reactions were terminated by adding RQ1-DNase (2 U) for 30 minutes at 37°C. Labeled probes were purified by phenol/chloroform extraction followed by ethanol precipitation. The purified probe mix was then dissolved in 50 μ l of hybridization buffer (80% formamide, 400 mmol/L NaCl, 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 40 mmol/L Pipes, pH 6.7) and diluted to an activity of 5×10^5 cpm/2 μ l. The probe set (2 μ l) was added to tubes containing 5 μ g of total RNA in 8 μ l of hybridization buffer, heated to 95°C for 2 minutes, then hybridized at 56°C overnight. Each sample was treated with 25 ng of RNaseA (Boehringer Mannheim, Indianapolis, IN) and 75 U of RNaseT1 (Promega) in 125 μ l of digestion buffer (10 mmol/L Tris, pH 7.5, 5 mmol/L EDTA, 30 mmol/L NaCl) for 45 minutes at 30°C. RNase digestion was followed by addition of 1.5 μ l of proteinase K (10 mg/ml) and 7 μ l of 10% sodium dodecyl sulfate and incubation for 15 minutes at 37°C. The protected probes were extracted, precipitated, and dissolved in loading buffer (80% formamide, 1 mmol/L EDTA, 50 mmol/L Tris-borate, pH 8.3), then separated by electrophoresis on 6% denaturing polyacrylamide gels. Gels were analyzed by autoradiography and the intensities of the specifically protected bands in gels were quantitated by PhosphorImager Analysis (Molecular Dynamics, Wayzata, MN). The expression level of each transcript species was determined relative to the L32 transcript.

Immunohistochemical Analyses of Cyclin D1 and MMP-7 Expression

Five- μ m sections of formalin-fixed, paraffin-embedded tissues were mounted on Probe-On slides (Fisher Scientific, Itasca, IL), deparaffinized in xylene, and then rehydrated into distilled H₂O through graded alcohols. Antigen retrieval was enhanced by microwaving the slides for 15 minutes in citrate buffer (pH 6.0; Biogenex, San Ramon, CA) for the MMP-7 immunohistochemistry and in 1 mmol/L EDTA (pH 8.0; Fisher Scientific, Pittsburgh, PA) for the cyclin D1 immunohistochemistry. Endogenous peroxidase activity was quenched by incubation with 6% hydrogen peroxide in methanol, then the sections were washed and blocked with 1.5% normal horse serum for 1 hour. Sections were then incubated with a mouse monoclonal anti-MMP-7 antibody (MAB3315; Chemicon International, Inc. Temecula, CA) at a dilution of 1:500, or anti-cyclin D1 antibody (NCL-cyclinD1-GM; Novocastra Laboratories, Newcastle, UK) at a dilution of 1:50 overnight at 4°C. Slides were washed in phosphate-buffered saline, then incubated with a biotinylated horse anti-mouse secondary antibody for 30 minutes at room temperature. Antigen-antibody complexes were detected with the avidin-biotin peroxidase method using 3,3'-diaminobenzidine as a chromogenic substrate (Vectastain ABC kit; Vector Laboratories, Burlingame, CA). Immunostained sections were lightly counterstained with hematoxylin then examined by light microscopy. Immunoreactivity for cyclin D1 and MMP-7 was interpreted

independently by two observers (YZ and RW). The results were scored semiquantitatively on the basis of cytoplasmic staining intensity for MMP-7 (–, no staining; $\pm$, focally positive; +, weak; ++, moderate; +++, strong) and the percentage of positive nuclei for cyclin D1 (–, no positive cells; $\pm$, <5%; +, 6 to 20%; ++, 21 to 50%; +++, >50%). For the rare cases with discordant scoring between the two observers, a final score was arrived at by consensus. Immunohistochemical analysis of β -catenin expression in this particular set of tumors has been previously reported.⁴¹

Statistical Analysis

The Student's *t*-test was used to determine the significance of differences in expression of candidate β -catenin/Tcf target genes in OEAs with and without Wnt pathway defects. Tumors with documented *CTNNB1* or *APC* mutations or unequivocally aberrant β -catenin localization were categorized as tumors with pathway defects.⁴¹ After quantitation of the signals in the RNase protection experiments and normalization of the values to *L32* expression, the mean expression and SD for each gene was assessed in the group of tumors with demonstrable defects in β -catenin regulation. Similarly, the mean expression and SD for each gene was determined in the group of tumors with intact β -catenin regulation. The difference in the expression of each candidate gene in the two groups of tumors was then assessed. Pearson correlation coefficients (*r*) were determined for comparisons between RNase protection and immunohistochemical expression data and between gene expression and β -catenin mutational and regulation status. All statistical analyses were conducted using GraphPad Prism software (GraphPad Prism, Inc., San Diego, CA). A *P* value <0.05 was considered statistically significant for all analyses.

Results

Expression of Candidate β -Catenin/Tcf Target Genes

As reviewed above, a number of candidate β -catenin/Tcf target genes have been suggested in the literature, including the *c-myc*, *CCND1*, *MMP-7*, *PPAR- δ* , and *CX43* genes.^{20,21,23,26,27} Another gene, *ITF2* (also known as *E2-2* or *SEF2*), which encodes a helix-loop-helix transcription factor, has recently been implicated as a potential β -catenin/Tcf target gene (Kolligs and colleagues, submitted). The expression of these six candidate β -catenin/Tcf target genes was evaluated using the ribonuclease (RNase) protection assay, a sensitive and quantitative assay of gene expression (Figure 1). Thirty-two of the 44 primary OEAs under study yielded RNA of suitable quality for the RNase protection analysis (Table 1). The 32 tumors included 15 OEAs with definitively established or ostensible Wnt/ β -catenin pathway defects [13 tumors with known *CTNNB1* mutations, and one tumor each with biallelic inactivation of the *APC* gene (OE-

Figure 1. Ribonuclease (RNase) protection assay of candidate β -catenin/Tcf target genes. Representative data from 22 tumors (11 tumors with altered β -catenin levels and localization and documented *CTNNB1* or *APC* gene mutations and 11 tumors with intact β -catenin regulation) are shown. Five μ g of total RNA from each tumor sample was hybridized to anti-sense 32 P-labeled riboprobes for *c-myc* (320 bp), *CCND1* (286 bp), *PPAR- δ* (255 bp), *MMP-7* (226 bp), *ITF2* (203 bp), *CX43* (180 bp), and *L32* (76 bp). After overnight hybridization, samples were treated with RNases. Protected probes were extracted, precipitated, and separated by electrophoresis on 6% polyacrylamide denaturing gels. The intensity of the protected fragments in each tumor sample was quantitated by PhosphorImager analysis and compared to the intensity of the *L32*-specific fragment.

32T), or diffuse strong cytoplasmic β -catenin immunoreactivity in the absence of membrane staining (OE-15T)]. The remaining 17 tumors had wild-type *CTNNB1* alleles and membranous and/or weak cytoplasmic β -catenin immunoreactivity. The expression of each candidate β -catenin/Tcf target gene was determined by comparing

the intensity of the specific protected fragment in the RNase protection assay to the intensity of the *L32*-specific fragment, using a phosphorimager to measure signal intensities. The mean expression of each candidate gene was then compared between the two groups of tumors (ie, those tumors with defective β -catenin regulation versus those tumors with intact β -catenin regulation). OEAs with deregulated β -catenin showed statistically significant increases in expression of the *MMP-7*, *CCND1*, *CX43*, *PPAR- δ* , and *ITF2* genes compared to tumors with intact β -catenin regulation (Figure 2). In contrast, no difference in *c-myc* expression was observed between the two tumor groups ($P = 0.64$). The results of real-time (quantitative) reverse transcriptase-polymerase chain reaction assays for *CCND1* and *ITF2* showed excellent correlation with the RNase protection results (data not shown).

Immunohistochemical Analysis of Cyclin D1 and MMP-7 Expression in OEAs

In an effort to determine whether the increased levels of transcripts for candidate β -catenin/Tcf target genes was associated with increased protein expression, we performed immunohistochemical staining of the primary

Table 1. RNase Protection Assay: Expression of Candidate β -Catenin/Tcf Target Genes Relative to *L32*

		β -cat/Tcf* pathway status	<i>CX 43</i>	<i>ITF2</i>	<i>MMP-7</i>	<i>PPAR-δ</i>	<i>CCND1</i>	<i>c-MYC</i>
1	OE-02T	β -cat mut	15.70	13.00	3.10	3.70	35.60	4.10
2	OE-13T	β -cat mut	0.04	5.87	11.05	2.75	10.26	11.26
3	OE-15T	ND [†]	3.90	21.70	1.50	3.30	18.60	30.60
4	OE-17T	β -cat mut	2.40	2.60	0.40	14.20	43.90	4.70
5	OE-18T	β -cat mut	3.90	18.60	129.70	9.71	23.40	12.80
6	OE-19T	β -cat mut	9.00	18.80	0.90	9.00	34.90	10.80
7	OE-21T	β -cat mut	23.40	6.90	40.10	7.40	3.20	18.20
8	OE-22T	β -cat mut	15.60	11.90	58.10	4.40	7.40	13.20
9	OE-32T	APC mut	28.90	9.80	15.70	4.60	49.10	8.40
10	OE-36T	β -cat mut	27.70	21.00	73.30	4.80	7.30	5.50
11	OE-37T	β -cat mut	8.00	4.00	36.20	5.56	27.80	14.40
12	OE-44T	β -cat mut	8.20	4.40	68.20	2.41	11.80	17.10
13	OE-45T	β -cat mut	6.80	9.20	7.10	4.40	41.80	1.90
14	OE-47T	β -cat mut	17.00	8.70	4.10	1.30	72.70	5.90
15	OE-48T	β -cat mut	7.60	8.30	18.90	6.60	22.80	4.80
16	OE-01T	ND	0.00	0.09	0.00	0.24	0.00	1.16
17	OE-06T	ND	0.01	1.10	0.73	0.95	0.00	0.00
18	OE-07T	ND	0.01	1.82	1.77	12.59	3.02	10.50
19	OE-08T	ND	0.02	0.93	2.61	0.00	0.00	0.00
20	OE-11T	ND	0.04	2.09	2.01	2.03	20.02	15.44
21	OE-12T	ND	12.30	11.80	3.50	3.10	4.90	5.20
22	OE-14T	ND	3.00	3.00	4.50	1.50	12.20	9.50
23	OE-16T	ND	9.90	3.60	7.10	5.00	9.10	22.90
24	OE-23T	ND	0.30	0.40	0.40	4.00	13.10	8.00
25	OE-24T	ND	0.20	1.00	2.10	0.60	2.40	10.20
26	OE-25T	ND	2.60	1.40	0.70	2.20	2.00	14.20
27	OE-31T	ND	1.50	0.90	0.01	1.20	8.30	0.90
28	OE-33T	ND	8.60	3.10	26.10	3.90	14.60	5.20
29	OE-38T	ND	4.60	10.80	2.90	1.70	23.90	61.10
30	OE-40T	ND	4.70	2.90	11.40	1.80	17.40	21.00
31	OE-42T	ND	4.30	2.70	7.00	1.40	0.06	16.20
32	OE-43T	ND	12.20	2.30	8.30	3.40	21.80	15.70

*Specific mutations reported in Wu et al.⁴¹ ND, not detected.

[†]Deregulated β -catenin based on aberrant cellular localization of β -catenin protein.

Figure 2. Average expression of candidate β -catenin/TCF target genes in OEAs with β -catenin regulation defects or intact β -catenin regulation. The data in Table 1 were used to determine the average expression of the indicated candidate genes across groups of tumors with and without known or presumptive Wnt pathway defects. The normalized data (normalized to L32 expression) were analyzed using a two-tailed Student's *t*-test assuming equal variance. All genes except *c-myc* showed significantly increased expression in the OEAs with defective β -catenin regulation.

OEA tissues to evaluate expression of the two genes with the highest transcript levels—MMP-7 and CCND1. All 44 tumors proved suitable for the immunohistochemical analysis. Based on our previous mutational and immunohistochemical analyses of Wnt pathway defects in the OEA specimens,⁴¹ 16 of the 44 OEAs were found to have persuasive evidence of defects in β -catenin regulation. These 16 tumors included 14 tumors with CTNNB1 mutations, 1 tumor with biallelic inactivation of APC (OE-32T), and 1 tumor with aberrant localization of β -catenin protein by immunohistochemistry (OE-15T). A tumor previously found to have a nonsense mutation in one allele of AXIN1 (OE-29T) was grouped with the 28 tumors with intact β -catenin regulation, because neither biallelic inactivation of AXIN1 nor aberrant localization of β -catenin protein could be demonstrated in the tumor. Cyclin D1 nuclear immunoreactivity scores ranging from + to +++ (see Methods and Materials for scoring) were observed in 11 of 16 (68.8%) tumors with deregulated β -catenin (Table 2 and Figure 3; A to D), including 9 of the 14 tumors with CTNNB1 mutations, OE-32T, and OE-15T. In contrast, only 2 of the 28 (7.1%) tumors with intact β -catenin regulation showed clearly positive reactivity for cyclin D1, with the remaining 26 tumors showing negative or limited focal cyclin D1 staining (Table 2 and Figure 3, E and F).

Cytoplasmic immunoreactivity for MMP-7 was observed in 40 of the 44 primary OEAs (Table 2). However, tumors displayed very different patterns depending on the status of β -catenin. Strong (+++) or moderate (++) staining for MMP-7 was seen in 9 of 14 tumors with documented CTNNB1 mutations, as well as in OE-32T and OE-15T (Table 2 and Figure 3, G and H). Conversely, moderate MMP-7 immunoreactivity was observed in only 5 of the 28 (17.8%) tumors with intact β -catenin regulation, with the remainder showing only weak (+) or negative MMP-7 expression (Table 2 and Figure 3, I and J).

In our previous study, nuclear immunoreactivity for β -catenin was observed in 14 of the 44 OEAs.⁴¹ Notably, tumors with nuclear accumulation of β -catenin and/or CTNNB1 mutations were always well or moderately dif-

ferentiated. Nuclear β -catenin accumulation was significantly correlated with elevated MMP-7 and cyclin D1 expression ($r = 0.50$, $P < 0.0006$ and $r = 0.77$, $P < 0.0001$, respectively). Elevated MMP-7 and cyclin D1 expression were also tightly correlated with CTNNB1 gene mutation ($r = 0.52$, $P < 0.0004$ and $r = 0.61$, $P < 0.0001$, respectively) (Table 2). Moreover, the membrane-associated β -catenin-staining pattern, which we inferred here to reflect intact β -catenin regulation, did not correlate with MMP-7 overexpression ($r = -0.23$) and showed a significant inverse correlation with cyclin D1 overexpression ($r = -0.44$, $P < 0.003$). Finally, we noted that, for both MMP-7 and cyclin D1, elevated expression of RNA and protein were highly concordant ($r = 0.75$, $P < 0.0001$ and $r = 0.80$, $P < 0.0001$, respectively).

Discussion

Comparison of gene expression patterns in cancers versus their matched normal (nonneoplastic) cellular counterparts is a potentially useful approach to uncover genes that may play an important role in cancer pathogenesis. Nonetheless, because of uncertainties regarding the specific cell of origin for most epithelial cancers and the many phenotypic differences observed on comparison of cancer and normal cells, it is often difficult to distinguish the genes most likely to play a causal role in cancer development from the genes with a nominal role in the process, through simple comparisons of expression differences between normal and cancer cells. As such, comparison of differential gene expression patterns in cancers of a single well-defined histological type, but which differ in genotype, may be a more robust initial strategy for ferreting out those genes with a critical role in pathogenesis. For instance, such an approach might be used to address the identity of downstream genes that are deregulated as a result of mutational defects in a specific pathway, such as the p53, Rb/E2F, *c-myc*, or Wnt/ β -catenin pathway. With this notion in mind, we performed studies to address the relationship between mutational defects in β -catenin regulation and the expression of candidate β -catenin/TCF target genes in ovarian carcinomas of a single histological type, namely OEAs. We have shown here that several presumptive β -catenin/TCF target genes do indeed show significantly increased expression in primary OEAs with known Wnt/ β -catenin pathway defects. Although direct regulation of these genes by β -catenin in OEAs is likely, we recognize that our studies have provided only circumstantial evidence supporting this assumption. Moreover, we have not directly demonstrated the functional significance of increased β -catenin/TCF target gene expression in OEAs.

The gene encoding cyclin D1 (CCND1) has been identified as a key target gene regulated by the Wnt/ β -catenin pathway in colorectal cancer cells and possibly also in breast cancer.^{25,26,43} Our findings support the view that increased expression of cyclin D1 may also play a vital role in the pathogenesis of some OEAs. Interestingly, previous work demonstrated increased cyclin D1 expression in 5 to 32% of ovarian carcinomas, often in the

Table 2. Immunohistochemical Analysis of β -Catenin, Cyclin D1, and MMP-7 in OEAs

Case	Tumor ID	Age	Clinical data		β -catenin [†]			MMP-7, C	Cyclin D1, N	β -cat/TCF pathway defect [‡]
			Stage	Grade*	N	C	M			
1	OE-02T	65	1	WD	+++			+	++	β -cat mut
2	OE-13T	39	1A	MD	+	+		+	+	β -cat mut
3	OE-17T	67	1C	WD	+	+		++	+	β -cat mut
4	OE-18T	35	2A	WD	++	++		+++	+/-	β -cat mut
5	OE-19T	50	1A	WD			++	+/-	-	β -cat mut
6	OE-21T	52	3B	WD	+	+		++	+/-	β -cat mut
7	OE-22T	53	2B	WD	+	+		++	+	β -cat mut
8	OE-36T	45	2A	WD	++	++		+++	+/-	β -cat mut
9	OE-37T	N/A	N/A	WD	+	+		++	++	β -cat mut
10	OE-44T	69	1	WD	+	+	+++	+++	-	β -cat mut
11	OE-45T	N/A	N/A	MD	++	++	+	++	++	β -cat mut
12	OE-47T	72	1A	WD	++			+	+++	β -cat mut
13	OE-48T	N/A	1C	WD	+	+	+	+	+	β -cat mut
14	OE-71T	49	1C	MD	++			++	++	β -cat mut
15	OE-32T	47	1C	WD	++	+		++	++	APC mut
16	OE-15T	61	1C	PD		++		++	+	ND
17	OE-01T	60	3	PD		++		-	-	ND
18	OE-06T	45	3	PD		++	++	-	-	ND
19	OE-07T	69	3C	PD		+	+	+/-	-	ND
20	OE-08T	73	3	PD		++	++	+	-	ND
21	OE-11T	43	2B	PD		+		+/-	-	ND
22	OE-12T	77	1C	WD			+	+/-	-	ND
23	OE-14T	50	4	MD		+	+++	+	+/-	ND
24	OE-16T	48	1	WD			+	+	-	ND
25	OE-20T	69	4	MD			++	++	-	ND
26	OE-23T	68	3C	PD		++	++	-	+/-	ND
27	OE-24T	66	3C	WD			++	+/-	-	ND
28	OE-25T	58	3C	PD		+	+	+	-	ND
29	OE-26T	48	3C	MD		+	+	-	-	ND
30	OE-28T	73	4	PD		+	+	+	-	ND
31	OE-29T	72	3C	MD		+	++	++	-	Axin1 mut [§]
32	OE-30T	51	1C	WD		+	+	+	-	ND
33	OE-31T	49	3C	PD			+	+	-	ND
34	OE-34T	48	2A	PD		+		+	-	ND
35	OE-35T	70	4	PD		+	+	+	-	ND
36	OE-38T	49	3C	MD		+	+	+	-	ND
37	OE-39T	73	3C	MD		++	+++	+/-	+/-	ND
38	OE-40T	58	3C	MD			+	+	+/-	ND
39	OE-41T	N/A	1B	PD		+	+	++	+/-	ND
40	OE-42T	61	3C	MD			+	+	-	ND
41	OE-43T	43	3C	MD		+	+	++	+	ND
42	OE-46T	N/A	N/A	PD		+	+	+	-	ND
43	OE-49T	53	3C	PD		+	++	+	-	ND
44	OE-72T	52	3	MD			+	++	+	ND

*PD, poorly differentiated; MD, moderately differentiated; WD, well differentiated.

[†] β -catenin staining reported previously.⁴¹ N, nuclear; C, cytoplasmic; M, membrane.

[‡]Mutational analyses reported previously.⁴¹ ND, not detected.

[§]Nonsense AXIN1 mutation (AAG[Lys] → TAG[stop]) affecting one allele.⁴¹

absence of *CCND1* amplification.^{44–47} Notably, in some of the studies, a predilection was observed for cyclin D1 overexpression in low-grade ovarian carcinomas and ovarian tumors of low malignant potential. The basis for this previous association may now be understood, at least in part, because OEAs with mutant β -catenin are more frequently low grade, exhibit squamous differentiation, and are associated with a favorable prognosis.^{14,37,38}

MMP-7 has been previously implicated as a β -catenin/TCF-regulated target gene, in large part because of studies in murine and human intestinal tumors.^{27,33} A previous study has described increased expression of *MMP-7* in ovarian carcinomas and tumors of low malignant potential, although no clear insights were offered into the basis for elevated *MMP-7* in the tumors.⁴⁸ In our analysis, primary OEAs with β -catenin defects showed higher ex-

Figure 3. Correlation of β -catenin staining with staining for cyclin D1 and *MMP-7* in representative OEAs. Serial sections of an OEA with mutant β -catenin (OE-47T) showed nuclear staining of β -catenin (A) and nuclear staining for cyclin D1 (B). A tumor with biallelic inactivation of *APC* (OE-32T) also showed nuclear β -catenin localization (C) and positive staining for cyclin D1 (D) in the neoplastic cells. In contrast, a tumor lacking β -catenin mutation (OE-20T) showed a membranous pattern of β -catenin staining (E) and lack of definitive immunoreactivity for cyclin D1 (F). Serial sections of an OEA with mutant β -catenin (OE-18T) and nuclear β -catenin staining (G) showed strong immunoreactivity for *MMP-7* (H). A tumor with wild-type β -catenin and membranous β -catenin immunoreactivity (I) showed no staining for *MMP-7* (J) (OE-23T). Original magnifications, $\times 400$.

pression of MMP-7 mRNA and protein than did OEAs with intact β -catenin regulation. However, a few (5 of 28) of the OEAs with intact β -catenin regulation did show definite MMP-7 protein expression. This is not an entirely surprising result, as MMP-7 expression is likely to be regulated by multiple different signaling pathways. For instance, epidermal growth factor signaling seems to increase MMP-7 levels in human colon adenocarcinoma cells.⁴⁹

Although the *CX43* and *PPAR- δ* genes have been identified as targets of the Wnt/ β -catenin signaling pathway in certain cell types and contexts,^{20,23} neither gene has been much studied in ovarian carcinomas. In fact, in one study, connexin 43 was noted to be expressed at reduced levels in ovarian carcinomas compared to normal ovarian surface epithelial cells.⁵⁰ Our data showing differences in the expression of *CX43* and *PPAR- δ* transcripts in OEAs with β -catenin defects versus OEAs with intact β -catenin regulation highlights the potential role of these two genes in ovarian cancer development. Yet another potential β -catenin/TCF target gene in OEAs is the *ITF2* gene (also known as *E2-2* or *SEF2*), which encodes a helix-loop-helix transcription factor that may oligomerize with itself and potentially other helix-loop-helix transcription factors, such as the E12 and E47 proteins and MyoD.^{51–53} *ITF2* has been implicated as a potential β -catenin/TCF target gene, primarily as the result of studies in tissue culture model systems (Kolligs and colleagues, submitted). The results of the studies presented here indicate *ITF2* deregulation may play a role in OEAs with defective β -catenin regulation and imply that further studies of the role of *ITF2* in other cancers with defects in β -catenin regulation may be of some interest.

c-myc was one of the first genes to be implicated as a β -catenin/TCF-regulated target gene in cancer.²¹ However, previous studies of the relationship between β -catenin deregulation in cancer and *c-myc* expression have yielded mixed results. For example, although a high degree of correlation between nuclear β -catenin accumulation and overexpression of *c-myc* protein was seen in colon tumors,³⁵ similar studies in hepatoblastoma and breast cancer found no such correlation.^{36,54} Studies in an *in vitro* model system in which mutant β -catenin induces neoplastic transformation of rat epithelial cells also failed to implicate *c-myc* as a critical β -catenin/TCF target gene.⁵⁵ Our RNase protection assay revealed no correlation between the level of *c-myc* transcripts and the status of β -catenin regulation in OEAs. In fact, on average, *c-myc* was expressed at modestly higher levels in the group of OEAs with intact regulation of β -catenin than in the tumors with deregulated β -catenin. Thus, despite the fact that elevated *c-myc* expression has been reported in upwards of 30% of ovarian cancers,^{56–58} it seems likely β -catenin/TCF plays at best a minor role in altered *c-myc* expression in ovarian cancer, with gene amplification of *c-myc* likely playing a more prominent role, particularly in advanced stage serous carcinomas.

It should be noted that although the *CCND1*, *MMP-7*, *PPAR- δ* , *CX43*, and *ITF2* genes were more highly expressed in the group of OEAs with β -catenin deregulation, the genes were not uniformly up-regulated in all OEAs with defective β -catenin regulation. Neither were

the genes uniformly expressed at low levels in all OEAs with intact β -catenin regulation. There are several possible explanations for these results. It is possible that the status of β -catenin regulation was misclassified in some tumors. We think this explanation is not a major factor in the cases under study, because we not only characterized β -catenin localization and levels by immunohistochemistry in all 44 tumors, but we also carefully assessed the mutational status of the Wnt/ β -catenin pathway genes known to be mutated in cancers with observed deregulation of β -catenin (ie, the *CTNNB1*, *APC*, *AXIN1*, and *AXIN2* genes).⁴¹ Another possible explanation for the absence of a strict correlation between β -catenin defects and gene expression is that variable expression of β -catenin-binding proteins, such as ICAT or Pin1,^{59,60} modulates β -catenin function and its ability to activate TCF-regulated target genes. Yet a third possibility is that expression of one or more of the β -catenin/TCF target genes under study was substantially impacted by alterations in other conserved signaling pathways and/or transcription factor complexes. This explanation seems reasonable for at least some of genes and tumors studied, as a number of candidate β -catenin/TCF target genes, including *c-myc*, *CCND1*, and *MMP-7*, presumably lie downstream of diverse signaling pathways and transcription factors. Further studies of gene expression profiles in the panel of OEAs studied here should assist with identification of novel downstream target genes in the Wnt/ β -catenin pathway as well as efforts to define critical factors in ovarian cancer pathogenesis.

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