Tumorigenesis and Neoplastic Progression

Oncogenic Regulators and Substrates of the Anaphase Promoting Complex/Cyclosome Are Frequently Overexpressed in Malignant Tumors

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The fidelity of cell division is dependent on the accumulation and ordered destruction of critical protein regulators. By triggering the appropriately timed, ubiquitin-dependent proteolysis of the mitotic regulatory proteins securin, cyclin B, aurora A kinase, and polo-like kinase 1, the anaphase promoting complex/cyclosome (APC/C) ubiquitin ligase plays an essential role in maintaining genomic stability. Misexpression of these APC/C substrates, individually, has been implicated in genomic instability and cancer. However, no comprehensive survey of the extent of their misregulation in tumors has been performed. Here, we analyzed more than 1600 benign and malignant tumors by immunohistochemical staining of tissue microarrays and found frequent overexpression of securin, polo-like kinase 1, aurora A, and Skp2 in malignant tumors. Positive and negative APC/C regulators, Cdh1 and Emi1, respectively, were also more strongly expressed in malignant versus benign tumors. Clustering and statistical analysis supports the finding that malignant tumors generally show broad misregulation of mitotic APC/C substrates not seen in benign tumors, suggesting that a “mitotic profile” in tumors may result from misregulation of the APC/C destruction pathway. This profile of misregulated mitotic APC/C substrates and regulators in malignant tumors suggests that analysis of this pathway may be diagnostically useful and represent a potentially important therapeutic target. (*Am J Pathol* 2007, 170:1793–1805; DOI: 10.2353/ajpath.2007.060767)

Tumor progression is characterized by misregulation of critical growth regulatory mechanisms. Typically, activation of growth factor pathways, eg, through tyrosine kinases or growth factors up-regulating cyclin D, and loss of growth regulatory tumor suppressors, eg, pRb, p16, and p53, directs unscheduled cell division.¹ In many tumors, neoplastic transformation is strongly linked to the development of chromosome instability, leading to activation of the aforementioned and additional oncogenic processes.

Recent studies have demonstrated that failure of normal chromosome segregation leading to subsequent mitotic catastrophe is a central mechanism among events leading to chromosome or genomic instability. Mitotic catastrophe is often linked to a failure of cytokinesis, giving rise to tetraploid or aneuploid cells. Tetraploidy is thought to provide a buffer against genetic loss in genomically unstable cells, having recently been shown to be the preferred pathway for cells that fail mitosis² and to otherwise independently lead to a tumorigenic state in p53-null cells.³ Mitotic catastrophe also leads to aneuploidy, possibly through tetraploid intermediates, and the genomic rearrangement typically seen in malignant tumors.

Misregulation of specific mitotic regulators can drive mitotic catastrophe in model genetic organisms, in cultured mammalian cells, and in mouse models. Notably, over- or underexpression of the mitotic kinases aurora A and polo-like kinase 1 (Plk1) and the chromosome segregation regulator securin can each give rise to mitotic catastrophe.^{4–7} Each of these proteins, along with the mitotic entry regulator Skp2,^{8,9} have been suggested to

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Figure 1. Model for pRb- and APC/C-dependent control of S phase and early mitosis. G₁ proliferation control genes upstream of Emi1 (shown in blue) regulate the E2F-dependent expression of Emi1 and certain APC/C substrates (cyclin A, Plk1, and securin). Accumulation of Emi1 stabilizes APC/C substrates vital for progression through S phase (blue) and mitosis (pink). When overexpressed Emi1, or the mitotic control APC/C substrates Skp2, Plk1, securin, or aurora A can induce mitotic catastrophe. In the absence of p53, these genomically unstable cells survive and lead to tumor formation.

be oncogenic, possibly by driving chromosomal rearrangement. Of interest, these proteins are substrates of the anaphase promoting complex or cyclosome (APC/C), the E3 ubiquitin ligase controlling destruction of mitotic cyclins, and other mitotic regulators, among other proteins.¹⁰ A biologically consistent model is that the carefully timed destruction of these proteins in mitosis reflects the importance of restricting their abundance and that their overexpression disrupts the timing of mitotic events.

The APC/C is a multisubunit ubiquitin ligase that recognizes critical RXXL or KEN amino acid motifs (degrons) within protein substrates to assemble polyubiquitin chains on these substrates, thereby targeting them to the 26S proteasome for proteolytic destruction. The APC/C exists in two forms depending on its associated activator protein, Cdc20 or Cdh1 (homologous to the *Drosophila* protein Fizzy-related and should not be confused with cadherin E, previously referred to as Cdh1 for cadherin 1). The APC/C^{Cdc20} functions in early mitosis to destroy cyclin A and securin and is regulated by the mitotic spindle assembly checkpoint (discussed below). The APC/C^{Cdh1} functions later in mitosis to direct the destruction of a host of mitotic regulators, thereby promoting mitotic exit.

To achieve the critical timing of substrate destruction, the APC/C itself must be tightly regulated. At the G₁/S transition, the APC/C^{Cdh1} ligase is inhibited by the zinc-binding protein Emi1^{11,12} (Figure 1). This allows APC/C substrate proteins important for progression of S phase and early mitosis to accumulate.^{13,14} In early mitosis, Emi1 is phosphorylated by Plk1,¹⁵ which triggers its ubiquitination by the SCF^{BT_{RC}P} E3 ubiquitin ligase.¹⁴ This in turn causes the activation of the APC/C in early prometaphase and cell cycle progression through early mitosis.

During late prometaphase and metaphase, a group of proteins comprising the mitotic spindle checkpoint inhibits APC/C^{Cdc20} activity. The function of the spindle checkpoint is to prevent chromosome segregation from occurring before the metaphase mitotic spindle has perfectly formed, to ensure the equal segregation of sister chro-

matids to each daughter cell.¹⁶ The APC/C activator Cdh1 is itself an APC/C substrate,¹⁷ further exemplifying the tight and complex regulation of the APC/C. For mitotic progression to occur smoothly, the APC/C initiates the sequential, timed destruction of cyclin A, securin, cyclin B, aurora A, aurora B, Plk1, and Cdh1 (Figure 1). The precise details of how these specific events are organized are currently the subject of intense study.

Given the exquisite timing of events that is necessary during mitosis, it is not surprising that misregulation of the APC/C appears linked to catastrophic events in mitosis. Illustrating this theme, misregulation of either of two critical regulators of the APC/C, the Mad2 spindle checkpoint protein and the APC/C inhibitor Emi1, induces mitotic catastrophe.^{14,18} Because inhibition of the APC/C can stabilize a host of key mitotic regulators, including aurora A, securin, Plk1, and cyclins, even subtle misregulation of the APC/C has the potential to cause genomic instability. The APC/C substrate Skp2 is also an important cell cycle control protein. Skp2 seems to regulate both the G₁/S and G₂/M transitions, where, as a subunit of an SCF E3 ligase, it is required for ubiquitination of the cyclin-dependent kinase inhibitor p27.¹⁹

Recent studies suggest that APC/C regulation and the control of cyclin accumulation may be linked to growth factor pathways frequently misregulated in cancer. Notably, both the Emi1 and Mad2 APC/C inhibitors have been shown to be targets of E2F transcription factors,^{13,20} potentially linking the frequent misregulation of the cyclin D/retinoblastoma/E2F pathway to APC/C misregulation. We previously found that Emi1 mRNA was up-regulated in several human tumors,¹³ which led us to suspect that APC/C misregulation might be a common event in cancer. We have also shown that Emi1 overexpression leads to unscheduled cell proliferation, tetraploidy, and chromosomal instability in p53-deficient cells.²¹ In p53 wild-type cells, the induction of tetraploidy and aneuploidy by overexpression of APC/C inhibitors like Emi1 typically leads to G₁ arrest or apoptosis. In p53 checkpoint-deficient cells, the continuation of unchecked proliferation in the face of severe chromosome rearrangement by mitotic catastrophe probably results in the striking aneuploidy seen in many malignant tumors.

Here, we examined the extent of APC/C pathway misregulation in human neoplasms by a broad survey of the protein expression of APC/C substrates and the APC/C regulators Emi1 and Cdh1 in all major types of human tumors using immunohistochemical analysis of tissue microarrays (TMAs).²² Analysis of more than 1600 benign and malignant tumors revealed over-accumulation of securin, Plk1, aurora A, Cdh1, and Emi1 in malignant tumors but generally not in benign tumors. Strikingly, the misregulation of these mitotic regulators was strongly linked in specific classes of highly malignant tumors. We propose that misregulation of the mitotic destruction pathway leads to a "mitotic APC/C substrate profile" of misregulation in malignant tumors and that this profile may be of predictive value in diagnosis and therapeutic response.

Figure 2. Validation of anti-Emi1 immunohistochemical staining. **A:** An Emi1-immunopositive ovarian clear cell carcinoma was stained with anti-Emi1 antibody showing characteristic cytoplasmic Emi1 immunoreactivity (top) or antibody preincubated with recombinant antigen eliminating Emi1-specific immunostaining (bottom). **B:** HCT116 cells transfected with control siRNAs specific for green fluorescent protein (top) or siRNAs specific for Emi1 (bottom) were fixed and immunostained for Emi1. Cells transfected with Emi1 siRNA express considerably less Emi1 as indicated by the decrease in red AEC chromagen labeling of the cells and elimination of the Emi1 band by Western blot (right).

Materials and Methods

Short Interfering RNA (siRNA)

siRNA for human Emi1 target sequence 5'-AAACU-UGCUGCCAGUUCUUC-3' and control siRNA for green fluorescent protein target sequence 5'-GGCTACGTC-CAGGAGCGCACC-3' (Dharmacon RNA Technologies, Lafayette, CO) were transfected into the HCT116 cells shown in Figure 2 using Oligofectamine (Invitrogen, Carlsbad, CA) in serum-free culture media for 4 hours.

Immunohistochemistry

Immunohistochemical staining was performed on 4- μ m paraffin-embedded tissue sections and TMA slides. An-

tigen retrieval was by citrate (pH 6.0), ethylenediamine tetraacetic acid (pH 8.0), or Tris (pH 10.0) buffer and microwave heating. Endogenous peroxidase and non-specific binding (when necessary) were blocked using 3% hydrogen peroxide and Power Block (Biogenix, San Ramon, CA), respectively. The secondary antibody was Envision Plus (Dako, Glostrup, Denmark) anti-mouse or anti-rabbit-horseradish peroxidase. 3,3-Diaminobenzidine was the chromagen. The counterstain was Mayer's hematoxylin. HCT116 cells in Figure 2 were fixed in acetone; 3-amino-9-ethyl carbazole was the chromagen, and Gills's hematoxylin was the counterstain. Primary antibodies were as follows: affinity-purified rabbit anti-human Emi1 as previously described¹³; anti-human aurora-A monoclonal antibody²³; anti-human cyclin E monoclonal antibody (Novacostra, Newcastle, UK); rabbit anti-human cyclin A (H-432), cyclin B1 (H-433), cyclin D1 (H-295), cyclin D2 (C-17), cyclin E (C-19) (used for the breast TMA only), and E2F-3 (C-18) (Santa Cruz Biotechnologies, Santa Cruz, CA); rabbit anti-human phospho-pRb (Ser 807/811) (Cell Signaling Technologies, Danvers, MA); anti-human β -catenin, p27^{KIP1} and Skp2 monoclonal antibodies, rabbit anti-human securin (PTTG1), anti-Plk1, and anti-Cdh1 antibodies (Zymed Laboratories, South San Francisco, CA); and anti-Ki67, anti-Bcl2, anti-HER2, and anti-ER α mouse monoclonal antibodies (Dako). Staining parameters for these antibodies are summarized in Table 1.

Tissue Microarrays

TMA's composed of 0.6-mm cores were constructed from formalin-fixed and paraffin-embedded human tissues (with the exception that the tissue was first fixed with ethanol for the breast TMA), immunostained, and scored by a pathologist as previously described.²⁴ TMA's were scored according to the number of tumor cells demon-

Table 1. Immunostaining Parameters for Primary Antibodies Used

Target	Species and type	Pretreatment and dilution	Staining pattern
Aurora A	Mouse monoclonal	Citrate 1:25	Cytoplasmic
Bcl2	Mouse monoclonal	Citrate 1:50	Nuclear
β -Catenin	Mouse monoclonal	Citrate 1:25	Nuclear and cytoplasmic
Cdh1	Rabbit polyclonal	Citrate 1:20	Cytoplasmic
Cyclin A	Rabbit polyclonal	Citrate 1:100	Nuclear
Cyclin B1	Rabbit polyclonal	EDTA 1:100	Nuclear
Cyclin D1	Rabbit polyclonal	Citrate 1:50	Nuclear and cytoplasmic
Cyclin D2	Rabbit polyclonal	Citrate 1:25	Nuclear and cytoplasmic
Cyclin E	Rabbit polyclonal	Citrate 1:250	Nuclear
Cyclin E	Mouse monoclonal	Citrate 1:30	Nuclear
E2F-3	Rabbit polyclonal	Citrate 1:400	Nuclear
Emi1	Rabbit polyclonal	Citrate 1:1000	Cytoplasmic
ER α	Mouse monoclonal	Citrate*	Nuclear
HER-2	Mouse monoclonal	Citrate 1:600	Entire membrane
Ki-67	Mouse monoclonal	Citrate 1:100	Nuclear
c-Myc	Mouse monoclonal	Citrate 1:200	Nuclear
Phos-pRb	Rabbit polyclonal	Citrate 1:50	Nuclear
Plk1	Rabbit polyclonal	Tris 1:20	Cytoplasmic
p27	Mouse monoclonal	Tris 1:1000	Nuclear
Securin	Rabbit polyclonal	Citrate 1:50	Cytoplasmic
Skp2	Mouse monoclonal	Tris 1:500	Nuclear

EDTA, ethylenediamine tetraacetic acid.
 *Prediluted manufacturer's kit.

Figure 3. Analysis of Emi1 protein expression in human tumors. Emi1 is highly expressed in retinoblastoma (eye whole mount and retinoblastoma rosette) and other major tumor types (TMA cores). The viable peripheral tumor tissue within the eye whole mount is Emi1 immunopositive, whereas the necrotic center is nonreactive. Normal lymph nodes and breast tissue exhibit less intense Emi1 staining than lymphomas and breast ductal cancer, respectively. Benign fibrocystic breast tissue and endometrial stroma show very little Emi1 staining in the ductal carcinoma *in situ* and endometrial cancer cores, respectively. Positive immunoreactivity is indicated by the brown diaminobenzidine chromogen.

strating specific immunoreactivity for a given primary antibody within a given sample core. Immunostaining was defined as negative (<3% of tumor cells positive), weak (3 to 29% of tumor cells positive), and strong (>30% tumor cells positive). Each tumor or tissue sample was represented once on the TMAs, except for the connective tissue tumor TMA where each sample was represented by two cores. In the latter case, the greatest percentage of positive cells of either core was scored. Data were processed, and complete-linkage hierarchical clustering was performed using samples in which 80 to 100% of marker data were available using Cluster and Tree View software.²⁴ To facilitate comparison of Emi1 and APC/C substrate accumulation with that of other markers, we weighted clustering on Emi1 expression, which ordered the tumors into Emi1-negative and -positive groups. This yielded identical cluster dendrograms of tumor markers compared with unweighted clustering. The neural TMA contained 180 tumors; the lymphoma TMA, 265 proliferative lesions; the breast TMA, 255 proliferative lesions; two cancer TMAs, 523 tumors of diverse tissues; and the connective tissue TMA, 460 tumors. TMAs contained variable numbers of control and normal tissue cores.

Statistical Analysis

To assess significance, we tabulated Pearson correlation coefficients and corresponding two-sided *P* values, based on normal theory,²⁵ for immunopositivity for protein markers relative to each other within TMAs (Supplemental Table 1, see <http://ajp.amjpathol.org>).

Results and Discussion

The APC/C Regulator Emi1 Is Highly Expressed in Tumors

To determine the possible extent of APC/C misregulation in human cancer, we used immunohistochemical staining to examine whether the APC/C regulators Emi1 and Cdh1 and several APC/C substrates were overexpressed in tumors. We began by extending our initial observation of Emi1 mRNA up-regulation in human tumors to the protein level. Validation of our anti-human Emi1 antibodies is shown in Figure 2.

Since Emi1 transcription is driven by E2F, we expected that Emi1 would be highly expressed in retinoblastomas, where E2Fs are not inhibited by pRb, and found strong Emi1 immunopositivity in retinoblastomas (Figure 3).

Because overexpression of Emi1 protein in tumors would be expected to lead to inappropriate APC/C inhibition and hyperaccumulation of APC/C substrates, we next used TMAs²² to screen a large sample of human tumors for Emi1 and APC/C substrate protein accumulation. Immunohistochemical staining of TMAs has several advantages over RNA-based methods for analyzing gene expression in tumors, particularly when examining components and substrates of the ubiquitin proteasome system.²⁶ Most importantly, relative message levels often do not accurately reflect relative protein levels in tumors, and TMAs allow semiquantitative measurement of protein levels within tumor cells specifically, without a confounding contribution from nontumorous stromal cells, as is the case with other methods.²⁶ Background staining can be a limitation of immunohistochemistry, but this can generally be minimized by careful optimization of antibody dilutions and antigen retrieval techniques and by interpretation performed by experienced pathologists using consistent criteria for immunopositivity between various tissues for a given antibody.

We found that Emi1 protein is highly expressed in a significant fraction of human neoplasms. Examples of TMA immunostaining for Emi1 and other markers are shown in Figure 3 and Supplemental Figures 1 and 2, see <http://ajp.amjpathol.org>. A summary of the immunohistochemical protein expression of Emi1, Cdh1, and the oncogenic APC/C substrates securin, Plk1, Skp2, and aurora A in many of the more common human tumors is presented in Figure 4. Here, tumors are grouped according to a classification system based on common developmental origin.²⁷ The figure depicts the percentage of immunopositive individual tumor specimens for each protein marker.

Notably, we found that 92% of renal cell carcinomas, 80% of cervical adenocarcinomas, 79% of hepatocellular carcinomas, 68% of oligodendrogliomas, 64% of lung adenocarcinomas, 62% of endometrial cancers, 55% of melanomas, and many lymphomas are Emi1 immunopositive. All germ cell tumors and all clear carcinomas of the ovary examined strongly expressed Emi1. A large fraction of other carcinomas, nonastrocytic neural tumors, and some sarcomas were also Emi1 immunopositive. Many astrocytomas, gastrointestinal adenocarcinomas, sarcomas, and most low-grade connective tissue tumors were Emi1 negative (Figure 4).

A number of trends were observed in specific classes of tumors. First, among neural and connective tissue neoplasms, benign tumors (indicated in blue in Figure 4) were typically Emi1-negative, whereas a subset of malignant tumors were Emi1-positive.

Second, in lymphomas, Emi1 expression generally paralleled increasing tumor grade. Here, 59% of World Health Organization grade I follicular lymphomas were Emi1 immunopositive compared with 82% of grade III follicular lymphomas and 81% of diffuse large B-cell lymphomas.

Peripheral T-cell lymphomas, a particularly aggressive lymphoma, were 100% Emi1 immunopositive.

Third, in some cancers, notably colon and breast cancer, substantial numbers of tumors lacked Emi1 immunoreactivity. Although some Emi1 immunoreactivity was present in corresponding normal tissues, it was of lower intensity and of a more compartmentalized manner within individual cells compared with staining in Emi1-positive tumors (Figure 3; Supplemental Figures 1 and 2, see <http://ajp.amjpathol.org>). The corresponding nonmalignant neoplasms, breast ductal papilloma and fibroadenoma, and premalignant neoplasm, colon tubular adenoma, showed Emi1 staining of intermediate intensity.

In breast cancer, more Emi1-positive tumors were low grade (43.8% grade 1, 31.3% grade 2, and 25.0% grade 3), whereas more Emi1-negative tumors tended to be higher grade (15.8% grade 1, 47.4% grade 2, and 36.8% grade 3). Emi1 (*Fbx05*) maps to chromosomal region 6q25,²⁸ close to the estrogen receptor α and Parkin genes, which frequently undergo loss of heterozygosity in breast cancers.²⁹ Likewise, papillary serous ovarian carcinomas, in which chromosomal region 6q25 is also frequently deleted, were Emi1 negative in approximately 50% of cases (Figure 4; Supplemental Figure 3, <http://ajp.amjpathol.org>). In these tumors, Emi1 is likely important at some early phase of tumor progression, but later Emi1 loss may provide a second step in tumor progression or may simply be a consequence of further genomic instability. Of biological note, the magnitude of differences in Emi1 protein levels between low- and high-Emi1-expressing ovarian tumors determined by Western blot (Supplemental Figure 3, <http://ajp.amjpathol.org>) was greater than the level of Emi1 overexpression that resulted in chromosomal instability in cell culture models.²¹

APC/C Substrates Are Frequently Overexpressed in Malignant Tumors

Because the extent of misregulation of APC/C substrates in human neoplasms is largely unknown, we surveyed APC/C misregulation in cancers by immunostaining TMAs for the APC/C substrates cyclin A, cyclin B, securin, aurora A, Plk1, Skp2, and Cdh1. To look for correlations, we analyzed these data using complete-linkage hierarchical clustering adapted for TMAs.²⁴ In many tumors with elevated Emi1 protein, several or all of the APC/C substrates clustered together, consistent with the model that Emi1 causes their stabilization. These include the following tumors: lung and cervical adenocarcinomas; lung, esophageal and head and neck squamous cell cancers; melanomas; lymphomas; urothelial transitional cell tumors; seminomas; ovarian clear cell carcinomas; several malignant neural tumors; and some sarcomas (Figures 4–6; Supplemental Tables 1 and 2, see <http://ajp.amjpathol.org>). Thus, it seems that APC/C misregulation by Emi1 overexpression or other factors may direct a broad program of APC/C substrate stabilization in tumors.

Specific APC/C substrates, notably Skp2, fail to follow the pattern in some tumors (Skp2 staining is not prevalent

EMBRYONIC						
Germ Cell		Emi1	Securin	Skp2	Plk1	Cdh1
Seminoma		100	91	20	100	82
						27
Primitive Differentiating						
PNET/Neuroblastoma		0	0	100	0	100
Nephroblastoma		0	67	33	33	33
Medulloblastoma		29	25	75	50	75
Ewing's Tumor		100	63	38	100	63
ENDODERM/ECTODERM						
Surface Epithelium		Emi1	Securin	Skp2	Plk1	Cdh1
Esoph. Adenocarcinoma		20	29	43	83	75
Gastric Adenocarcinoma		23	62	21	76	32
Colon Adenoca. Grade I		20	60	80	25	0
Colon Adenoca. Grade II		24	39	86	67	50
Colon Adenoca. Grade III		25	27	82	100	71
Esoph. Squamous Ca.		25	63	50	86	43
Lung Squamous Cancer		53	77	69	92	83
Head/Neck Squamous		58	74	65	64	67
Lung Adenocarcinoma		64	71	18	90	57
Breast Fibroadenoma		83	100	17	71	17
Breast C/S		44	75	10	92	38
Breast Ductal Cancer		28	79	29	87	70
Breast Lobular Cancer		17	86	8	83	91
Parenchymal Epithelium		Emi1	Securin	Skp2	Plk1	Cdh1
Prostate Cancer		19	15	20	88	67
Pancreas Adenoca		30	25	13	75	57
Hepatic Adenocarcinoma		79	75	7	92	82
Endocrine		Emi1	Securin	Skp2	Plk1	Cdh1
Thyroid Papillary Ca.		47	12	0	100	27
MESODERM						
Lymphoma		Emi1	Securin	Skp2	Plk1	Cdh1
Splenic Marginal Zone		40	0	20	100	0
CLL/SLL		50	38	0	71	57
Follicular, Grade I		59	72	28	72	64
Follicular, Grade II		60	81	55	80	76
Follicular, Grade III		82	77	34	79	84
Diffuse Large B-Cell		81	80	55	46	63
Lymphoblastic T-Cell		60	100	100	86	100
Peripheral T-Cell		100	70	70	100	100
Connective Tissue		Emi1	Securin	Skp2	Plk1	Cdh1
Granular Cell Tumor		0	0	0	100	0
Leiomyoma		0	0	0	100	0
Atypical Lipoma		0	0	33	63	0
Solitary Fibrous Tumor		0	0	50	67	100
DFSP		10	11	11	88	67
Myxofibrosarcoma		0	25	33	33	25
GI Stromal Tumor		8	39	16	92	57
Synovial Sarcoma		0	23	32	52	77
Osteosarcoma		17	7	64	69	71
Malig. Fibrohistiocytoma		19	25	34	97	73
Liposarcoma		20	38	30	83	83
Leiomyosarcoma		41	36	23	79	44
Rhabdomyosarcoma		60	41	86	94	100
Sub-Coelomic		Emi1	Securin	Skp2	Plk1	Cdh1
Renal Cell Carcinoma		92	7	7	73	62
Transitional Cell Cancer		44	36	32	74	67
Ovarian Papillary Cancer		52	76	96	85	71
Ovarian Clear Cell Ca.		100	33	67	83	100
Adrenocortical Cancer		50	50	75	100	50
Coelomic		Emi1	Securin	Skp2	Plk1	Cdh1
Endometrial Cancer		62	35	88	93	24
Cervical Adenocarcinoma		80	75	100	75	25
Cervical Squamous Ca.		50	100	80	100	0
NEUROECTODERM						
Neural Tube		Emi1	Securin	Skp2	Plk1	Cdh1
Astrocytoma Grade I		0	0	0	25	0
Astrocytoma Grade II		29	44	0	40	25
Astrocytoma, Grade III		20	43	40	0	40
Glioblastoma, Grade IV		35	55	50	76	71
Ependymoma		86	89	43	43	86
Oligodendroglioma		68	80	45	90	95
Neural Crest		Emi1	Securin	Skp2	Plk1	Cdh1
Neurofibroma		0	0	0	0	0
Schwannoma		0	0	0	18	0
Meningioma, Grade I		70	89	67	10	10
Meningioma, Grade II-III		100	100	100	13	38
MPNST		29	14	79	93	50
Melanoma		55	64	71	89	62

Figure 4. Summary of TMA analysis of Emi1 and APC/C substrate protein expression in human tumors. TMAs representing different classes of tumors, grouped according to developmental tissue origin, were analyzed by immunohistochemical staining. Numbers in boxes indicate the percentage of positive tumors (specific numbers of immunopositive and total tumors surveyed are summarized in Supplemental Table 2, see <http://ajp.amjpatbol.org>). Green indicates a low percentage of immunopositive tumors (<33% of cases); dark red, an intermediate percentage (33 to 66%); and bright red, a high percentage of Emi1-positive tumors (>66%). Benign tumors are highlighted by the blue boxes and less aggressive malignant tumors by yellow boxes. World Health Organization grades are indicated for astrocytomas and follicular lymphomas. Histopathological grades are also listed for colon adenocarcinomas. Adenoca, adenocarcinoma; Ca, carcinoma; CIS, carcinoma *in situ*; DFSP, dermatofibrosarcoma protuberans; esoph., esophageal; GI, gastrointestinal; HN, head and neck; MPNST, malignant peripheral nerve sheath tumor; PNET, primitive neuroectodermal tumor.

in hepatic, pancreatic, or renal carcinomas, despite strong involvement of other APC/C substrates), possibly because of genomic rearrangement or regulatory differences, eg, tissue-specific signaling factors or repressors. This is supported by the fact that Skp2 was infrequently overexpressed in all of the different types of breast tumors and all of the tumors derived from organ parenchymal epithelium (Figure 4).

APC/C misregulation by Emi1 is closely linked to the pRb/E2F transcriptional activation pathway.¹³ This may explain the lack of a uniform up-regulation of all APC/C substrates in some tumor types. Up-regulation of E2F-mediated transcription due to pRb loss or hyperphosphorylation (eg, from cyclin E up-regulation) may dominate over the effects of altered protein stability due to APC/C misregulation. Securin and Plk1 are both E2F targets; therefore, they would tend to cluster together when pRb transcription repression is misregulated. The effects of their overexpression on genomic instability would remain no matter what the mechanism of their misregulation. pRb is characteristically altered in seminoma, lung cancer, and transitional cell carcinoma. This would explain increased expression of Emi1 and/or Plk1 and securin in these tumors without concomitant aurora or Skp2 overexpression (Figure 4). It is important to note that overexpression of a single oncogenic APC/C substrate, such as aurora A, is sufficient to cause chromosomal instability or morphological transformation *in vitro*.⁵⁻⁹

Another parameter that might explain some of the non-uniformity of the APC/C cluster data are the inherent limitations of immunohistochemical analysis. Immunohistochemistry detects various thresholds of protein expression for different markers due to a variety of factors, including differences in expression ranges for different proteins, differences in the efficiencies of primary antibodies to bind target proteins, and differences in efficiencies of detecting target proteins in different cellular compartments (nucleus versus cytoplasm) in formalin-fixed, paraffin-embedded tissues.²⁶

Our data also support the incidence of overexpression of each APC/C substrate as a strong predictor of malignancy, whereas the absence of overexpression of APC/C substrates in most cases correlates with benign lesions. Using nearest shrunken centroids analysis,²⁵ the predictive value of having a single APC/C marker up-regulated is only slightly improved by including additional markers (Supplemental Figure 4, see <http://ajp.amjpathol.org>). This analysis suggests that misregulation of APC/C substrate accumulation is a fairly uniform program downstream of APC/C misregulation and that Emi1 overexpression is linked to a high percentage of APC/C substrate-positive tumors.

Within specific tumors, varying percentages of tumors are APC/C substrate positive but Emi1 negative, including a percentage of neural tumors, gastrointestinal tumors, breast cancers, primitive differentiating embryonic tumors, sarcomas, and lymphomas (Figures 4–6). In colon tumors, decreased or absent Emi1 may be explained by high levels of Plk1³⁰ and/or β -TrCp,³¹ the kinase¹⁵ and SCF substrate adapter¹⁴ that trigger ubiquitin-dependent destruction of Emi1, respectively.

Accumulation of APC/C substrates such as Skp2, Plk1, and aurora A is common in colon cancer (Figure 4). Here, we suspect that another form of APC/C misregulation may be occurring, such as APC/C subunit mutation, alterations of other APC/C regulators including spindle checkpoint proteins such as Mad2, or direct transcriptional activation or amplification of specific APC/C substrates (discussed below).

Activation of the G₁/S Cyclin/pRb/E2F Pathway Correlates with Emi1 and APC/C Substrate Protein Levels in Malignant Tumors

To test our hypothesis that activation of the G₁/S cyclin program is linked to oncogenic APC/C substrate positivity, we examined the status of other proliferation pathways including proteins critical for G₁/S control. Accordingly, we immunostained TMAs for several regulators of cellular proliferation including cyclins D and E, phosphorylated pRb, E2F3, p27, Bcl2, c-Myc, and β -catenin (Figures 5 and 6). Here, we looked for linkages between these G₁/S programs and misregulation of the APC/C.

We first considered the status of pRb and its regulatory partners. Tumors with a high incidence of pRb loss including lung and hepatic adenocarcinomas showed a similarly high incidence of Emi1 overexpression and APC/C substrate positivity, as did cervical adenocarcinomas in which pRb is inactivated by human papillomavirus E7 protein.

In addition to cyclin D/cdk4/6, cyclin E/cdk2 maintains pRb phosphorylation in S phase through early mitosis,³² and cyclin E overexpression can induce chromosome instability, similarly to Emi1.³³ We found that cyclin E expression clustered with Emi1 in several malignant tumor types including ovarian, lung, breast, and bladder cancers; oligodendroglial and meningeal neural tumors; leiomyosarcoma, rhabdomyosarcoma, and malignant fibrous histiocytoma connective tissue tumors; and rare lymphomas (Figures 5 and 6). Cyclin E immunopositivity statistically correlated with Emi1 expression in the breast, connective tissue, and cancer TMAs ($P < 0.001$) (Supplemental Table 1, see <http://ajp.amjpathol.org>). Likewise, cyclin D1 expression correlated with Emi1 in the breast, connective tissue, and lymphoma TMAs ($P < 0.002$), and phosphorylated-pRb correlated with Emi1 in all of four TMAs in which it was examined (breast, connective tissue, cancer, and neural) ($P < 0.001$). Tumors with a high incidence of loss of the cyclin D/cdk4 inhibitor p16, an important inhibitor of pRb phosphorylation, including melanoma, ovarian clear cell carcinoma, transitional cell cancer, and head and neck cancer, also showed strong correlations with Emi1 and APC/C substrate positivity. Thus, Emi1 protein was strongly expressed in tumors expected to highly express Emi1 mRNA due to biological alterations leading to increased pRb phosphorylation, namely increased cyclin D or E expression, or loss of p16.

Emi1 protein expression also clustered with β -catenin in a large cross section of tumors (Figures 5 and 6). This correlation was statistically significant across all of the TMAs ($P < 0.001$) and was strongest for the cancer

and connective tissue tumor arrays ($P < 0.05$ and 0.001 , respectively; Supplemental Table 1, see <http://ajp.amjpathol.org>). Wnt signals through β -catenin to induce S phase.³⁴ Interestingly, both Emi1 and β -catenin are regulated by β -TrCP, which can restrain cell-nonautonomous signaling through the Wnt and Hedgehog pathways³⁵ as well as the cell autonomous overduplication of centrosomes with overexpression of Emi1.¹⁴

APC/C Mitotic Substrate Misregulation Is Distinct from Up-Regulation of Proliferation Proteins and Is a Marker of Aggressive Tumors

In many tissue types, including neural and connective tissue tumors, lymphomas, and a subset of carcinomas, general S phase markers including Ki67, cyclin A, Bcl2, phosphorylated-pRb, and E2F3 form a "proliferation" cluster distinct from that containing the mitotic regulatory and oncogenic APC/C substrates securin, aurora A, Plk1, Skp2, and the APC/C-activating subunit Cdh1, "mitotic APC/C cluster." With few exceptions, proliferative but benign World Health Organization grade I neural tumors, such as neurofibromas, schwannomas, and pilocytic astrocytomas, expressed the proliferation cluster but were generally negative for the APC/C cluster, whereas malignant tumors, such as grade II–IV gliomas, were nearly uniformly immunopositive for both clusters (Figure 5A).

This trend also occurred for lymphoid and connective tissue tumors. World Health Organization grade I to II follicular lymphomas are generally proliferation cluster positive and APC/C cluster negative, whereas grade III follicular and diffuse large B-cell lymphomas are immunopositive for both clusters (Figure 5B). In addition, all of the diffuse large B-cell lymphomas expressing cyclin D2, a marker of poor prognosis,³⁶ were in the Emi1-positive subset. Benign connective tissue tumors are generally Emi1, securin, and Skp2 immunonegative. Malignant connective tissue tumors (sarcomas) are only occasionally Emi1 immunopositive but frequently highly express securin and Skp2 (Figure 6).

In contrast to most carcinomas, prostate and thyroid tumors, which are usually less aggressive cancers, were often mitotic APC/C mitotic cluster negative (Figure 4). Thus in some cases, overexpression of Emi1 and in many cases, overexpression of the oncogenic mitotic control APC/C substrates seem to be markers of tumor aggressiveness.

A high percentage of malignant tumors appeared to markedly accumulate the APC/C activator subunit Cdh1,

whereas most benign and some low-grade tumors were Cdh1 immunonegative (Figure 4). This suggests that Cdh1 overexpression may be a response to APC/C inhibition. Cdh1 overexpression in tumors with reduced APC/C activity could represent a compensatory feedback loop. It may seem counterintuitive that both the APC/C activator Cdh1 and APC/C inhibitor Emi1 are concomitantly overexpressed in some tumor types; however, this is not surprising when one considers that Cdh1 is itself an APC/C substrate¹⁷ and that Emi1 knockdown by siRNA decreases Cdh1 levels (N.L.L., unpublished data). Furthermore, Emi1 overexpression can override the effects of Cdh1 overexpression *in vitro*,¹³ possibly because of stabilization of cyclin A, because Cdh1 is inactivated by phosphorylation via cyclin A/cdk.³⁷ Specifically, Emi1 overexpression has been shown to relieve the transient cell cycle block caused by Cdh1 overexpression.¹³ Again, this is not unexpected because Emi1 can bind either free Cdh1 or the APC/C core subunit complex and block APC/C activity.¹²

Cdh1 overexpression may thus reflect an imbalance of positive and negative APC/C regulation in tumors. Notably, the APC/C activator Cdc20 has been reported to be overexpressed at the mRNA level in gastric and lung cancers.^{38,39} Although Cdh1 normally acts to induce cell cycle exit and thus limit cell cycle progression, Cdh1 overexpression can result in massive over-replication of the genome⁴⁰; therefore Cdh1 overexpression could lead to genomic instability in some circumstances.

In some tumor types, particularly in mesoderm-derived tissue tumors, Plk1 seems to be broadly expressed in both malignant and benign neoplasms (Figure 4). Because Plk1 is an E2F target gene⁴¹ that is involved in numerous phosphorylation reactions during G2 and mitosis,⁴² it may thus be more of a general marker of proliferation, perhaps specifically in hematolymphoid and connective tissue (mesoderm-derived) tumors. In support of this, we found that Plk1 protein expression strongly correlated with phosphorylated pRb in the connective tissue and cancer TMAs with correlation coefficients of 0.267 ($P < 0.000$) and 0.440 ($P < 0.000$), respectively (Supplemental Table 1, see <http://ajp.amjpathol.org>). In contrast to mesoderm-derived tumors, Plk1 expression correlated with increasing histopathological grade in colon adenocarcinomas and neuroectoderm-derived tumors (Figure 4).

The timely destruction of cyclins and other central mitotic regulators by the APC/C is essential to the accurate segregation of chromosomes and the maintenance of genomic stability. Several mechanisms may lead to

Figure 5. Hierarchical clustering analysis of Emi1, proliferation control, and mitotic control proteins in human tumors. For each tumor type, immunopositivity for Emi1, proliferation control (cyclins D1, D2, E, and A; β -catenin; p27^{Kip1}; Bcl2; and Ki67), and mitotic control/APC/C (aurora A, Plk1, securin, Cdh1, and Skp2) substrates were identified. Dendrograms for neural tumor (A) and lymphoma (B) TMAs are shown. Green rectangles indicate a tumor showing no immunopositivity, dark red indicates moderate positivity (3 to 29% of tumor cells positive), and bright red indicates high positivity (>30% tumor cells positive). Gray rectangles indicate unscorable TMA cores. The dendrograms on the top horizontal axes show clusters of proliferation control proteins and APC/C substrate proteins. Oncogenic APC/C substrates securin, aurora A, Plk1, and Skp2 (red) form a cluster with Emi1 distinct from broader proliferation markers such as Ki67, cyclin A, and Bcl-2 in malignant neural tumors and lymphomas. The pink rectangles on the right vertical axes represent Emi1-positive tumors, which are largely malignant and positive for both proliferation and APC/C clusters. The yellow rectangles indicate mostly malignant tumors that are largely proliferation and APC/C cluster positive but Emi1 negative. The blue rectangles represent mostly benign or low-grade tumors that are predominantly proliferation cluster positive and APC/C cluster negative. ALCL, anaplastic large cell lymphoma; DLBCL, diffuse large B-cell lymphoma; FL1–3, follicular lymphoma grades 1 through 3; NKC, natural killer cell (lymphoma); NLP, nodular lymphocyte predominant Hodgkin's lymphoma; CLL/SL, chronic lymphocytic leukemia/small lymphocytic lymphoma; PTLD, posttransplant lymphoproliferative disorder.

Figure 6. Hierarchical clustering analysis of Emi1, proliferation control, and mitotic control proteins in human connective tissue tumors. Data are represented as in Figure 4. DFSP, dermatofibrosarcoma protuberans; DSRCT, desmoplastic small round cell tumor; GIST, gastrointestinal stromal cell tumor; MFH, malignant fibrous histiocytoma; MPNST, malignant peripheral nerve sheath tumor; NOS, type not otherwise specified; PNET, primitive neuroectodermal tumor; SFT, solitary fibrous tumor.

APC/C misregulation in tumors including inactivating mutations or down-regulation of APC/C subunits^{43,44}; mutation, loss, or overexpression of spindle checkpoint proteins such as Mad2,^{18,20} Bub1,⁴⁵ or BubR1^{46,47}; overexpression of Emi1; or inactivation of the potential APC/C inhibitor RASSF1A.⁴⁸ In addition, some APC/C substrates may themselves be misexpressed through genomic alterations, eg, aurora A gene amplification in breast, gastric and colon cancers.⁴⁹ Because Plk1 and securin are E2F transcriptional targets,^{41,50} they can also be overexpressed at the mRNA level by pRb misregulation or loss.

A small number of studies to date have suggested that specific substrates of the APC/C are misregulated in certain tumors.^{5-7,50-56} However, no general survey of the extent or uniformity of APC/C substrate misregulation in all classes of human tumors has been published. Our analysis of more than 1600 tumor samples, representing more than 80 tumor classes, provides the first systematic analysis of APC/C substrate misregulation. We found that protein levels of mitotic APC/C substrates are frequently and coordinately elevated in malignant human tumors, in many cases with concomitant overexpression of the APC/C inhibitor Emi1. Overaccumulation of APC/C substrates could be, in some cases, directly because of their increased transcription. In other cases, inappropriate protein stability, secondary to APC/C misregulation by APC/C mutations, Emi1, or Mad2 misexpression may be an important primary or contributing factor. The latter is supported by the ability of overexpressed Emi1 and Mad2 to stabilize APC/C substrates and cause genomic instability *in vitro*.²⁰⁻²¹ In addition, because APC/C substrates are substantially regulated by ubiquitin-dependent proteolysis, it would not be unexpected that altered degradation plays a role in their overexpression in neoplasia.

Our analysis demonstrates a strong correlation between APC/C misregulation and malignancy and an anti-correlation with nonmalignancy. This profile is independent of misregulation of G₁/S phase cell cycle markers (cyclin A, Ki67, and E2F), which are also strongly accumulated in benign tumors. Importantly, in malignant tumors, both the G₁/S phase cell cycle markers and the APC/C markers are correlated. This distinction between the prevalence of G₁/S phase cell cycle misregulation and mitotic APC/C substrate misregulation suggests several conclusions. First, it suggests that G₁/S phase cell cycle regulation, presumably linked to increased proliferation, is representative of a broader class of hyperproliferative processes and may be linked to the activation of growth signaling pathways. Second, it suggests that the misregulation of mitotic APC/C substrates occurs through an independent mechanism, not strictly linked to G₁/S phase control. A strong candidate for this independent mechanism is the stabilization control pathway regulated by APC/C inhibitors including Emi1 and Mad2. Our present data support that Emi1 overexpression is linked to a sizable fraction of cases where mitotic APC/C substrates are misregulated in tumors but not to all cases. Functional tests from our laboratory show that overexpression of Emi1 is sufficient to stabilize APC/C substrates, creating a state much like that seen in tumors.^{14,21} For those tumors lacking Emi1 overexpression, it may be that 1) Emi1

is reduced by loss of heterozygosity following an event of genomic instability, 2) other APC/C regulators such as Mad2 can direct APC/C misregulation, or 3) independent control mechanisms determine the increased accumulation of these mitotic regulators. Additional studies to look at Mad2 in tumors are ongoing.

Besides Mad2, misexpression or mutation of other spindle checkpoint proteins, such as BubR1, may be linked to genomic instability. BubR1 is necessary for apoptosis after prolonged spindle damage and is significantly decreased in approximately 30% of colon adenocarcinomas,⁴⁶ and biallelic mutations in *BUB1B*, which encodes BubR1, have recently been reported in families affected by Mosaic Variegated Aneuploidy syndrome, which manifests in mosaic aneuploidy and predisposition to childhood malignancies.⁴⁷

In addition to Emi1 misregulation, misregulation of other APC/C regulators that are E2F targets, such as Mad2 and BubR1, and/or E2F-regulated APC/C substrates, such as securin or Plk1, could be contributing or alternate mechanisms of mitotic APC/C substrate misregulation in cancers, independent of Emi1. Thus, both increased E2F-mediated transcription and inappropriate protein stability probably work in concert in the pathway to genomic instability downstream of pRb/E2F misregulation.

We observed that an increase in the protein expression levels of the mitotic control APC/C substrates Skp2, securin, aurora A, and Plk1; the APC/C regulator Cdh1; and in many cases the APC/C inhibitor Emi1, strongly correlates with malignancy. Furthermore, in some tumors, such as lymphomas, increased accumulation of these proteins may correlate with tumor aggressiveness. We suggest that this is due to an increased propensity for mitotic catastrophe and genomic instability. However, despite the *in vitro* data showing that overexpression of Emi1 and APC/C substrates results in chromosomal instability, the ultimate biological consequences of APC/C substrate overexpression in tumors are unclear. The question of whether APC/C substrate overexpression is causal or only a downstream consequence of tumorigenesis and genomic instability cannot be answered by the present study.

Nevertheless, the observation of up-regulation of the APC/C pathway in tumors could prove to be of clinical importance. The pathway may be therapeutically exploitable through pharmacological targeting of Emi1 or other APC/C regulators or substrates. The propensity for genomic instability in tumors may also be related to sensitivity to antimetotics; APC/C substrate profiles might serve as a predictive marker for cancers responsive or resistant to agents that target the spindle checkpoint, eg, taxanes,⁵² or other regulators of mitosis. Last, because the mitotic APC/C expression profile not only distinguishes nonmalignant from malignant proliferations in certain tumor classes, such as connective tissue neoplasms and a large subset of neural tumors, but also is nearly uniform in certain specific tumor types, such as seminomas and clear cell carcinomas of the ovary, individual APC/C substrates and/or Emi1 may have broader utility in diagnostic pathology. The near uniformity of Cdh1 overexpression in malignant versus benign tumors

may prove to be a valuable diagnostic tool. Further studies will clearly be necessary to explore these important clinical possibilities.

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