

# Adrenocortical Carcinoma: Clinical, Morphologic, and Molecular Characterization

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**Purpose:** To define multimolecular phenotypes of adrenocortical carcinoma (ACC) and to correlate outcome with morphologic and molecular parameters.

**Patients and Methods:** Clinical data were analyzed for 124 patients, histopathologic slides for 67 primary tumors, and tissue specimens for 74 patients (38 primary and 36 metastatic tumors) with ACC and for 38 normal adrenal tissue samples. Molecular expression profiles were investigated by immunohistochemistry. The prognostic significance of 12 gross and histologic parameters in 67 primary ACCs was evaluated. Morphologic and protein expression patterns were correlated with disease-specific survival (DSS). Univariate influence of prognostic factors on DSS was analyzed by log-rank test and multivariate analysis by Cox regression.

**Results:** The median follow-up period was 4.7 years. Significant predictors of DSS included distant metastasis at time of initial presentation; venous, capsular, and

adjacent organ invasion; tumor necrosis, mitotic rate, atypical mitosis, and mdm-2 overexpression. Five-year DSS by number (one to six) of adverse histologic parameters was as follows: one to two, 84%; three to four, 37%; more than four, 9% ( $P = .005$ ). The phenotype Ki-67(-) p53(-) mdm-2(+) cyclinD1(-) Bcl-2(-) p21(-) p27(+) was observed in 83% of normal and 3% of malignant adrenal tissue ( $P = .01$ ). Molecular phenotypic expression was more heterogeneous in malignant than in normal (10 v five phenotypes) adrenal tissue.

**Conclusion:** Meticulous morphologic evaluation, mitotic count, and tumor stage are essential in determining prognosis for patients with ACC. Multimolecular phenotyping demonstrates that the molecular complexity and heterogeneity of these neoplasms are such that targeted therapy needs to be patient specific.

*J Clin Oncol* 20:941-950. © 2002 by American Society of Clinical Oncology.

ADRENOCORTICAL carcinoma (ACC) is a rare malignancy associated with dismal prognosis; a significant proportion (30% to 85%) of patients have distant metastasis at the time of initial presentation.<sup>1</sup> Disease stage and completeness of resection are primary determinants of outcome for this disease.<sup>2,3</sup> The widely applied, multifactorial scoring system proposed by Weiss et al<sup>4</sup> is based on 11 histopathologic features, of which tumor mitotic activity has been indicated to be the most significant determinant of survival. Despite these well-defined morphologic criteria, the biology of ACC remains poorly understood.

The importance of mitotic rate as a stratifying variable for outcome has stimulated interest in cellular proliferation and molecular mechanisms of adrenocortical tumorigenesis. The role of cell cycle regulatory proteins such as the p53 tumor suppressor and Ki-67 proliferative index in adrenal carcinoma remains controversial because the rarity of the disease limits adequate study population size to correlate molecular expression with prognosis. The patterns of p53 expression and those of related molecules, Bcl-2, mdm-2, and p21, along with other molecular markers, have not been collectively studied in ACC. Multimolecular expression profiles for cell growth promoters and inhibitors in the same tumor are unavailable because of the complexity of conventional full-section immunohistochemical (IHC) analysis. A novel technique, tissue microarray with IHC analysis, has enabled multimolecular profiling.<sup>5,6</sup>

In the current study, we apply tissue microarrays and IHC analysis to a group of pathologically well-characterized ACCs to define the molecular phenotypes of normal and malignant adrenal tissue. Morphologic and molecular parameters are compared and correlated with patient outcome.

## PATIENTS AND METHODS

The Memorial Sloan-Kettering Cancer Center (MSKCC) Endocrine Tumor Database was created in 1982 and has prospectively maintained clinical, pathologic, treatment, and outcome data on all adult patients with primary and recurrent malignant endocrine neoplasms that received inpatient treatment at our institution. We reviewed the clinical and pathologic records and updated the follow-up of 124 patients, including children, with ACC treated and observed at MSKCC from January 1982 to November 1999.

### Patients

This study consists of 78 female patients (63%) and 46 male patients (37%) with histologically confirmed ACC. Adrenal adenomas were not

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Submitted August 9, 2001; accepted October 22, 2001.

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0732-183X/02/2004-941/\$20.00

included. Median age of the study cohort was 46 years (range, 2 to 77 years). Fifty (40%) of the 124 patients presented with synchronous distant metastatic disease. For the purpose of molecular comparison, 38 patients with normal adrenal tissue and no history of ACC served as controls. The median follow-up period was 4.7 years (range, 1.2 to 16.9 years) for those patients alive at the time of the last follow-up visit. Clinical follow-up, telephone interview, and mailed questionnaires were used to update collected data.

Seventy-four patients (60%) underwent curative resection of primary ACC in the absence of synchronous distant metastases. Sixty-two patients later experienced recurrence of disease; the precise date of recurrence could not be determined in 10 patients because they returned to the care of their private doctors or to remote sites of residence. All 10 patients died as a result of disease. We have previously reported the clinical behavior, role of repeat resection, and clinical prognostic indicators of outcome for ACC in a smaller cohort of patients treated at MSKCC.<sup>3</sup> The results of that study are not repeated here because the primary purpose of this analysis is to characterize the molecular phenotypes of normal and cancerous adrenal tissue, and to define and compare morphologic and molecular prognostic factors for ACC.

### *Pathologic Review and Inclusion Criteria*

An endocrine pathologist (R.A.G.) reviewed a mean of 14 hematoxylin and eosin-stained slides per patient. Histopathologic review was conducted without the pathologist's knowledge of the patients' clinical characteristics or outcomes. The diagnosis of ACC was based on the 11-tiered system of pathologic features proposed by Weiss et al.<sup>4</sup> Gross tumor size and weight were recorded. Tumor size was measured as the maximum diameter of the fresh resected tumor specimen. Tumors were classified into four size categories: 0 to 5 cm, 6 to 10 cm, 11 to 20 cm, and more than 20 cm. Nuclear grade was based on the Fuhrman grading system used in renal cell carcinomas.<sup>7</sup> Mitotic rate was determined by counting 50 high-power fields ( $\times 400$ ) with an Olympus microscope (U-DO model) in the areas of greatest concentrations of mitotic figures. Each 10 high-power fields was counted on a different slide whenever possible. The presence or absence of atypical mitotic figures was recorded.

Tumor cell cytoplasm was categorized as 0% to 25% and 26% to 100% clear. When sheets of cells without a characteristic pattern of growth characterized more than 33% of the tumor, the tumor was classified as having diffuse architecture; in the absence of this diffuse architecture, the tumor was considered nondiffuse. The presence or absence of tumor necrosis was recorded. Venous or sinusoidal invasion was evident when a vein or sinusoid within or external to the adrenal tumor contained tumor cells within its lumen and adherent to the vessel wall. Veins were defined as endothelial lined vessels with muscle as a component of the vessel wall and sinusoids as endothelial lined vessel with little supporting tissue. Unequivocal capsular invasion was characterized by complete capsular penetration by tumor. Microscopic margins were defined at the time of histopathologic assessment and confirmed again at the time of review. Microscopic resection margins were categorized as positive (tumor at the inked margin) or negative (no tumor at the inked margin).

No single morphologic characteristic was found in all tumors examined. All primary tumors reviewed as part of this study demonstrated three or more of the histopathologic criteria needed for the diagnosis of ACC as defined by Weiss et al<sup>4</sup> and Weiss.<sup>8</sup> This extends both in number and intensity our previous pathologic analysis<sup>9</sup> of curatively resected primary ACC that was not based on the criteria of Weiss et al and Weiss.

### *Histopathologically Confirmed ACC*

Clinical, pathologic, and outcome data were available for 124 patients. Histopathologic slides of the primary tumor were available for 67 patients who underwent adrenalectomy with or without concomitant solid organ (eg, pancreas, spleen, kidney, liver) resection. Twenty-four of these 67 patients experienced synchronous distant metastases at time of initial presentation. Patients for whom slides of the primary tumor were not available were included in the analysis because their diagnoses were confirmed by review of pathology reports and by their clinical courses. Paraffin-embedded tissue samples were available for 38 patients, each with normal adrenal glands and primary ACC. Paraffin-embedded distant metastasis tumor blocks were available for 36 cases.

### *Tissue Microarray Construction and IHC*

Normal adrenal tissue and tissue from ACCs were embedded in paraffin. Five-micron sections stained with hematoxylin and eosin were obtained to confirm the diagnosis and to identify viable, representative areas of the specimen. From these defined areas, core samples were taken with a precision instrument for biopsy (Beecher Instruments, Silver Springs, MD), as previously described.<sup>5</sup> Tissue cores with a diameter of 0.6 mm from each specimen were punched and arrayed in triplicate on a recipient paraffin block.<sup>6</sup> Five-micron sections of these tissue array blocks were cut and placed on charged polylysine-coated slides. These sections were used for IHC analysis.

Tissues and cell lines known to express the antigens under study were used as positive controls. Arrayed normal tissues served as baseline controls. Paraffin was removed from sections from tissue arrays; sections were rehydrated in graded alcohols and processed by means of the avidin-biotin immunoperoxidase method. Briefly, sections were submitted to antigen retrieval by microwave oven treatment for 15 minutes in 0.01 M citrate buffer at pH 6.0. This procedure was performed for all antibodies under study. For Ki-67 antibody, an additional step of incubation in preheated 0.05% trypsin and 0.05% CaCl<sub>2</sub> in Tris-HCl (pH 7.6) for 5 minutes at 37°C before microwave treatment was performed. Slides were subsequently incubated in 10% normal horse serum for 30 minutes, then incubated overnight at 4°C in appropriately diluted primary antibody. Mouse antihuman monoclonal antibodies to p53 (Ab-2, clone 1801, 1:500 dilution; Calbiochem, Cambridge, MA), mdm-2 (clone 2A10, 1:500 dilution, provided by A. Levine, PhD, Rockefeller University, New York, NY), p21 (WAF-1 [Ab-1], clone EA10, 1:100 dilution; Calbiochem), p27 (kip-1 [Ab-2], clone DCS72, 1:500 dilution; Oncogene Research Products, Cambridge, MA), cyclin D1 (CD1) (Ab-3, clone DCS-6, 1:100 dilution; Calbiochem), Ki-67 (Mib-1, 1:1,000 dilution; Dako, Glostrup, Denmark) and Bcl-2 (clone 124, 1:154 dilution; Dako) were used. The anti-p53 antibody detects wild-type and mutated p53.

Samples were then incubated with biotinylated antimouse immunoglobulins at 1:500 dilution (Vector Laboratories, Inc, Burlingame, CA), followed by avidin-biotin peroxidase complexes (1:25, Vector Laboratories) for 30 minutes. Diaminobenzidine was used as the chromogen and hematoxylin as the nuclear counterstain.

Tissue loss is a significant factor for tissue array-based analysis, with previously reported rates of tissue damage ranging from 15% to 33%.<sup>6,10-12</sup> In these analyses, rates of lost cases attributable to tissue damage ranged between 1% and 9% for the different markers. Immunoreactivity was classified as continuous data (undetectable levels, 0% to homogeneous staining, 100%) for all markers. Two investigators (R.A.G., A.S.) reviewed and scored slides by estimating the percentage of tumor cells showing characteristic staining. The percentage of tumor

cell staining was determined by consensus among the reviewing investigators. The cutoff values for tumor cell staining used in the present study were defined on the basis of previously established cutoff values used in clinicopathologic studies of different solid tumors by using identical reagents.<sup>6,13</sup> These established cutoff values were used as the basis for the present analysis and were modified according to clinicopathologic characteristics of ACC.

The cutoff values for tumor cell staining used in this study were defined as follows: high Ki-67 proliferative index if more than 5% tumor nuclei stained; p53 nuclear overexpression if more than 5% tumor nuclei stained; mdm-2 overexpression if more than 50% tumor nuclei stained; p21 nuclear overexpression if more than 10% of tumor nuclei stained; p27 nuclear overexpression if more than 30% nuclei stained; Bcl-2 overexpression if more than 50% of tumor cells demonstrated cytoplasmic staining; and CD1 overexpression if more than 5% of tumor nuclei stained. Tumors were then grouped into two categories defined as follows: normal (negative) expression, or neoplasms below the defined cutoff value of immunoreactivity; and abnormal (positive) expression, or neoplastic tissues above defined cutoff values of immunoreactivity. Identical cutoff values were applied for IHC-stained normal adrenal tissue.

#### *Data and Definitions*

Primary end points included disease-free survival (DFS) after primary tumor resection and disease-specific survival (DSS). Clinical variables correlated with study end points were distant metastases at time of initial presentation and disease-free interval (DFI). Some received adjuvant radiation, chemotherapy, or both, at the discretion of the treating doctor. Because adjuvant therapy was not prospectively randomized or uniformly applied, it was excluded from the statistical analysis. Patients were treated according to the standard of care at MSKCC.

A primary tumor was defined as a localized lesion previously untreated or sampled for biopsy before surgical therapy and was categorized according to presence or absence of synchronous distant metastases. Locoregional recurrence was defined by clinical, pathologic, or radiologic evidence of disease recurrence in the region of the previously resected tumor bed, and distant metastasis was defined by pathologic, clinical, or radiologic evidence of systemic disease spread outside the primary tumor site.

#### *Statistical Analysis*

Summary statistics were obtained by use of established methods. Associations between categorical factors were studied with the  $\chi^2$  test. The clinical outcomes studied were DFS and DSS. Outcome was classified according to sites of first disease recurrence. Time to first locoregional or distant recurrence, DFS, and DSS were calculated from time of primary tumor resection. In defining disease-related mortality, only deaths that were confirmed to be related to disease were considered in disease-related mortality calculations; all others were censored at date of last follow-up. Follow-up was calculated from the time of first operation for the primary tumor to date of the last follow-up.

The rate of recurrence or death was estimated by the Kaplan-Meier product limit method. Univariate influence of prognostic factors on study end points was analyzed by the log-rank test. Comparison of rates of tumor-related mortality according to DFI considered time of death from time of recurrence, not primary tumor resection. Multivariate analysis based on Cox's proportional hazards regression model was used to associate covariates to time-dependent end points. Statistical

analysis was performed by JMP software (SAS Institute, Cary, NC).  $P < .05$  was considered significant.

## RESULTS

### *Clinical*

The clinical variables of the study population are listed in Table 1. Forty percent of patients had distant metastasis evident at initial presentation. Patients with distant metastatic disease had worse DSS than those with no systemic disease at time of initial diagnosis (5-year DSS, 17% v 51%,  $P < .001$ ). Median survival for those with systemic disease at initial diagnosis was 13.7 months, as opposed to 62.0 months for those patients without initial distant metastases. Thirty patients (58%) experienced recurrence within 2 years of primary tumor resection (DFI  $< 24$  months). The proportion of distant disease recurrence among patients with DFI less than 24 and  $\geq 24$  months was not significantly different (83% v 82%).

### *Histopathology*

Pathologic features of primary ACC are demonstrated in Table 1. Forty-three (78%) of 55 primary tumors were larger than 10 cm in size. Histologic evidence of venous, capsular, and adjacent organ invasion and necrosis was identified in 42%, 53%, 8%, and 90% of primary adrenal carcinomas reviewed. Mitotic rate exceeded five per 50 high-power fields in 79% and atypical mitoses identified in 69% of cases. Grade 3 or 4 nuclei were observed in 99% of tumors.

### *Summary Outcome Data*

Recurrence and survival data are defined in Table 2. The median follow-up period was 4.7 years (range, 1.2 to 16.9 years) for patients alive at time of the last clinic visit. Of the 74 patients treated for localized primary ACC, 84% ( $n = 62$ ) experienced recurrence of disease. Fifty-eight percent of patients experienced relapse within 2 years of primary treatment. The time to first recurrence after complete primary tumor resection ranged from 4 months to 16.5 years (median, 25 months). Median survivals for DFIs less than 24 and  $\geq 24$  months after treatment of recurrence were 21 and 36 months, respectively ( $P = .13$ ). Five-year DSS after early (DFI  $< 24$  months) and late (DFI  $\geq 24$  months) first recurrence was 17% and 30%, respectively. Overall median and 5-year DSS for the entire study cohort was 34 months and 39%, respectively. At the time of last follow-up, 11% (14 of 124) of patients were alive and free of disease, and 75% (93 of 124) had died as a result of disease.

**Table 1. Clinical and Pathologic Features of Primary ACC (N = 124)**

Variable	No.	%
<b>Sex</b>		
Female	78	63
Male	46	37
<b>Age</b>		
< 45 years	57	46
≥ 45 years	67	54
Median, years	46	
Range, years	2-77	
<b>Distant metastasis at presentation</b>		
Present	50	40
Absent	74	60
<b>DFI</b>		
< 24 months	30/52	58
≥ 24 months	22/52	42
<b>Tumor size</b>		
≤ 5 cm	1/55	2
6-10 cm	11/55	20
> 10-20 cm	38/55	69
> 20 cm	5/55	9
<b>Tumor weight</b>		
0-100 g	4/44	9
> 100-250 g	6/44	14
> 250-1,000 g	25/44	57
> 1,000 g	9/44	20
<b>Venous invasion</b>		
Present	28/66	42
Absent	38/66	58
<b>Sinusoidal invasion</b>		
Present	63/67	94
Absent	4/67	6
<b>Capsular invasion</b>		
Present	34/64	53
Absent	30/64	47
<b>Adjacent organ invasion</b>		
Present	5/66	8
Absent	61/66	92
<b>Necrosis</b>		
Present	60/66	90
Absent	6/66	10
<b>Mitotic rate per 50 high-power fields</b>		
≤ 5	14/67	21
6-20	22/67	33
21-50	24/67	36
> 50	7/67	10
<b>Atypical mitotic figures</b>		
Present	46/67	69
Absent	21/67	31
<b>Nuclear grade</b>		
1	0/67	0
2	2/67	1
3	18/67	27
4	48/67	72
<b>Architecture</b>		
Diffuse	55/67	82
Nondiffuse	12/67	18
<b>Cytoplasm</b>		
0%-25% clear	47/67	70
26%-100% clear	20/67	30
<b>Microscopic resection margin</b>		
Positive	14/39	36
Negative	25/39	64

NOTE. Numbers < 67 indicate only those variables that could be determined with certainty after review of the histopathology slides and pathology reports.

**Table 2. ACC Recurrence, Follow-up, and Survival (N = 124)**

Characteristic	No.	%
<b>Recurrence</b>		
Yes	62/124	50.0
No	12/124	10.0
M1 at presentation	50/124	40.0
<b>DFS</b>		
Median, months	24.9	
Range, months	4.0-198.0	
At 5 years, %	25.6	
<b>Status at last follow-up</b>		
NED	14	11.3
AWD	16	12.9
DOD	93	75.0
DOC	1	0.8
<b>DSS</b>		
Median, months	33.9	
Range, months	6.2-234.9	
At 5 years, %	38.5	

Abbreviations: NED, alive with no evidence of disease; AWD, alive with disease; DOD, disease-related death; DOC, death unrelated to adrenal carcinoma; M1, distant metastases.

### IHC Profiling of Cell Cycle Regulatory Proteins

The molecular expression profiles of all markers in normal adrenal and tumor tissues are presented in Table 3. A significant variation in cell cycle regulatory protein expression was identified within adrenal carcinoma and between tumor and normal tissue. The highest rate of expression in adrenal carcinoma was for p27, which was evident in 94.4% of tumors. The lowest rates of expression were observed with Bcl-2 and CD1; both proteins could not be detected above the defined cutoff levels of 10% and 5% tumor cells in any of the investigated tissues. IHC analysis of the cellular proliferation marker Ki-67 demonstrated absence of nuclear staining in all normal adrenal tissue. High Ki-67 proliferative index was observed in 35.5% of adrenal carcinomas. Immunostaining for p53 represents accumulation of protein products potentially from a mutated p53 gene. p53 overexpression was observed in only 5.4% of carcinomas, and no normal adrenal demonstrated more than 5% nuclear staining for p53. More diverse observations were made for other cell cycle regulatory molecules in the p53 pathway.

The majority of normal adrenal tissues (91.7%) demonstrated nuclear staining for mdm-2, whereas only 20% of carcinomas overexpressed this marker ( $P < .001$ ). Seventy-two percent of carcinomas demonstrated less than 30% nuclear mdm-2 expression. Normal adrenal tissue was predominantly p21 negative, whereas nuclear p21 overexpression was present in 69.4% of adrenal carcinomas ( $P < .001$ ). The cyclin-dependent kinase inhibitor p27 was over-

**Table 3. Cell Cycle Regulator Expression in Primary ACC**

Molecular Marker (positive % cutoff)	Normal (n = 38)		ACC (n = 38)		P
	No.	%	No.	%	
Ki-67 (5%)					< .001
Negative	38	100	20	64.5	
Positive	0	0	11	35.5	
p53 (5%)					.09
Negative	38	100	35	94.6	
Positive	0	0	2	5.4	
mdm-2 (50%)					< .001
Negative	3	8.3	28	80	
Positive	33	91.7	7	20	
p21 (10%)					< .001
Negative	35	94.6	11	30.6	
Positive	2	5.4	25	69.4	
p27 (30%)					.54
Negative	1	2.7	2	5.6	
Positive	36	97.3	34	94.4	
Bcl-2 (10%)					NA
Negative	38	100	38	100	
Positive	0	0	0	0	
Cyclin D1 (5%)					NA
Negative	38	100	38	100	
Positive	0	0	0	0	

NOTE. Number of patients in subgroup less than the total number in respective group reflects tissue loss during specimen microarray processing. Abbreviation: NA, not applicable.

expressed in nearly every normal and malignant adrenal tissue (97.3% and 94.4%). The frequency of nuclear expression of the cell cycle regulator CD1 was uniformly less than 5% for both adrenal carcinoma and normal adrenal tissue. Overexpression of the antiapoptotic Bcl-2 protein was defined as more than 10% cell cytoplasmic staining. The Bcl-2-negative phenotype was identified in all normal and malignant adrenal tissue. Differential molecular ex-

pression allowed us to distinguish between the benign and malignant adrenal tissue. There was statistically significant differential expression of the following cell cycle regulatory proteins among normal adrenal tissue and ACC, Ki-67, mdm-2, and p21.

#### Multimolecular Phenotypes

The differential expression of cell cycle regulators that promote and inhibit cell growth in normal and malignant tissue was analyzed according to multimolecular phenotypes. There was significantly different expression between tissue types for these molecular markers (Table 4). One phenotype, Ki-67(-)p53(-)mdm-2(+)-CD1(-)Bcl-2(-)p21(-)p27(+), distinguished normal from malignant adrenal tissue. This phenotype was observed in 83.3% of normal adrenal glands and in only one (3.2%) of 31 adrenal carcinomas ( $P < .01$ ). Multimolecular phenotypic expression was more heterogeneous for malignant than normal adrenal tissue. The most common phenotype identified in only eight (25.8%) of 31 primary adrenal carcinomas was Ki-67(-)p53(-)mdm-2(-)CD1(-)Bcl-2(-)p21(+)-p27(+). No normal adrenal tissue demonstrated this multimolecular pattern of expression. Four of eight patients with primary adrenal carcinoma phenotype Ki-67(-)p53(-)mdm-2(-)CD1(-)Bcl-2(-)p21(+)-p27(+) and three of the remaining 23 patients, each with a unique phenotype remain, were alive and free of disease at the time of the last follow-up examination.

Molecular phenotypic expression varied between primary and metastatic adrenal carcinoma across and within the same patients. The most common multimolecular profile for metastatic adrenal carcinoma was Ki-67(+)-p53(-)mdm-2(+)-CD1(-)Bcl-2(-)p21(+)-p27(+). This phenotype was

**Table 4. Multimolecular Phenotypes in Normal Adrenal and Primary and Metastatic ACC**

Phenotype	Normal (n = 36)		Primary (n = 31)		Metastatic (n = 28)	
	No.	%	No.	%	No.	%
Ki67(-)p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(-)p27(-)	1	2.8	1	3.2	1	3.6
Ki67(-)p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(-)p27(+)	2	5.5	5	16.1	2	7.1
Ki67(-)p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(+)-p27(+)*	0	0	8	25.8	0	0
Ki67(-)p53(-)mdm2(+)-CD1(-)Bcl-2(-)p21(-)p27(+)*	30	83.3	1	3.2	0	0
Ki67(-)p53(-)mdm2(+)-CD1(-)Bcl-2(-)p21(+)-p27(+)	2	5.5	4	12.9	0	0
Ki67(+)-p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(-)p27(+)	0	0	3	9.7	5	17.9
Ki67(+)-p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(+)-p27(-)	0	0	0	0	1	3.6
Ki67(+)-p53(-)mdm2(-)CD1(-)Bcl-2(-)p21(+)-p27(+)	0	0	4	12.9	7	25
Ki67(+)-p53(-)mdm2(+)-CD1(-)Bcl-2(-)p21(-)p27(-)	1	2.8	1	3.2	0	0
Ki67(+)-p53(-)mdm2(+)-CD1(-)Bcl-2(-)p21(-)p27(+)	0	0	0	0	1	3.6
Ki67(+)-p53(-)mdm2(+)-CD1(-)Bcl-2(-)p21(+)-p27(+)	0	0	2	6.4	9	32.1
Ki67(+)-p53(+)-mdm2(+)-CD1(-)Bcl-2(-)p21(+)-p27(+)	0	0	2	6.4	0	0
Ki67(+)-p53(+)-mdm2(-)CD1(-)Bcl-2(-)p21(-)p27(+)	0	0	0	0	1	3.6
Ki67(+)-p53(+)-mdm2(-)CD1(-)Bcl-2(-)p21(+)-p27(-)	0	0	0	0	1	3.6

NOTE. In these analyses, rates of lost cases attributable to tissue damage ranged between 1% and 9% for the different markers. Cases without complete molecular profiles as a result of tissue loss during processing were excluded from the comparisons.

\* $P < .05$ .

observed in only two primary adrenal carcinomas (6.4%) and in none of the normal adrenal tissues. Identical multi-molecular phenotypes in primary and metastatic tumors were observed in only one of five patients with complete molecular data available for both primary and metastatic tumor specimens.

#### *Analysis of Prognostic Factors*

Univariate and multivariate comparisons were performed for study end points. In the univariate analysis, tumor necrosis ( $P = .01$ ), mitotic rate more than five of 50 high-power fields ( $P = .004$ ), and atypical mitotic figures ( $P = .008$ ) were associated with reduced DFS. On multivariate analysis, mitotic rate was an independent predictor of DFS (mitotic rate  $\leq 5$ ; relative risk, 0.28; 95% confidence interval, 0.10 to 0.68;  $P = .009$ ). Univariate comparisons were made between clinical, histopathologic, and molecular categorical variables for the end point, DSS, for the entire study cohort. Factors predicting outcome by univariate analysis included distant metastasis at time of initial presentation; venous, capsular, and adjacent organ invasion; tumor necrosis and mitotic rate; atypical mitosis; and mdm-2 overexpression. The 5-year DSSs are listed in Table 5. DSS rates were calculated on the basis of the number of the six adverse histopathologic variables; venous, capsular, and adjacent organ invasion; tumor necrosis and mitotic rate; and atypical mitosis categorized into one to two, three to four, and more than four factors. There was a statistically significant survival difference between the three groups ( $P = .005$ ). Five-year DSS for patients with one to two, three to four, and more than four adverse primary tumor morphologic features was 83.5%, 36.8%, and 8.6%, respectively (Fig 1). When the same survival analysis was conducted on patients undergoing resection of primary adrenal carcinoma in the absence of synchronous distant metastasis, the corresponding 5-year DSS rates were 88.9% (one to two factors,  $n = 10$ ), 48.8% (three to four factors,  $n = 26$ ) and 0% ( $> 4$  factors,  $n = 7$ ), respectively ( $P = .01$ ).

Multivariate analysis was used to associate factors found to correlate with outcome by univariate analysis to the end point, DSS. Distant metastasis at initial presentation, mitotic rate (more than five per 50 high-power fields), and adjacent organ invasion emerged as independent predictors of tumor-related mortality. When the multivariate analysis was conducted for patients with primary adrenal carcinoma in the absence of synchronous metastasis who underwent curative resection, only mitotic rate was found to independently predict survival (mitotic rate  $\leq 5$ ; relative risk, 0.36; 95% confidence interval, 0.12 to 0.84;  $P = .03$ ).

#### DISCUSSION

Although more than 50% of patients with ACC die within 2 years of diagnosis, some patients with this disease have experienced prolonged survival.<sup>1,9,14</sup> The presence of distant metastases, older age ( $\geq 45$  years), and incomplete (R1) resection were indicated to be determinants of poor survival.<sup>1,2</sup> However, there are currently no well-established prognostic criteria based on histologic or IHC analysis of the primary tumors. High mitotic activity was found to be a predictor of poor outcome when all cases of ACC (localized and metastatic) were analyzed as a single group.<sup>4,15,16</sup> Evans and Vassilopoulou-Sellin<sup>16</sup> did not find the mitotic index to be prognostically significant when patients with and without metastases were analyzed separately. In contrast, Harrison et al<sup>9</sup> were able to predict survival by using mitotic rate in patients who underwent curative adrenalectomy for primary disease. In the current study, we were able to predict survival on the basis of mitotic count in the entire patient population as well as in patients without synchronous metastases.

Nuclear grade was found to be predictive of survival in some studies.<sup>17,18</sup> However, we and others have not found this variable to be of prognostic value.<sup>4,16</sup> In the current series, we evaluated the prognostic significance of 12 gross and histologic parameters in the primary tumors. Eleven of those parameters were analyzed according to the criteria established by Weiss et al<sup>4</sup> and Weiss.<sup>8</sup> The only prognostic marker that we added was the presence or absence of a positive microscopic margin.

In comparison to all other previous reports, we found a higher number of microscopic variables to be of prognostic significance. In addition to high mitotic rate, we found the presence of tumor necrosis, atypical mitosis, capsular, venous, and adjacent organ invasion to be adverse predictors of tumor-related mortality in univariate analysis. The presence of atypical mitosis and capsular invasion approached statistical significance in the study of Weiss et al.<sup>4</sup> Tumor size categorization according to Weiss et al was not a significant factor in predicting survival. However, if primary tumor size is stratified into two categories by size ( $\leq 12$  cm and  $> 12$  cm), patients with completely resected large ( $> 12$  cm) tumors have significantly reduced survival ( $P = .03$ ), as we demonstrated in a previous report.<sup>9</sup>

DSS was estimated according to the number of these six adverse histopathologic variables identified in the primary adrenal tumor. Five-year DSS for the entire study cohort according to one to two, three to four, and more than four adverse morphologic factors was 84%, 37%, and 9%, respectively ( $P = .005$ ). The same statistically significant correlation was observed when the group of patients without

Table 5. Analysis of DSS in 124 Patients With ACC

Characteristic	5-year DSS (%)	Univariate <i>P</i>	Multivariate <i>P</i>	RR	95% CI
Sex		.23			
Male (n = 46)	41				
Female (n = 78)	35				
Age		.38			
< 45 years (n = 57)	39				
≥ 45 years (n = 67)	34				
Initial metastasis		< .001		1.95	1.40-2.68
Present (n = 50)	17		< .001		
Absent (n = 74)	51				
DFI		.13			
< 24 months (n = 30)	17				
≥ 24 months (n = 22)	30				
Tumor size		.57			
≤ 5 cm (n = 1)	—				
6-10 cm (n = 11)	50				
10-20 cm (n = 38)	35				
> 20 cm (n = 5)	60				
Tumor weight		.64			
0-100 g (n = 4)	100				
101-250 g (n = 6)	33				
251-1,000 g (n = 25)	40				
> 1,000 g (n = 9)	22				
Venous invasion		.01	.09		
Present (n = 28)	28				
Absent (n = 38)	49				
Capsular invasion		.03	.93		
Present (n = 34)	26				
Absent (n = 30)	57				
Adjacent organ invasion		.004		1.89	1.10-3.12
Present (n = 5)	0		.03		
Absent (n = 61)	42				
Necrosis		.046	.16		
Present (n = 60)	36				
Absent (n = 6)	69				
Mitotic rate per 50 high-power fields		.04	.005	0.43	0.21-0.79
≤ 5 (n = 14)	63				
6-10 (n = 22)	50				
21-50 (n = 24)	25				
> 50 (n = 7)	0				
Atypical mitotic figures		.01	.35		
Present (n = 46)	27				
Absent (n = 21)	66				
Nuclear grade		.05			
2 (n = 2)	—				
3 (n = 18)	34				
4 (n = 48)	45				
Architecture		.71			
Diffuse (n = 55)	38				
Nondiffuse (n = 12)	46				
Cytoplasm		.44			
0%-25% clear (n = 47)	40				
26%-100% clear (n = 25)	38				
Microscopic margin		.10			
Positive (n = 14)	33				
Negative (n = 25)	56				
Ki-67		.10			
Negative (n = 20)	48				
Positive (n = 11)	25				
p53		.74			
Negative (n = 35)	39				
Positive (n = 2)	—				
mdm-2		.048	.15		
Negative (n = 28)	46				
Positive (n = 7)	17				
p21		.51			
Negative (n = 11)	35				
Positive (n = 25)	46				
p27		.54			
Negative (n = 2)	—				
Positive (n = 34)	47				
Bcl-2		NA			
Negative (n = 38)	42				
Positive (n = 0)	—				
Cyclin D1		NA			
Negative (n = 38)	40				
Positive (n = 0)	—				

NOTE. DFI refers to time from primary tumor resection to first local, regional, or distant recurrence. Univariate *P* value refers to log-rank test of no difference v any difference between categories. Multivariate *P* refers to Cox proportional hazards model evaluating significant factors identified by univariate analysis. For DFI comparison, DSS is calculated from time of first recurrence.

Abbreviation: RR, relative risk; 95% CI, 95% confidence interval.

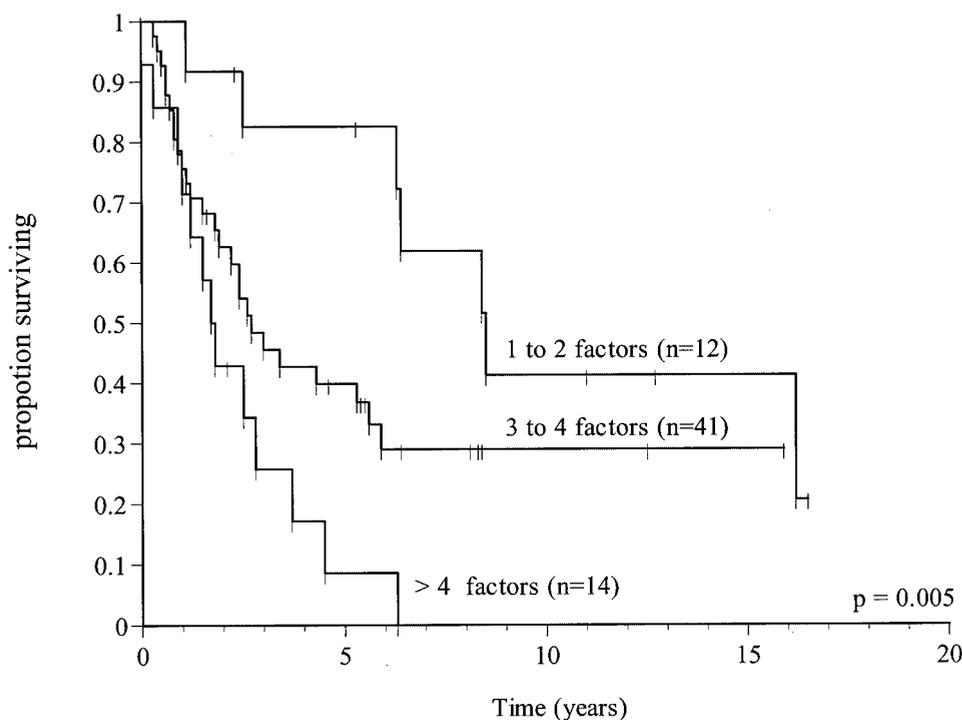


Fig 1. Graph indicating DSS for patients with primary ACC displaying 1 to 2, 3 to 4, and > 4 of 6 adverse histologic prognostic factors, including high mitotic rate (> 5 of 50 high-power fields); atypical mitoses; venous, capsular, and adjacent organ invasion; and tumor necrosis.

metastatic disease was analyzed separately. The presence of tumor necrosis, high mitotic rate (more than five of 50 high-power fields), and atypical mitosis also correlated with decreased DFS in univariate analysis. The fact that the prognosis of this tumor can be refined and predicted on the basis of multiple conventional histologic parameters is encouraging.

The diagnosis and morphologic evaluation of prognostic factors remains challenging for adrenocortical neoplasms. The appraisal of findings such as mitotic index and atypical mitosis depends on the diligent scrutiny of many permanent sections by an experienced pathologist. Interobserver variability may arise in the counting of mitosis and in the identification of atypical mitotic figures. Appraisal of other morphologic features as capsular and vascular invasion is often subjective and may lead to discrepant results. Reliable diagnostic and prognostic criteria are needed for primary adrenal tumors.

Several investigators have analyzed cell cycle proteins in ACC by use of IHC.<sup>19-23</sup> Because ACC may have multiple molecular alterations in their cellular machinery, the molecular characterization of these tumors requires investigation of multiple molecules in the same tissues. Molecules that play a pivotal role in the p53 pathway, such as mdm-2 and p21, have not been studied in ACC.

By use of a multimolecular profiling approach, we studied important components of the p53 pathway. p53

overexpression was detected in only 5% of primary ACC. Other authors have found a higher percentage (22% to 50%) of p53 positivity.<sup>19,21,23</sup> However, all authors agree on the lack of prognostic significance for p53 overexpression in these carcinomas. The mdm-2 protein inhibits the function of p53 and is overexpressed in human cancers. This protein's expression was upregulated in 20% of ACC in this study. Overexpression of mdm-2 significantly correlated with tumor-related mortality. This finding is consistent with its role in tumor cell proliferation. However, mdm-2 proved to be of little clinical value because it did not emerge as a significant factor in multivariate analysis. In this context, it is important to note that mdm-2 was overexpressed in most (92%) of the normal adrenal tissues investigated. This finding is contrary to findings in other diseases, where mdm-2 expression, investigated with the same reagents as in the present study, was low in normal (compared with malignant) tissues.<sup>13</sup> Thus, the role of mdm-2 in adrenocortical carcinogenesis remains elusive and warrants further study.

p21 is a cyclin-dependent kinase inhibitor that can be induced by p53. Overexpression of this protein triggers cell cycle arrest in proliferating tumor cells. p21 was overexpressed in 70% of ACC samples assessed in the study we report here. However, downregulation of p21 did not have any prognostic significance for patients with this disease. p21 was inversely expressed in normal adrenal tissues: most

normal tissues (95%) demonstrated low expression of p21. This may be explained by the variety of different mechanisms that can trigger p21 expression.<sup>24</sup>

p53 mediates apoptosis through the Bcl-2/Bax pathway. Bcl-2 has an antiapoptotic function that may promote tumor growth.<sup>25</sup> The Bcl-2-negative phenotype was identified in all normal and malignant adrenal tissue in this study, implying that Bcl-2 does not play a role in adrenocortical tumorigenesis. This finding also emphasizes the strong difference of expression of this molecule as compared with other endocrine organs, such as thyroid, where Bcl-2 is strongly expressed.<sup>13</sup>

The cyclin-dependent kinase inhibitor p27 regulates cell cycle progression from G1 to S phase of the cell cycle and is considered a potent tumor suppressor gene. The loss of p27 protein as detected by IHC constitutes a true reduction in p27 levels.<sup>26</sup> By use of 30% of immunopositive cells as a cutoff value, we found p27 immunopositivity in more than 94% of cases of ACC and normal adrenal tissues. Nakazumi et al<sup>20</sup> investigated the presence of p27 in 12 ACC and were able to detect more than 30% immunopositive cells in all cases. Neither they nor we could identify any significant correlation between p27 tumor expression and outcome.

CD1 is another regulator of the G1 to S transition phase of the cell cycle. CD1 gene amplification has been implicated in the progression of many neoplasms. The absence of CD1 expression in both normal and malignant adrenal tissue suggests the absence of a role for this molecule in adrenocortical tumorigenesis. Cellular proliferation can be viewed as an end point of several cell cycle regulatory pathways. Various studies have assessed tumor cell proliferative activity by immunostaining for the Ki-67 nuclear antigen.<sup>27,28</sup> In this study, Ki-67 immunopositivity was found in 36% of ACC. Patients positive for Ki-67 had a lower survival rate than did patients with Ki-67-negative tumors (5-year DSS, 25% v 48%;  $P = .10$ ). Nakazumi et al<sup>20</sup> found the same correlation. In their study, as well as in ours, statistical significance was not reached.

The findings of this study indicate that the cell cycle proteins analyzed have no significant prognostic value in ACC when each variable is analyzed alone. We therefore conducted a multimolecular analysis including all cell cycle proteins. One phenotype, Ki-67(-)p53(-)mdm-2(+)CD1(-)Bcl-2(-)p21(-)p27(+), distinguished normal from malignant adrenal tissue. This phenotype was observed in 83% of normal adrenal glands and in only one (3%) of 31 adrenal carcinomas ( $P = .01$ ). However, this phenotype is of limited clinical use because conventional microscopy can readily differentiate normal from malignant adrenocortical tissue. The most common malignant phenotype identified in 26% of primary adrenal carcinomas was Ki-67(-)p53(-)mdm-2(-)CD1(-)Bcl-2(-)p21(+)

(+). No normal adrenal tissue or metastases demonstrated this multimolecular pattern of expression. The most common multimolecular profile for metastatic adrenal carcinoma was Ki-67(+)/p53(-)/mdm-2(+)/CD1(-)/Bcl-2(-) p21(+)/p27(+) and was present in 32% of the lesions. This phenotype was observed in only two primary adrenal carcinomas (6%) and in none of the normal adrenal tissues. The only difference evident between the most frequent "primary" and "metastatic" phenotypes was an upregulation in cell proliferation reflected by Ki-67, and mdm-2 overexpression with higher stage. These findings are purely descriptive and require further study.

Multimolecular phenotypic expression was more heterogeneous in malignant than in normal adrenal tissue; five separate phenotypes were identified for 35 normal adrenals, 10 different phenotypes for 31 primary ACC, and nine dissimilar ones for 28 metastases. Another interesting finding is the variation in molecular phenotypes between primary and metastatic disease among individuals and within the same patients. Although the data presented here provide a comprehensive characterization of this rare disease, they are descriptive in nature and need to be verified in future studies. These results emphasize the molecular complexity of these neoplasms and emphasize the need for targeted molecular therapies tailored to the individual patient. These data also argue in favor of targeted therapy based not only on the primary tumor, but also on the metastatic deposit.

To our knowledge, this study is the first to simultaneously analyze a large number of morphologic and molecular parameters of outcome in ACC and to perform multivariate analysis on the data. After including clinical, histologic, and molecular parameters in the multivariate analysis, distant metastases, increased mitotic rate, and adjacent organ invasion emerged as the only independent predictors of survival in the study population. When data from patients without metastases who underwent curative resection were subjected to multivariate analysis, only mitotic activity (more than five of 50 high-power fields) was found to be an independent predictor of survival. This emphasizes the need to count mitoses carefully (50 high-power fields should be examined) and to report mitotic activity in pathologic reports on ACC for diagnostic and prognostic purposes.

In conclusion, careful morphologic evaluation and diligent mitotic count are essential components in the assessment of prognosis for patients with ACC, along with stage and completeness of resection. The stratification of patients with ACC may help guide and critically evaluate current and future adjuvant therapies. The multimolecular phenotyping performed in this study demonstrates that the molecular complexity and heterogeneity of these neoplasms mean that formulating targeted therapy will be challenging.

## REFERENCES

1. Luton J, Cerdas S, Billaud L, et al: Clinical features of adrenocortical carcinoma, prognostic factors, and the effect of mitotane therapy. *N Engl J Med* 322:1195-1201, 1990
2. Icard P, Chapuis Y, Andreassian B, et al: Adrenocortical carcinoma in surgically treated patients: A retrospective study on 156 cases by the French Association of Endocrine Surgery. *Surgery* 112:972-980, 1992
3. Schulik RD, Brennan MF: Long-term survival after complete resection and repeat resection in patients with adrenocortical carcinoma. *Ann Surg Oncol* 6:719-726, 1999
4. Weiss LM, Medeiros LJ, Vickery AL: Pathologic features of prognostic significance in adrenocortical carcinoma. *Am J Surg Pathol* 13:202-206, 1989
5. Kononen J, Bubendorf L, Kallioniemi A, et al: Tissue microarrays for high-throughput molecular profiling of tumor specimens. *Nat Med* 4:844-847, 1998
6. Hoos A, Urist MJ, Stojadinovic A, et al: Validation of tissue microarrays for immunohistochemical profiling of cancer specimens using the example of human fibroblastic tumors. *Am J Pathol* 158:1245-1251, 2001
7. Fuhrman SA, Lasky LC, Limas C: Prognostic significance of morphologic parameters in renal cell carcinoma. *Am J Surg Pathol* 6:655-663, 1982
8. Weiss LM: Comparative histologic study of 43 metastasizing and nonmetastasizing adrenocortical tumors. *Am J Surg Pathol* 8:163-169, 1984
9. Harrison LE, Gaudin PB, Brennan MF: Pathologic features of prognostic significance for adrenocortical carcinoma after curative resection. *Arch Surg* 134:181-185, 1999
10. Mucci NR, Akdas G, Manley S, et al: Neuroendocrine expression in prostate cancer: Evaluation of high throughput microarrays to detect heterogeneous protein expression. *Hum Pathol* 31:406-414, 2000
11. Schraml P, Kononen J, Bubendorf L, et al: Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* 5:1966-1975, 1999
12. Richter J, Wagner U, Kononen J, et al: High throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* 157:787-794, 2000
13. Hoos A, Stojadinovic A, Singh B, et al: Clinical significance of molecular expression profiles of Hürthle cell tumors of the thyroid gland analyzed via tissue microarrays. *Am J Pathol* (in press)
14. Tritos NA, Cushing GW, Heatley G, et al: Clinical features and prognostic factors associated with adrenocortical carcinoma: Lahey Clinic Medical Center experience. *Am Surg* 66:73-79, 2000
15. Slooten H, Schaberg A, Smeenk D, et al: Morphologic characteristics of benign and malignant adrenocortical tumors. *Cancer* 55:766-773, 1985
16. Evans HL, Vassilopoulou-Sellin R: Adrenal cortical neoplasms: A study of 56 cases. *Am J Clin Pathol* 105:76-86, 1996
17. Nakano M: Adrenal cortical carcinoma: A clinicopathological and immunohistochemical study of 91 autopsy cases. *Acta Pathol Jpn* 38:163-180, 1988
18. Hogan T, Gilchrist KW, Westring DW, et al: A clinical and pathological study of adrenocortical carcinoma: Therapeutic implications. *Cancer* 45:2880-2883, 1980
19. Edgren M, Eriksson B, Wilander, et al: Biological characteristics of adrenocortical carcinoma: A study of p53, IGF, EGF-r, Ki-67 and PCNA in 17 adrenocortical carcinomas. *Anticancer Res* 17:1303-1310, 1997
20. Nakazumi H, Sasano H, Iino K, et al: Expression of cell cycle inhibitor p27 and Ki-67 in human adrenocortical neoplasms. *Mod Pathol* 11:1165-1170, 1998
21. McNicol AM, Nolan CE, Struthers MA, et al: Expression of p53 in adrenocortical tumors: Clinicopathological correlations. *J Pathol* 181:146-152, 1997
22. Sasano H, Imatani A, Shizawa S, et al: Cell proliferation and apoptosis in normal and pathologic human adrenal. *Mod Pathol* 8:11-17, 1995
23. Reincke M, Karl M, Travis WH, et al: p53 mutations in human adrenocortical neoplasms: Immunohistochemical and molecular studies. *J Clin Endocrinol Metab* 78:790-794, 1994
24. El-Deiry WS, Tokino T, Velculescu VE, et al: WAF1, a potential mediator of p53 tumor suppression. *Cell* 75:817-825, 1993
25. Chao DT, Korsmeyer SJ: BCL-2 family: Regulators of cell death. *Ann Rev Immunol* 16:395-419, 1998
26. Catzavelos C, Bhattacharya, Ung YC, et al: Decreased levels of the cell-cycle inhibitor p27 protein: Prognostic implications in primary breast cancer. *Nat Med* 3:227-230, 1997
27. Gerdes J, Li L, Schlueter C, et al: Immunobiochemical and molecular biologic characterization of the cell proliferation-associated nuclear antigen that is defined by monoclonal antibody Ki-67. *Am J Pathol* 138:867-873, 1991
28. Hoos A, Stojadinovic A, Mastorides S, et al: High Ki-67 proliferative index predicts disease-specific survival in patients with high-risk soft tissue sarcomas. *Cancer* 92:869-874, 2001