Amphiregulin Is a Prognostic Factor in Colorectal Cancer

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Abstract. *Amphiregulin is an epidermal growth factor (EGF) which is a ligand of epidermal growth factor receptor (EGFR). Amphiregulin is the most enhanced EGFR ligand in colon cancer. Here we report on the expression of Amphiregulin using immunohistochemical staining in primary colorectal cancer, and the correlations between prognosis and various clinicopathological factors. We examined 174 consecutive patients who underwent curative resection of colorectal cancer, from January 2002 to December 2004. Amphiregulin was positive in 156 (90%) patients. Amphiregulin was found to be an independent predictor of overall survival [hazard ratio=6.25 (95% confidence interval=1.3-111.5; p=0.0144)] and relapse-free survival [hazard ratio=6.94 (95% confidence interval=1.5-123.2; p=0.0075)]. We conclude that the expression of Amphiregulin in a primary colorectal tumor is useful as an indicator of prognosis and as a predictor of recurrence.*

Amphiregulin is a member of the epidermal growth factor (EGF) family and is a ligand of epidermal growth factor receptor (EGFR). Several kinds of molecules such as EGF, amphiregulin, transforming growth factor (TGFα), epigen, epiregulin (EPR), betacellulin (BTC), heparin-binding EGF-like growth factor (HB-EGF) andor neuregulin (NRG) are reported to belong to the EGF family (1). EGFR forms a homo- or heterodimer and, through signal transduction, is involved in cancer development and progression (2). Anti-EGFR antibodies (cetuximab, panitumumab) have been used in colorectal cancer and have led to good results. Malignant transformation of EGFR and its cognate ligands, and autocrine/paracrine production of EGFR ligands, as well as overexpression of EGFR, are often implicated in cancer development and progression (3). In colon cancer, amphiregulin has been recognized to be the EGFR ligand with the most enhanced expression (2). The expression of amphiregulin in the primary tumor has been reported to be a possible predictor of liver metastasis (4), but it is not yet clearly understood how the expression of amphiregulin contributes to colorectal cancer. Here we examined the expression of amphiregulin in primary colorectal cancer tumors and the correlations with clinicopathological factors and prognosis.

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Materials and Methods

Human tissues. The study population consisted of 174 consecutive patients who underwent curative resection of colorectal cancer at the Department of Surgery, Kurume University Hospital from January 2002 to December 2004. Patients with a second cancer were not included. The patients' characteristics are described in Table I. The median follow-up duration was 80 months (ranging from 2 to 118 months). Patients had stage 0 to IIIC disease (TNM 7th). There were 18 cases of recurrence, seven cases of liver metastasis, seven cases of lung metastasis, and seven cases of local recurrence. Adjuvant chemotherapy was used in 61 cases (35%).

Immunohistochemistry. For amphiregulin immunostaining with goat polyclonal antibodies, tissue sections (3 μm) were deparaffinized in xylene and rehydrated in an ethanol series. The sections were then treated for 5 min with 3% hydrogen peroxide to block endogenous peroxidase activity. The sections were unmasked in Tris/EDTA buffer, pH 9 (DAKO Target Retrieval Solution) in an autoclave for 10 min, at 105˚C, and were subsequently washed with phosphate-buffered saline (PBS). The sections were incubated with rabbit serum for 30 min at room temperature and were then incubated with the primary antibody [goat polyclonal antibody to amphiregulin (1/200): R&D: McKinley Place NE Minneapolis, MN, USA] for 30 min, at room temperature. The bound primary antibodies were detected by adding anti-goat secondary antibodies and an avidin/biotin/horseradish peroxidase complex (VECTOR: Ingold

Table I. *Patients' profiles*.

Pathological factor		Value
Age, years	Median (range)	67 (29-87)
Gender, n	Male	105
	Female	69
Adjuvant chemotherapy, n	Present	61
	Absent	113
CEA, ng/ml	Median (range)	3.65 (0-129)
Location, n	Rectum	64
	Colon	110
Differentiation, n	Well	112
	Moderate	50
	Poor	12
Depth of invasion, n	T0	10
	T1	14
	T2	27
	T3	68
	T4	55
Lymph node metastasis, n	Present	62
	Absent	112
Stage, n	0	9
	I	36
	IIA	47
	IIB	22
	IIIA	2
	IIIB	40
	IIIC	18
Lymphatic invasion, n	Positive	76
	Negative	98
Vascular invasion, n	Positive	126
	Negative	48
Budding, n	Positive	100
	Negative	74
Max dimension, cm	Median (range)	45 (12-128)
Recurrence, n	Present	18
	Absent	156
Organ of recurrence	Liver	7
	Lung	7
	Local	7
	Peritoneum	0
	Lymph node	5

CEA: Carcinoembryonic antigen.

Road, Burlingame, CA, USA) for 30 min, at room temperature. The sections were visualized using solid diaminobenzine diluted in PBS, counterstained with Mayer's hematoxylin, and were finally mounted.

Evaluation of immunostaining. The slides were graded according to the staining intensity and the percentage of immunopositive cells, as previously described (5). Specific staining with postimmune serum was semiquantitated by assigning a score of 0 to 3 based on the color intensity of the brown diaminobenzidine precipitate, as 1, representing light brown staining; 2, a moderately brown color; or as 3, an intense brown color. The number of positive cells per slide was stratified into three groups based on the percentage of positive cells: group 1 with <33%; group 2 with 33 to 67%; and group 3 with >67%. Semiquantitative scores ranging from 1 to 9 for the specific staining of each specimen were obtained by multiplying the staining intensity by the number of the group that represented the percentage of positive cells within each specimen. A score of zero represents no specific staining (Figure 1).

Data analysis. The association between the results of the amphiregulin staining and each of the clinicopathological factors were tested using the chi-squared test or *t*-test. Cox proportional hazard model was employed to examine the effect of amphiregulin on overall survival (OS) and relapse-free survival (RFS). Before accessing the effect of amphiregulin on OS and RFS, we used the survival tree model (6) to stratify the study sample into a number of clinically meaningful and homogeneous subgroups based on the clinicopathological factors. These strata were included in the Cox model to control confounding. When the number of events is low with a large number of potential confounding variables (7), the tree-based method such as the survival tree model may be a practical method for controlling confounding. The R (version 2.14.1) package RPART (recursive partitioning and regression trees) by Terry Therneau and Beth Atkinson (http://www.mayo.edu/biostatistics) was used to conduct the survival tree modeling. Other analyses were carried out by JMP 9.0.0 (SAS: roppongi, minatoku, Tokyo, Japan).

Results

Immunohistochemistry. The expression of amphiregulin was found in the cytoplasm and the membrane (Figure 1). There was no expression in 18 cases (10%), light expression in 48 (28%), moderate in 75 (48%), and intense in 33 (19%). According to the criteria mentioned above, 156 (90%) patients were determined to be amphiregulin-positive. Amphiregulin and its correlations with the clinicopathological factors are shown in Table II. The amphiregulin-positive group was significantly younger than the negative group (p=0.0341). The amphiregulin- positive rate was significantly higher with moderately or well-differentiated tumor types (p=0.0237), and in vascular invasion-negative cases (p=0.0272). No significant difference was found concerning amphiregulin and other clinicopathological factors.

Survival time analysis. Stratification of the study samples was carried out for OS and RFS separately, and results are shown in Figure 2 and Figure 3, respectively. These strata were included in the Cox proportional hazard models. Tables III and IV show the results of multivariate analyses. The effect of amphiregulin on OS was significant (p=0.0144) with an adjusted hazard ratio (HR) of 6.25 (95% confidence interval, CI=1.3-111.5). This finding suggests that the expression of amphiregulin may be an independent prognostic factor for colorectal cancer. The effect of amphiregulin on RFS was significant (p=0.0075), with an adjusted HR of 6.94 (95% CI=1.5-123.2). These findings indicate that amphiregulin may be an independent predictor of recurrence.

Table II. *Relationship between amphiregulin (AR) expression and clinicopathological factors.*

		AR expression				
		Positive (n=156)		Negative (n=18)		*p*-Value
Age, years		64.6±0.92		69.8±2.71		0.0341
Gender, n	Male	94	(90%)	11	(10%)	0.944
	Female	62	(90%)	7	(10%)	
Location, n	Rectum	55	(86%)	9	(14%)	0.2193
	Colon	101	(92%)	9	(8%)	
Depth, n	T0, 1, 2	50	(98%)	1	(2%)	0.2159
	T3, 4	106	(86%)	17	(13%)	
Lymph node metastasis, n	Present	56	(90%)	6	(10%)	0.8297
	Absent	100	(89%)	12	(11%)	
Differentiation, n	Poor	8	(67%)	4	(33%)	0.0237
	Well, moderate	148	(91%)	14	(9%)	
Lymphatic invasion, n	Positive	67	(88%)	9	(12%)	0.5679
	Negative	89	(91%)	9	(9%)	
Vascular invasion, n	Positive	109	(86%)	17	(13%)	0.0272
	Negative	47	(98%)	1	(2%)	
Budding, n	Positive	90	(90%)	10	(10%)	0.8622
	Negative	66	(89%)	8	(11%)	
Recurrence, n	Yes	18	(100%)	0	(0%)	0.2223
	No	138	(88%)	18	(12%)	
Max dimention, cm		49.5±1.88		53.17±5.54		0.1263
CEA, ng/ml		8.33±1.29		7.73±3.78		0.105
Adjuvant chemotherapy, n	Yes	56	(92%)	5	(8%)	0.6069
	No	100	(88%)	13	(12%)	

Discussion

Data analyses revealed that the expression of amphiregulin in colorectal cancer primary tumor may be an important predictor of prognosis and recurrence. However, these findings were not without a caveat. There are two concerns regarding the data analyses. The first is the highly unbalanced proportion of amphiregulin-positive (90%) and -negative (10%) cases. The second concern is the low number of events in both OS and RFS. Conducting a study with a larger number of patients is required in order to confirm these preliminary findings.

Human amphiregulin is an 84-amino acid glycoprotein discovered and characterized in the late 1980s by Shoyab *et al*. (1). Amphiregulin was originally isolated from the conditioned medium of phorbol 12-myristate 13-acetate (PMA)-stimulated MCF-7 human breast carcinoma cells (1). The human amphiregulin gene (gene ID 374) spans about 10 kb of genomic DNA and is located on the q13–q21 region of chromosome 4. It is composed of six exons encoding a 1.4 kb pre-protein mRNA transcript. The amphiregulin protein is synthesized as a 252-amino acid transmembrane precursor, pro-amphiregulin. At the plasma membrane, pro-amphiregulin is subjected to sequential proteolytic cleavages within its ectodomain and is then released as the soluble amphiregulin protein. Depending on the cell type and microenvironment, amphiregulin can be produced in multiple cellular and mature forms using alternative pro- amphiregulin cleavage sites and glycosylation motifs, thus impacting the biological activity of amphiregulin (8). Amphiregulin shedding is essentially mediated by tumor-necrosis factor-alpha converting enzyme (TACE), a member of the

Table III. *Multivariate analysis for overall survival (OS).*

	df	χ^2	*p*-Value	HR	95% CI
Amphiregulin	1	6.26	0.0124	7.24	1.42-132.39
OSST	11	70.4	<0.0001		

df: Degree of freedom. HR: Hazard ratio. CI: Confidence interval. OSST: Strata from survival tree for OS.

Table IV. *Multivariate analysis for relapse-free survival.*

	df	χ^2	*p*-Value	HR	95% CI
AR	1	5.85	0.0155	6.69	1.34-121.52
RFSST	12	67.37	<0.0001		

df: Degree of freedom. HR: Hazard ratio. CI: Confidence interval. RFSST; Strata from survival tree for RFS.

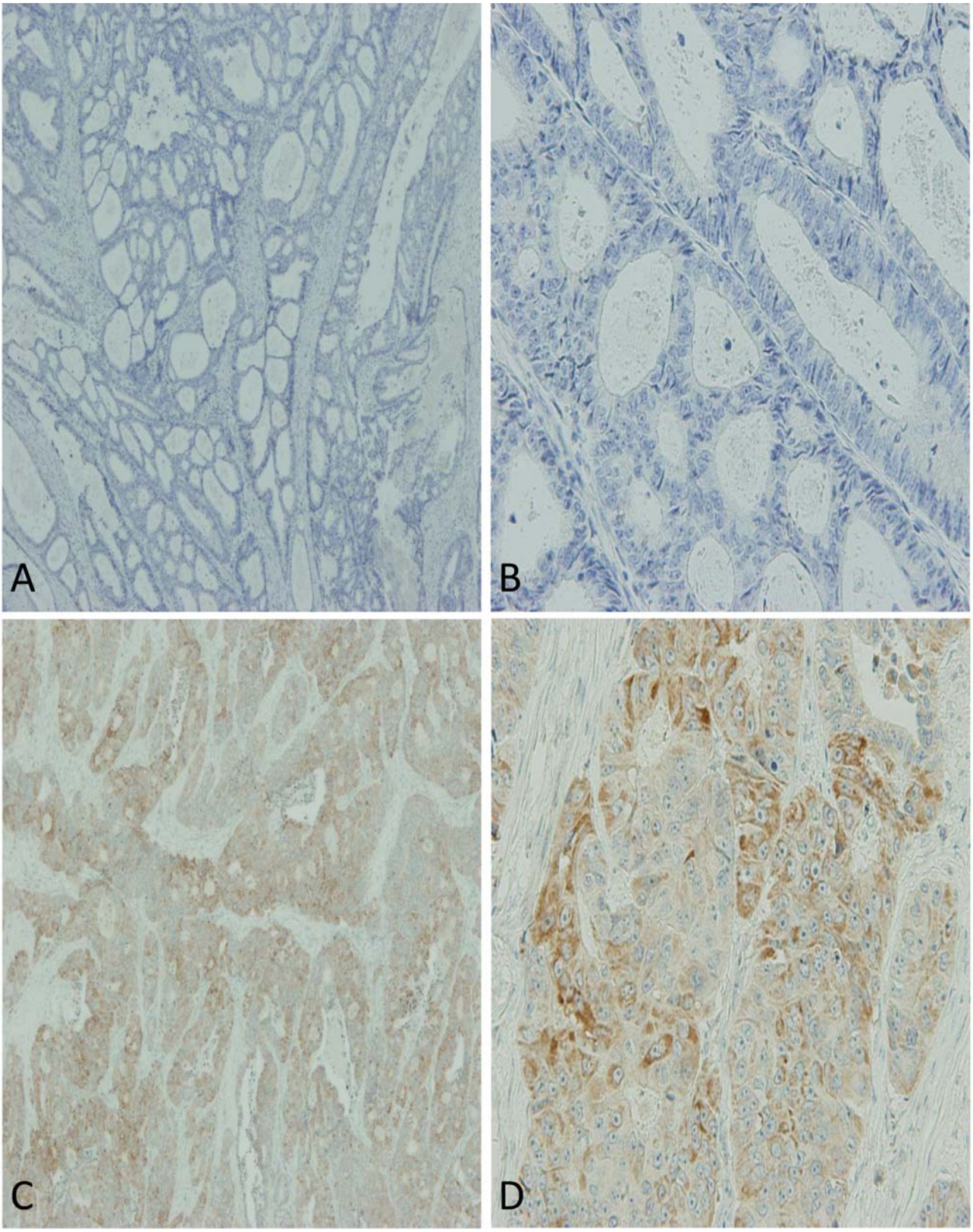

Figure 1. *Immunohistochemical analysis for primary tumors of colorectal cancer. A: Amphiregulin-negative case (×40). B: Amphiregulin-negative case (×200). C: Amphiregulin-positive case (×40). D: Amphiregulin-positive case (×200).*

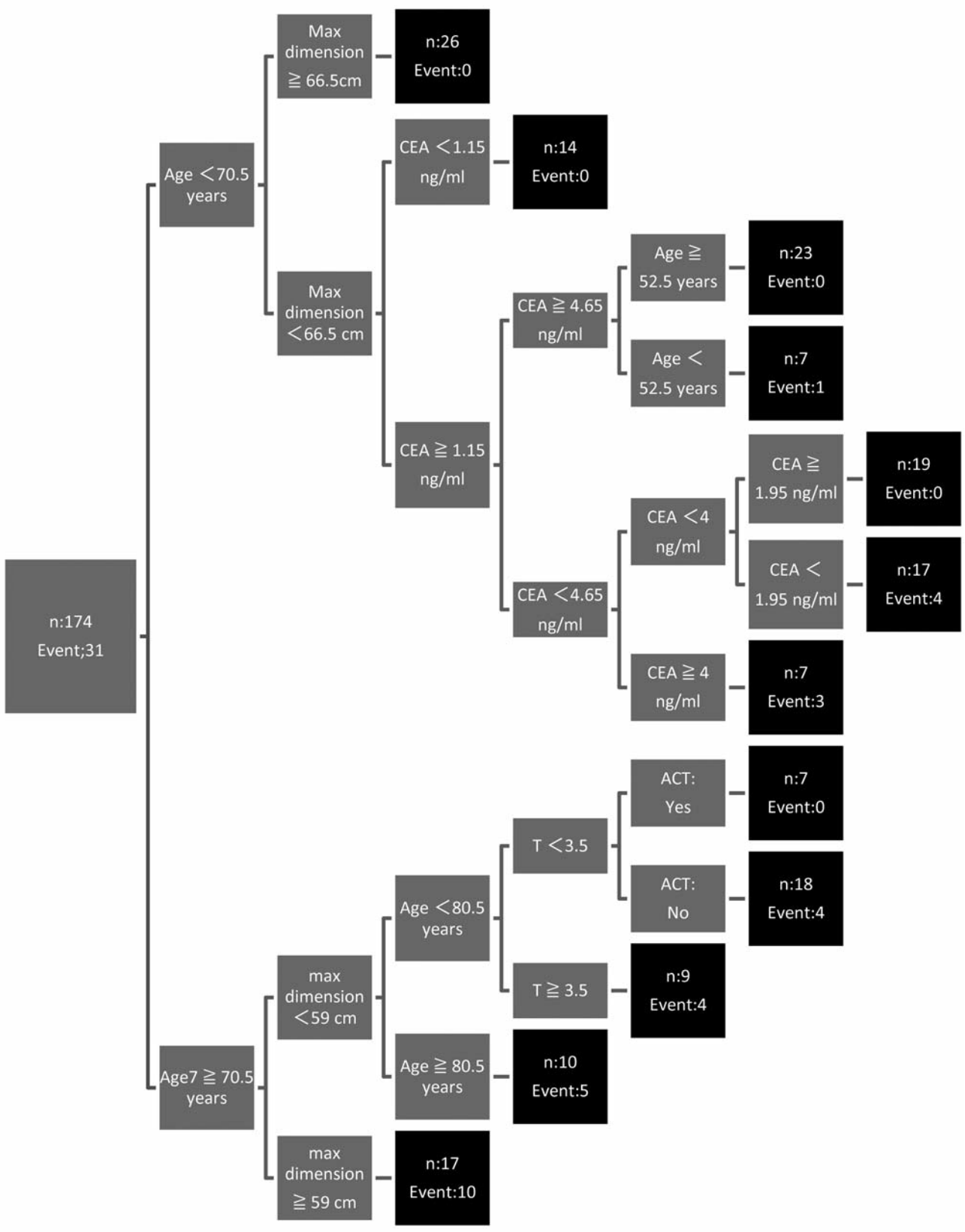

Figure 2. *Survival tree model partition of the dataset into strata, based on the value of clinicopathlogical factors; patients belonging to the same stratum share homogeneous overall survival.*

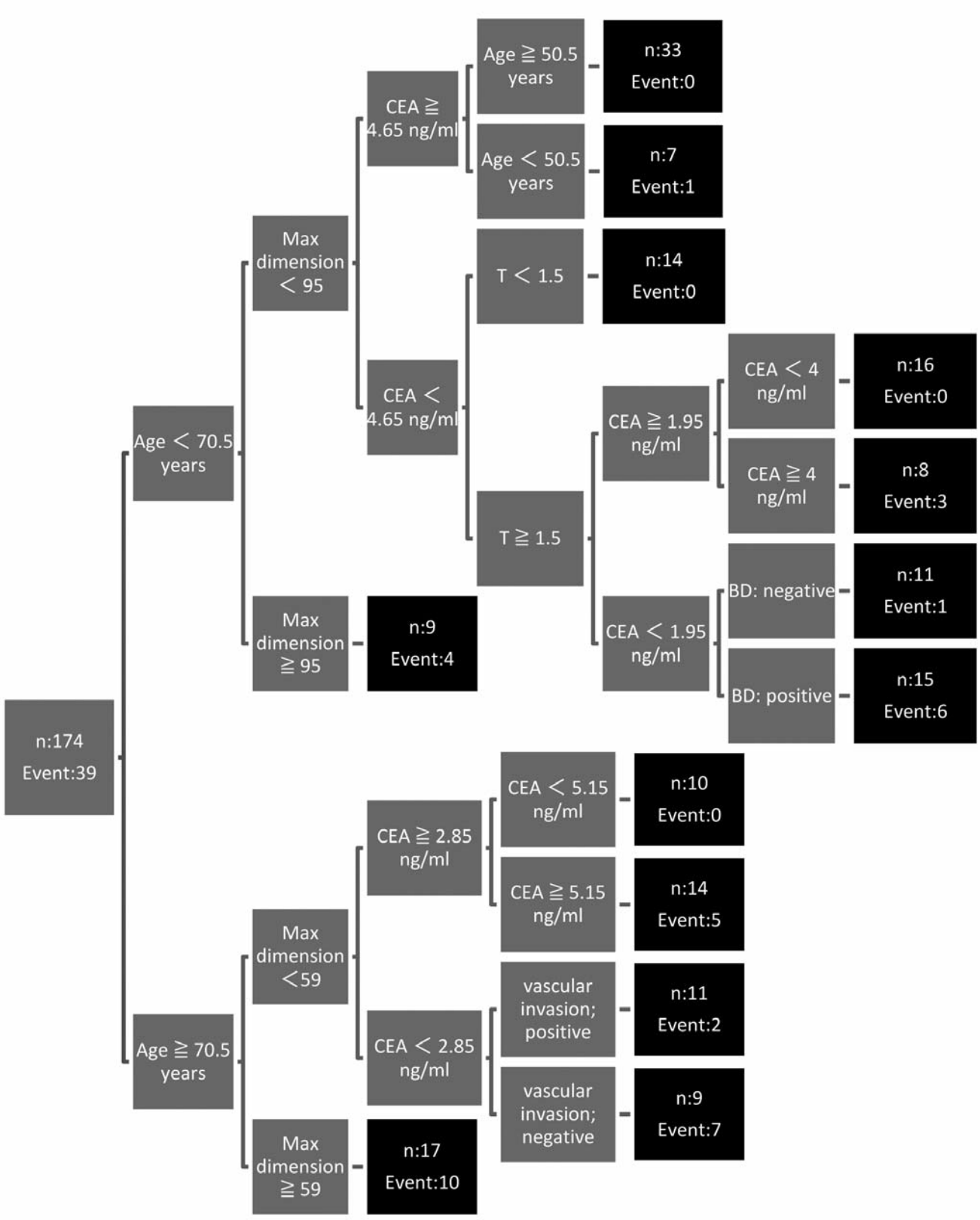

Figure 3. *Survival tree model partition of the dataset into strata, based on the value of clinicopathlogical factors; patients belonging to the same stratum share homogeneous relapse-free survival.*

disintegrin and metalloproteinase (ADAM) family (also known as ADAM-17) (9).

The EGFR is a tyrosine kinase receptor that is involved in fundamental signaling pathways and is therefore a major target in oncology. EGFR belongs to the ERBB/human epidermoid receptor (HER) family, which contains the following four members: (EGFR/HER1/ERBB1), HER2/neu (ERBB2), HER3 (ERBB3) and HER4 (ERBB4). These receptors share important structural homology and are composed of an intracellular domain with tyrosine kinase activity, a hydrophobic alpha-helix transmembrane domain, and an extracellular domain required for ligand recognition and binding. The extracellular domain is the least conserved among the four ERBrbB members, allowing distinct specificities and selectivities for ligands (10).

Reports on amphiregulin in colorectal cancer suggest the following: the amphiregulin and cripto expression may discriminate normal from malignant colonic epithelium and may provide a selective growth advantage for colorectal carcinomas (11). Similarly, amphiregulin and epiregulin were not expressed in the normal colonic mucosa but were clearly detectable in adenomas and carcinomas (12). High serum and tissue levels of amphiregulin were found to be predictors of poor prognosis (13) and were related to disease-free survival and hepatic metastasis-free survival for patients with colorectal carcinoma (4). These reported findings may not be comparable to our results due to differences in the patients' background factors. These differences might be attributed to our study design. Both are observational and retrospective studies. Although our exploratory findings may contribute to establishing new hypotheses regarding the effects of amphiregulin on the prognosis of colorectal cancer, the conduction of an appropriate randomized controlled trial is necessary to confirm any role of amphiregulin expression in the prognosis of colorectal cancer.

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