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Adiponectin, Leptin, and Insulin-Pathway Receptors as Endometrial Cancer Subtyping Markers

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Abstract

Developing a system of molecular subtyping for endometrial tumors might improve insight into disease etiology and clinical prediction of patient outcomes. High body mass index (BMI) has been implicated in development of endometrial cancer through hormonal pathways and might influence tumor expression of biomarkers involved in BMI-sensitive pathways. We evaluated whether endometrial tumor expression of 7 markers from BMI-sensitive pathways of insulin resistance could effectively characterize molecular subtypes: Adiponectin Receptor 1, Adiponectin Receptor 2, Leptin Receptor, Insulin Receptor (beta subunit), Insulin Receptor Substrate 1, Insulin-Like Growth Factor 1 Receptor, and Insulin-Like Growth Factor 2 Receptor. Using endometrial carcinoma tissue specimens from a case-only prospective sample of 360 women from the Nurses' Health Study, we scored categorical immunohistochemical measurements of protein expression for each marker. Logistic regression was used to estimate associations between endometrial cancer risk factors, especially BMI, and tumor marker expression. Proportional hazards modeling was performed to estimate associations between marker expression and time to all-cause mortality as well as time to endometrial cancer-specific mortality. No association was observed between BMI and tumor expression of any marker. No marker was associated with time

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare that they have no conflict of interest. Informed consent was obtained from all individual participants included in the study. The Institutional Review Board of Brigham and Women's Hospital in Boston, MA, USA approved the analysis. All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1975 Helsinki Declaration and its later amendments or comparable ethical standards. This article does not contain any studies with animals performed by any of the authors.

to either all-cause mortality or endometrial cancer-specific mortality in models with or without standard clinical predictors of patient mortality (tumor stage, grade, and histologic type). It did not appear that any of the markers evaluated here could be used effectively to define molecular subtypes of endometrial cancer.

Keywords

endometrial cancer; subtypes; etiology; mortality; prediction; insulin resistance

INTRODUCTION

Endometrial tumor subtypes are generally defined histologically [1,2]. Supplementing this approach by defining molecular subtypes might improve understanding of disease etiology, prognosis, and response to therapy, which can lead to better prevention efforts and treatment decisions. Several recent studies of molecular subtypes of endometrial cancer have identified markers that could be used to define subtypes, for example, mutations in the *POLE* gene [3–6]. The proposed classification schemes have gaps, such as not clearly classifying cases with multiple alterations among classification markers [7] and including an ambiguous subtype characterized as “no specific molecular profile” [4]. These observations suggest that molecular subtyping of endometrial tumors might be improved by the inclusion of one or more markers from additional molecular pathways.

Body mass index (BMI) has been associated with risk of developing endometrial cancer [8–10]. Mechanistically, this association might be due to BMI influencing endogenous hormone levels, such as greater BMI increasing estrogen levels, which in turn could stimulate excessive endometrial tissue proliferation [8,11,12]. Given the hormone-mediated carcinogenic effects of BMI on endometrial tissue, variation in BMI might correspond to variation in endometrial tumor expression of molecular markers involved in BMI-sensitive pathways. Two pathways implicated in insulin resistance, the adiponectin/leptin [13–15] and insulin [16–19] pathways, have been associated with risk of endometrial cancer. If tumor expression of a marker from one of these pathways is related to endometrial cancer risk factors and patient outcomes, then the marker could serve as a useful basis to define molecular subtypes.

In endometrial cancer cases from the Nurses’ Health Study (NHS), we evaluated the potential for tumor expression of 7 markers from BMI-sensitive pathways to define molecular subtypes: 3 markers from the adiponectin/leptin pathway (Adiponectin Receptor 1 [ADIPOR1], Adiponectin Receptor 2 [ADIPOR2], and Leptin Receptor [OBR]) and 4 markers from the insulin pathway (Insulin Receptor, β -subunit [IR], Insulin Receptor Substrate 1 [IRS1], Insulin-Like Growth Factor 1 Receptor [IGF1R], and Insulin-Like Growth Factor 2 Receptor [IGF2R]). We hypothesized that greater BMI at diagnosis would be associated with greater tumor expression of OBR, IR, IRS1, and IGF1R, and with lower tumor expression of ADIPOR1, ADIPOR2, and IGF2R [16,17,13,14,18,19,15]. We also evaluated whether tumor expression of any of the markers might improve clinical prediction

of patient mortality outcomes beyond what is already achieved by standard predictors (tumor stage, grade, and histologic type).

MATERIALS AND METHODS

Detailed descriptions of the study sample, participant flow, and case ascertainment procedures have been published [20]. Briefly, the NHS prospective cohort study enrolled 121,700 female registered nurses from the United States who were aged 30–55 at baseline in 1976 [21–23]. Biennially after baseline, participants completed questionnaires updating reproductive, lifestyle, and other health characteristics, including disease diagnoses. For participants who self-reported a diagnosis of endometrial cancer on a questionnaire, an NHS physician reviewed the participant's medical records to confirm an International Classification of Diseases 8 code starting with 182. Medical records were also used by a pathologist (G.L. Mutter) to update tumor histologic classifications to 2009 standards [24]. Through 2013, 2,035 confirmed cases of primary endometrial carcinoma had been diagnosed in the cohort. Endometrial tumor tissue specimens were acquired for 472 cases, of which 360 had tissue adequate to be incorporated into tissue microarrays (TMAs). The present study evaluated marker expression in cases from the TMAs. The NHS protocol was approved by the Institutional Review Board of Brigham and Women's Hospital, Boston, MA, and all participants provided informed consent.

Protein expression of each marker was measured in endometrial primary carcinoma specimens using immunohistochemistry as described previously [20]. The antibodies and dilutions were anti-ADIPOR1 rabbit antibody vs human peptide aa357-375 (Phoenix Pharmaceuticals, H-001-44) at 1:500 dilution; anti-ADIPOR2 rabbit antibody vs human peptide aa374-386 (Phoenix Pharmaceuticals, H-001-23) at 1:500 dilution; anti-OBR polyclonal vs human peptide aa541-840 (Santa Cruz H-300, sc-8325) at 1:50 dilution; anti-IR mouse monoclonal vs human, C-terminus of β subunit (EMD Millipore CT-3, 05-1104) at 1:50 dilution; anti-IRS1 rabbit polyclonal vs C-terminus human peptide (Santa Cruz Biotechnology C-20, sc-559) at 1:50 dilution; anti-IGF1R rabbit polyclonal raised vs C-terminus of human IGF-1R β -subunit (Santa Cruz Biotechnology C-20, sc-713 IGF-1R β) at 1:100 dilution; and anti-IGF2R goat polyclonal vs. human C-terminus peptide (Santa Cruz Biotechnology C-15, sc-14410) at 1:50 dilution.

Most participants had 3 tissue cores. For each marker, immunohistochemistry staining was manually scored by a single pathologist (G.L. Mutter) on a categorical scale, with the final score for an individual being a composite of 2 duplicate reads of the 3 cores. ADIPOR1, ADIPOR2, and OBR were scored for cytoplasmic staining. IR, IGF1R, and IGF2R were scored for a combination of cytoplasmic and membrane staining. IRS1 was scored for a combination of cytoplasmic and nuclear staining. ADIPOR1 and ADIPOR2 staining were scored on a 0–3 scale (0=none, 1=low, 2=moderate, 3=high), while staining for the rest of the markers was scored on a 0–2 scale (0=none, 1=low, 2=high). Specimens subject to tissue dropout, or for which background and signal staining could not be distinguished, were excluded. This meant that the final available sample size for each marker was less than 360, with the exact count varying by marker. The intra-observer scoring agreement of the 2 duplicate reads per marker was as follows: ADIPOR1 95%, ADIPOR2 83%, OBR 95%, IR

86%, IRS1 87%, IGF1R 87%, and IGF2R 91%. Discrepancies were resolved by a third review by G.L. Mutter.

Associations between BMI and tumor marker expression were evaluated using unconditional logistic regression. The dependent variable was marker expression, which was dichotomized by a cut point distinguishing expression above the cut point from expression at or below the cut point. For each marker, we created every dichotomous expression variable that the categorical data permitted, e.g. for 0–2 scale we created 2 dichotomous variables: 2 vs. 0/1, and 1/2 vs. 0. The independent variables were endometrial cancer risk factors of BMI (categorical <25, 25–<30, 30+ kg/m²), years of unopposed estrogen hormone therapy use (continuous), years of estrogen–progesterone combination hormone therapy use (continuous), years of other hormone therapy use (continuous), years of oral contraceptive use (continuous), whether the participant was a current smoker (yes/no), whether the participant was a former smoker (yes/no), number of pack-years smoked (continuous), diabetes status (yes/no), family history of endometrial cancer (yes/no), parity (continuous), years from menarche to menopause (continuous), and years since menopause (continuous). BMI was categorized because continuous measurements were not normally distributed, with category boundaries of 25 and 30 being chosen based on World Health Organization standards [25]. For tumor markers, to assess the impact of different cut points to define high versus low expression, multiple model runs for each marker were performed, one for each marker expression cut point.

Associations between tumor marker expression and mortality outcomes were evaluated using Cox proportional hazards models. Two different outcomes were assessed: time from diagnosis to all-cause mortality and time from diagnosis to endometrial cancer-specific mortality. Each outcome was censored at 10 years after diagnosis or in June 2012, whichever came first. Mortality outcomes were identified using the National Death Index, report from relatives, or the United States Postal Service. To evaluate whether addition of a marker to standard clinical predictors of patient mortality outcomes would improve the performance of the panel of predictors, independent variables were dichotomous tumor marker expression and endometrial cancer prognostic factors of tumor stage (III/IV vs I/II), tumor grade (poorly-differentiated vs well/moderately-differentiated), and histologic type (non-endometrioid vs endometrioid). Tumor stage in statistical models was the participant's classification according to standards set in 2009.

Given that the models in a cut point analysis are not independent [20], models were not adjusted for multiple comparisons [26]. The study had 80% power to detect etiologic odds ratios of at least 1.71 and survivorship hazard ratios of at least 2.31. Power calculations for etiologic odds ratios were performed using Power V.3.0.0 (National Cancer Institute, Bethesda, MD). All other analyses were performed using SAS 9.4 (SAS Institute, Cary, NC).

RESULTS

Table 1 presents demographic, reproductive, behavioral, medical history, and tumor characteristics for the 360 endometrial cancer cases incorporated into TMAs. Previously, we

showed that descriptive statistics for cases in the TMAs were similar to those for all 2,035 confirmed NHS endometrial cancer cases [20].

Table 2 presents expression distributions for each marker. The proportion of participants with no staining was 11% or less for every marker except IR. For some markers—ADIPOR1, OBR, IRS1, and IGF2R—at least 70% of the tumors had low expression. The remaining markers had a more even distribution of expression from low to high. Table 2 also shows estimates of associations between BMI and tumor marker expression. BMI at diagnosis was not associated with expression of any of the tumor markers, regardless of how high versus low marker expression was defined. Apart from considerations of statistical significance, the magnitudes of point estimates were generally close to the null value of 1.00.

Table 3 shows estimates of associations between tumor marker expression and mortality outcomes when marker expression was adjusted for standard prognostic factors. Tumor marker expression was not associated with either time to all-cause mortality or time to cancer-specific mortality, regardless of the cut point used to define high versus low expression. Although not statistically significant, the magnitude of several point estimates did diverge notably from 1.00, such as those in the range of about 0.50–0.60 or around 2.00. Similar results were observed when the markers were not adjusted for standard prognostic factors (data not shown).

DISCUSSION

In a case-only study of several insulin-resistance markers to define endometrial cancer subtypes, we found that BMI at diagnosis was not associated with tumor expression of any of the markers. We also did not find any statistically significant associations between any marker and patient mortality outcomes, although several point estimates diverged notably from the null value. Given that the markers were chosen in part based on their role in BMI-sensitive hormonal pathways, the null associations between BMI and marker expression suggest that measurements of these markers would not distinguish different etiologies for biologically-different endometrial tumors.

The lack of association between marker expression and patient mortality outcomes implies that the markers are unlikely to improve clinical prediction of these outcomes beyond what is already achieved using the standard predictors of tumor stage, grade, and histologic subtype. However, the suggestive point estimates for associations between some tumor markers and patient mortality outcomes indicates the possibility of associations that would be more clearly detected in larger samples. While these data do not rule out the possibility of a useful role for one or more of the markers evaluated here—for example, one of them might predict an outcome we could not evaluate, such as response to therapy—overall we observed no firm evidence that any of these markers would provide a useful basis for defining molecular subtypes of endometrial cancer, either for preventive or clinical purposes. Furthermore, in relation to the panels of markers previously proposed to define molecular subtypes of endometrial cancer [3–6], our results suggest that it is unlikely that any of the markers we evaluated would improve the performance of those panels.

Prior work suggested a possible pathway from BMI to hormone level changes to development of endometrial cancer. For example, greater BMI has been associated with greater endogenous estrogen levels, and elevated estrogen has a carcinogenic influence on endometrial tissue, thereby increasing risk of endometrial cancer [8,9,11,10,12]. Previously, we found that, compared to the non-obese, obesity could be associated with greater endometrial tumor expression of estrogen receptor (ER) [20]. Thus, estrogen levels might be associated not just with overall risk of developing endometrial cancer, but might further influence the biological variation among endometrial tumors. We observed that tumor expression of ER was inversely associated with endometrial cancer-specific mortality.

These findings suggested that levels of other hormones that are also influenced by BMI could likewise contribute to biological variation among endometrial tumors, and that such tumor variation could be related to patient outcomes. Given that insulin resistance has been associated with risk of endometrial cancer, through both adiponectin/leptin and insulin pathways [16,17,13,14,18,19,15], we evaluated whether endometrial tumor expression of 7 receptors related to these pathways were associated with BMI and with patient mortality outcomes, but detected no associations.

The present results should be viewed in the context of previous work on the relationship of endometrial cancer to circulating levels of adiponectin, leptin, and insulin-pathway molecules. Low circulating adiponectin [13,15] and high circulating leptin [13] have both been associated with increased risk of endometrial cancer. The role of leptin in stimulating endometrial tissue proliferation has been noted [16]. For the insulin pathway, IGF1 has been associated with cell proliferation [16], and both IGF1 and IGF2 have been implicated in endometrial carcinoma progression, especially with regards to tumor aggressiveness [19]. It may be that circulating levels of adiponectin, leptin, and insulin-pathway molecules exert carcinogenic effects on endometrial tissue, but not in a way that influences the expression levels of the corresponding receptors of these molecules in the resulting tumors.

Among the strengths of the analysis, first, our evaluation of every possible cut point for each marker to define high versus low expression allowed us to assess whether the results were sensitive to the definition of marker positivity. Second, different kinds of modeling were used for the two halves of the investigation, each appropriate to the research question it addressed [20]. We used causal modeling in our etiologic investigation of association between BMI and tumor marker expression because the interest in this context was in population-level prevention efforts. This meant that our BMI-marker models were adjusted for other risk factors of endometrial cancer as potential confounders of the association of interest. We used predictive modeling to investigate the relationship between marker expression and mortality outcomes because, in that case, the interest would be in clinical outcomes prediction at the level of the individual [27]. Besides marker expression, the survivorship prediction models included as independent variables the standard clinical prognostic factors of mortality outcomes, not to control confounding, but rather to assess whether adding marker expression to the panel of standard prognostic factors might improve prediction. Had we found associations between marker expression and mortality outcomes, this would have justified additional prediction analyses using more clinically-relevant measures such as risk reclassification statistics (e.g. event and non-event Net

Reclassification Indices, Reclassification Calibration Statistic). However, this was unnecessary since the markers did not meet the lowest standard of evidence, i.e. association.

The study had several limitations. First, given that protein expression is naturally continuous, the use of categorical scoring meant that our measurement technique may have glossed over some of the existing expression variation. This reduced the number of marker-positivity cut points that could be assessed and might also have resulted in some degree of misclassification. Second, the sample size was not large, which suggests that the null associations found here should not be regarded as definitive. However, we did find positive associations in the same sample for a different set of markers using similar models [20], and some survivorship point estimates in this analysis had suggestive magnitudes. Third, no information was available on cancer treatments. Therefore, our survivorship analysis could not take this information into account. Fourth, the only available clinical outcomes were overall and endometrial cancer-specific mortality, which meant that we could not evaluate associations between tumor markers and other outcomes of interest, such as response to therapy.

To conclude, the potential of tumor expression of adiponectin, leptin, and insulin-pathway receptors to define endometrial cancer subtypes might warrant further study in a larger sample, particularly for relationships with clinical outcomes. However, based on the present results, it appears that none of them would be effective subtyping markers.

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Table 1**Participant characteristics (n=360)**

Characteristic	Mean	SD
Age (years)	69.0	7.3
Years from menarche to menopause	38.9	4.1
Years since menopause	17.7	8.5
E-only HT use of ever users (years) ^a	5.1	4.9
E+P HT use of ever users (years) ^a	6.2	5.0
Other HT use of ever users (years) ^a	5.1	8.1
Oral contraceptive use of ever users (years)	3.6	3.7
Pack-years of smoking	11.6	17.7
Parity among parous	3.0	1.4
	N	%
Year of diagnosis		
1976–1985	0	0
1986–1995	34	9
1996–2005	190	53
2006–2013	136	38
Race		
White	356	99
African-American	2	1
Native American	0	0
Asian	2	1
Hispanic ethnicity	0	0
Body mass index (kg/m ²)		
Normal weight (<25)	97	29
Overweight (25≤BMI<30)	109	32
Obese (≥30)	134	39
E-only HT never users	281	78
E+P HT never users	238	66
Other HT never users	224	62

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Characteristic	Mean	SD
Oral contraceptive never users	208	58
Nulliparous	28	8
Smoking status		
Never smoker	170	47
Former smoker	177	49
Current smoker	13	4
Diabetic	48	13
Family history of endometrial cancer	22	6
Tumor stage		
I	313	87
II	18	5
III	5	1
IV	23	6
Tumor histologic type		
Endometrioid	349	97
Non-endometrioid	11	3
Tumor grade		
Well-differentiated	178	51
Moderately differentiated	109	31
Poorly differentiated	65	18

NOTE: Variables are as of time of endometrial cancer diagnosis. Participants are confirmed Nurses' Health Study endometrial carcinoma cases who provided tissue specimens adequate to be incorporated into tissue microarrays. Table 1 reproduced from American Association for Cancer Research *Cancer Epidemiology, Biomarkers & Prevention*, Endometrial Cancer Risk Factors, Hormone Receptors, and Mortality Prediction, Volume 26 (5), May 2017, Table 1: Characteristics of endometrial cancer cases from the NHS (page 730, —In tissue microarrays column) by Evan L. Busch, Marta Crous-Bou, Jennifer Prescott, Maxine M. Chen, Michael J. Downing, Bernard A. Rosner, George L. Mutter, and Immaculata De Vivo. Copyright 2017 American Association for Cancer Research.

Abbreviations: E-only, unopposed estrogen; E+P, estrogen plus progesterone; HT, hormone therapy.

^aOrally ingested pills only.

^bIncludes E-only, E+P, and progesterone-only vaginal creams, as well as progesterone-only orally-ingested pills.

Table 2

Endometrial primary carcinoma marker expression distributions and association with BMI at diagnosis (n=360)

Marker	Expression Scoring ^a	N (%)	Marker Coding in Model	N in Model	Model N (%)	High Expression	BMI	
							OR ^b	95% CI
Adiponectin Receptor 1	0	2 (1)	1/2/3 vs 0	337		335 (99)	-- ^c	--
	1	316 (93)	2/3 vs 0/1			20 (6)	0.89	0.48, 1.67
	2	15 (4)	3 vs 0/1/2			5 (1)	0.90	0.26, 3.09
	3	5 (1)						
Adiponectin Receptor 2	0	1 (0)	1/2/3 vs 0	332		331 (100)	-- ^c	--
	1	154 (46)	2/3 vs 0/1			178 (54)	1.07	0.80, 1.44
	2	161 (48)	3 vs 0/1/2			17 (5)	0.72	0.36, 1.41
	3	17 (5)						
Leptin Receptor	0	30 (9)	1/2 vs 0	333		303 (91)	1.10	0.66, 1.84
	1	266 (80)	2 vs 0/1			38 (11)	0.83	0.52, 1.32
	2	38 (11)						
	0	164 (50)	1/2 vs 0	330		166 (50)	0.92	0.68, 1.25
Insulin Receptor, beta subunit	1	128 (39)	2 vs 0/1			39 (12)	1.06	0.67, 1.67
	2	39 (12)						
	0	2 (1)	1/2 vs 0	337		335 (99)	-- ^c	--
	1	241 (71)	2 vs 0/1			95 (28)	1.04	0.74, 1.46
Insulin-Like Growth Factor-1 Receptor	2	95 (28)						
	0	1 (0)	1/2 vs 0	329		328 (100)	-- ^c	--
	1	140 (42)	2 vs 0/1			188 (57)	0.98	0.72, 1.32
	2	189 (57)						
Insulin-Like Growth Factor-2 Receptor	0	37 (11)	1/2 vs 0	329		292 (89)	1.33	0.83, 2.13
	1	231 (70)	2 vs 0/1			62 (19)	1.04	0.71, 1.51
	2	62 (19)						
	0	1						

^a Categorical 0–3 expression scale interpreted as 0=none, 1=low, 2=moderate, 3=high. Categorical 0–2 expression scale interpreted as 0=none, 1=low, 2=high. Differences between 360 and distribution totals were missing/non-informative measurements.

c Not estimable.

BMI=body mass index, E-only=unopposed estrogen, E+P=estrogen and progesterone, HT=hormone therapy

Table 3

Associations between endometrial primary carcinoma marker expression and time to patient mortality

Marker	Time-to-Mortality Outcome									
	N	Marker Coding in Model ^a	N (%) High Expression	# Deaths	All-Cause			Endometrial Cancer-Specific		
					HR ^b	95% CI	# Deaths	HR ^b	95% CI	
Adiponectin Receptor 1	330	1/2/3 vs 0	328 (99)	54	-- ^c	--	24	-- ^c	--	
		2/3 vs 0/1	20 (6)		1.24	0.49, 3.16		1.25	0.35, 4.39	
		3 vs 0/1/2	5 (2)		0.51	0.07, 3.96		0.51	0.06, 4.14	
Adiponectin Receptor 2	325	1/2/3 vs 0	324 (99)	53	-- ^c	--	24	-- ^c	--	
		2/3 vs 0/1	176 (54)		1.07	0.60, 1.91		1.16	0.46, 2.92	
		3 vs 0/1/2	17 (5)		1.27	0.49, 3.27		1.23	0.34, 4.39	
Leptin Receptor	326	1/2 vs 0	296 (91)	54	1.13	0.40, 3.17	24	2.62	0.34, 20.02	
		2 vs 0/1	37 (11)		1.16	0.57, 2.36		1.57	0.59, 4.13	
		1/2 vs 0	161 (50)	54	0.69	0.40, 1.21	24	1.61	0.63, 4.08	
Insulin Receptor, beta subunit	323	2 vs 0/1	39 (12)		0.41	0.15, 1.16		0.59	0.17, 2.07	
		1/2 vs 0	328 (99)	55	-- ^c	--	24	-- ^c	--	
		2 vs 0/1	95 (29)		0.91	0.52, 1.61		0.56	0.23, 1.38	
Insulin Receptor Substrate 1	330	1/2 vs 0	321 (99)	54	-- ^c	--	24	-- ^c	--	
		2 vs 0/1	185 (57)		1.28	0.72, 2.28		1.82	0.72, 4.63	
		1/2 vs 0	285 (89)	54	0.51	0.24, 1.10	24	0.54	0.16, 1.89	
Insulin-Like Growth Factor-1 Receptor	322	2 vs 0/1	60 (19)		0.84	0.43, 1.65		0.62	0.22, 1.77	
		1/2 vs 0								
Insulin-Like Growth Factor-2 Receptor	322	1/2 vs 0								
		2 vs 0/1								

^a Categorical 0–3 expression scale interpreted as 0=none, 1=low, 2=moderate, 3=high. Categorical 0–2 expression scale interpreted as 0=none, 1=low, 2=high.^b Each HR is the marker estimate from a model with a dependent variable of time to mortality (all-cause or endometrial cancer-specific) censored 10 years after cancer diagnosis and with independent variables of dichotomous marker status at the indicated cut point (high vs low expression), tumor stage, tumor grade, and histologic type.^c Not estimable.