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RESEARCH

Prognostic Significance of Carbonic Anhydrase IX (CA-IX), Endoglin (CD105) and 8-hydroxy-2′-deoxyguanosine (8-OHdG) in Breast Cancer Patients

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Abstract The aim of this study was to examine the prognostic significance of carbonic anhydrase IX (CA-IX), an endogenous marker for tumor hypoxia; endoglin (CD105), a proliferation-associated and hypoxia-inducible glycoprotein and 8-hydroxy-2′-deoxyguanosine (8-OHdG), an oxidative DNA lesion, in breast cancer patients. Immunohistochemical expressions of CA-IX, CD105 and 8-OHdG, analyzed on paraffin-embedded tumor tissues from forty female breast cancer patients, were used to assess their prognostic implication on overall survival (OS) and relapse-free survival (RFS). Patients with high CA-IX expression (above cut-off value) had a higher occurrence of relapse (P=0.002). High CA-IX expression was significantly associated with shorter RFS (P<0.001, hazard ratio (HR) 0.21) and shorter OS (P<0.001, HR 0.19). Lymph node negative patients with high CA-IX expression had worse RFS (P=0.031, HR 0.14) and OS (P=0.005, HR 0.05). Patients with grade I&II tumors and high CA-IX expression showed shorter RFS (P=0.028, HR 0.28) and OS (P=0.008, HR 0.20). Worse OS (P=0.046, HR 0.28) was found in subgroup of patients with grade II tumors and high CA-IX expression. Among all three markers, only high CA-IX expression was strong independent prognostic indicator for shorter OS (HR 4.14, 95% CI 1.28–13.35, P=0.018) and shorter RFS (HR 3.99, 95% CI 1.38–11.59, P=0.011). Elevated expression of CA-IX was an independent prognostic factor for decreased RFS and OS and a significant marker for tumor aggressiveness. CD105 had week prognostic value; whereas, 8-OHdG, in this study, did not provide sufficient evidence as a prognostic indicator in breast cancer patients.

Keywords 8-OHdG · Breast cancer · CA-IX · CD105 · Survival analysis

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Abbreviations

8-OHdG	8-hydroxy-2′-deoxyguanosine
CA-IX	carbonic anhydrase IX
CD105	endoglin
MVD	microvascular density
OS	overall survival
RFS	relapse-free survival

Introduction

Breast carcinoma is the most frequent malignancy in women and the second leading cause of cancer-related deaths in women in the Western world [1]. Although,

improvements in early detection and treatment strategy have resulted in decreased breast cancer mortality, reliable prognostic and predictive markers of breast cancer are still widely needed. Beside the established prognostic value of histological factors such as axillary lymph node status, tumor size, tumor grade, and hormonal receptor status [2–5], additional indicators which can refine the risk of relapse, predict individual patient prognosis and allow for treatment adjustment are still to be identified.

Oxygenation, an indicator of tumor physiologic status, has potentially important prognostic value. Tumor necrosis has also been shown to be a prognostic indicator [6]; however, it is nonspecific and may not always be associated with severe hypoxia [7–9]. Hypoxia is one of the hallmarks of cancer [10], whose presence has been established in different types of solid tumors, including breast cancer [11]. Tumor hypoxia is caused by lack of functional blood vessels, as well as increased oxygen consumption in proliferating tumor tissue. Malignant cells can undergo genetic and adaptive changes to promote their survival under hypoxic conditions. These changes enhance tumor resistance to chemotherapy and radiotherapy, resulting in poor treatment outcome [12, 13]. Hypoxia-inducible genes, such as HIF-1α, along with their downstream gene products (i.e. VEGF), have been examined for their ability to serve as prognostic indicators [14–16].

One such downstream target of HIF-1α is carbonic anhydrase IX (CA-IX), belonging to the family of zinc metalloenzymes [17]. CA-IX is involved in the hydration of carbon dioxide to carbonic acid, thereby, affecting cell metabolism and pH regulation. Although present in normal tissue, several clinical studies have revealed that elevated CA-IX expression has prognostic value in breast cancer [18–20], cervical cancer [21, 22], non-small cell lung cancer [23–25], nasopharyngeal cancer [26], and renal cell carcinoma [27, 28]. CA-IX expression has been found to be strongly associated with regions of necrosis, an indicator of intratumoral hypoxia [29]. This data suggests that CA-IX may serve as an endogenous marker for tumor hypoxia [30].

Endoglin (CD105), part of the TGF-β receptor complex, is a proliferation-associated and hypoxia-inducible protein abundantly expressed in endothelial cells of tumor blood vessels, but not in most normal tissue [31, 32]. CD105, as a measure of intratumoral microvessel density, could also be used in terms of prognostic significance [33]. Two studies [33, 34] have shown that CD105 expression is associated with poor survival.

8-Hydroxy-2′-deoxyguanosine (8-OHdG) is an oxidized DNA nucleoside that belongs to reactive oxygen species and can attack lipid, protein and nucleic acid in living cells [35, 36]. Nagashima et al. [37] reported no significantly different 8-OHdG levels in breast cancer patients from those of non-malignant samples. But other studies used different measurement techniques and revealed higher levels of 8-OHdG in patients with breast cancer [38–40]. So far, no significant associations of 8-OHdG with survival or disease-free interval have been shown [41].

The purpose of this study was to use immunohistochemical analysis of CA-IX, CD105 and 8-OHdG to evaluate these hypoxia, neoangiogenesis and oxidative stress markers as potential prognostic factors for overall survival and relapse-free survival in breast cancer patients. Special attention was put on lymph node negative patients and those with grade I&II tumors, whose survival prognosis and therapeutic treatments, could be potentially assessed according to the extent of markers expression.

Patients and Methods

Study Population

Forty female breast cancer patients operated at the University Hospital for Tumors, Zagreb in the period from year 1999 to 2005 were included in this study. All patients had invasive ductal carcinoma and other patients' characteristics are listed in Table 1. The median follow-up of surviving patients at the time of analysis was 55.8 months (range: 10.3–83.5 months). Follow-up data were obtained from medical records. Survival times were measured from the date of surgery to the time of death or last follow-up observation. All tumor samples were collected following the ethical principles approved by Institutional Ethical Board, and in concordance with the Declaration of Helsinki. Informed consent was obtained from each patient.

Immunohistochemistry

Immunohistochemistry for CA-IX, CD105, and 8-OHdG was performed on consecutive 5-μm sections of formalin-fixed, paraffin-embedded tissues placed onto positively charged glass slides. Tissue sections were deparaffinized and rehydrated using xylene and ethanol (100-80%). Endogenous peroxidase activity was quenched with 3% hydrogen peroxide for 15 min. For microwave antigen retrieval, slides were heated twice on high power for 5 min each. Tissue sections were rinsed with phosphate-buffered saline, blocked with 10% donkey serum, and incubated with primary antibodies to 8-OHdG (1:2000, JaICA, Shizuoka, Japan), a marker of oxidative stress, CA-IX (1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA), a marker for hypoxia, and CD105 (1:20, Dako, Glostrup, Denmark) to assess angiogenesis, overnight at 4°C. Simultaneous incubation of slides in which primary antibody was omitted served as negative control. Slides were rinsed three times with phosphate-buffered saline (PBS) and incubated with the appropriate biotinylated

Table 1 Patients' characteristics

Characteristics	No. (%)
Age (years)	
Median	61.5
Range	33–84
Histological grade	
I	5 (12.5)
II	21 (52.5)
III	14 (35)
Tumor size	
<= 5 cm	35 (87.5)
> 5 cm	5 (12.5)
Lymph node status	
Negative	18 (45)
Positive	22 (55)
ER status	
ER−	6 (15)
ER+	34 (85)
PR status	
PR−	11 (27.5)
PR+	29 (72.5)
Her2 status	
Her2−	14 (35)
Her2+	6 (15)
Not determined	20 (50)
Survival	
Alive	16 (40.0)
Dead	24 (60.0)
Recurrence	
Non-recurrence	15 (37.5)
Recurrence	25 (62.5)

secondary antibody (1:1000, v/v) for 30 min at room temperature. Again, slides were washed three times with PBS, followed by incubation with ABC-Elite kit (Vector Lab, Inc., Burlingame, CA). Slides were again washed in PBS and color was developed using five-minute incubation with 3,3′-diaminobenzidine (DAB) solution (Laboratory Vision, Fremont, CA). Slides were counterstained with Harris hematoxylin (Fisher Scientific, Pittsburgh, PA). To assess variability of immunostaining, we included a positive sample in each batch of 20 tumor samples.

Image analysis was performed as previously described [24]. For image analysis, two independent investigators, blinded to clinical outcome, evaluated all slides and differences between the two observers were resolved by consensus. The degree of positive CA-IX staining was assessed at high magnification (200x) using a semiquantitative scale of 1 to 3 and the percentage of tumor cells staining positive for CA-IX were measured at low magnification (40x). The mean value of the examined fields was the final value. The CA-IX score was derived from the product of the percentage of tumor cells staining for CA-IX and the average intensity of that staining [24, 25].

Angiogenesis was accessed using CD105 positivity, a marker for microvascular density (MVD). Tumor sections were evaluated for MVD at 40x magnification. Microvessel density was expressed as the number of vessels per field. The mean value from three fields was recorded as the MVD for each tumor.

Oxidative stress was quantified by determining the percent area of positive 8-OHdG expression per tumor section.

Assessment of Tumor Necrosis

Tumor necrosis was assessed on both CA-IX-stained sections and hematoxylin and eosin (H&E)-stained sections. The extent of necrosis was assessed at low magnification (40x), and cases were divided into two groups according to the extent of necrosis: minimal necrosis group, with necrosis in less than 5% of the optical fields; and moderate to severe necrosis group, with necrosis in more than 5% of the optical fields.

Statistical Analysis

Fisher's exact test was used to assess the association and distribution of categorical variables. Non-parametric Spearman rank correlation coefficient (ρ) was used to assess the correlation between expressions of markers. Recurrence-free survival and overall survival curves were calculated with the Kaplan–Meier Method and were compared by the Log-Rank test. The Cox proportional hazards regression model with forward stepwise variable selection was used for multivariate analysis. Two-tailed P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using MedCalc for Windows, version 7.2.0.2 (MedCalc Software, Mariakerke, Belgium).

Results

Levels of CA-IX, CD105 and 8-OHdG Expressions and Its Relation to Clinicopathologic Variables

Expression of CA-IX, CD105 and 8-OHdG were primarily focal and membranous with a varying degree of cytoplasmic reactivity (Fig. 1).

ROC (Receiver Operating Characteristic) curve analysis was used to define cut-off point for categorizing expression data into two groups, "low" and "high". Cut-off values for CA-IX, CD105 and 8-OHdG expressions were 52.5, 11 and 20, respectively. We analyzed correlation of these three markers with pathologic variables in breast cancer patients (Table 2).

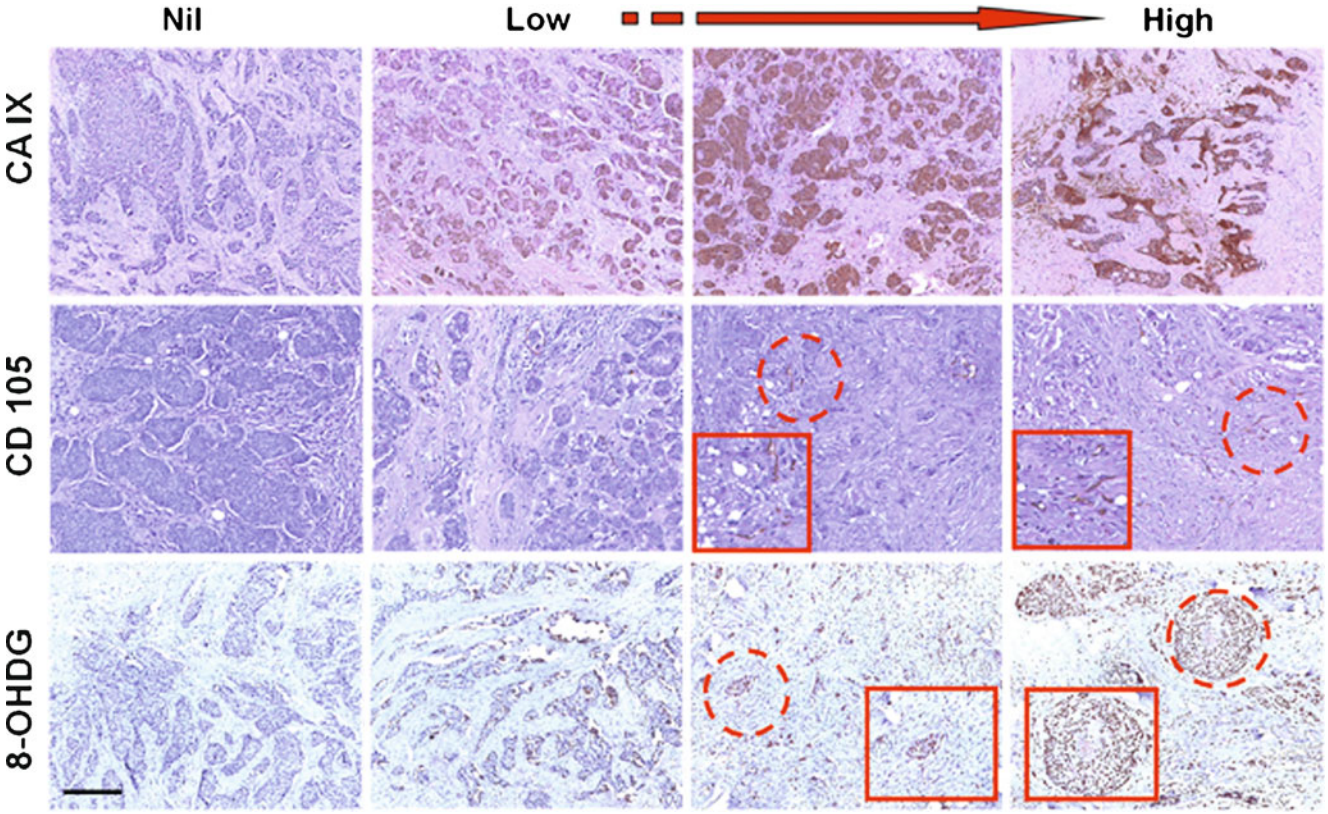


Fig. 1 Representative photomicrographs of CA-IX, CD105 and 8-OHdG immunohistochemical staining. Nil presents negative control, consecutive slides show areas with ascending staining intensity. Squares represent magnified encircled areas. Original magnification 200×, bar represents 100 μm

High expression of CA-IX was associated with moderate to severe necrosis (P=0.004) and positive lymph nodes (P=0.003). High expression of CD105 was also associated with moderate to severe necrosis (P=0.046) and positive lymph nodes (P=0.005). High expression of 8-OHdG was significantly associated only with moderate to severe necrosis (P=0.006). There was no significant association between expression of any marker and tumor size,

Table 2 Levels of markers' expression in relation to clinicopathologic variables

Variables	CA-IX expression			CD105 expression			8-OHdG expression		
	LOW	HIGH	P-value[a]	LOW	HIGH	P-value[a]	LOW	HIGH	P-value[a]
	<=52.5	>52.5		<=11	>11		<=20	>20	
Necrosis									
Minimal	7	1	0.004	7	1	0.046	7	1	0.006
Moderate to severe	9	23		14	18		10	22	
Lymph node status									
Negative	12	6	0.003	14	4	0.005	8	10	1.000
Positive	4	18		7	15		9	13	
Tumor Size									
<= 5 cm	15	20	0.631	19	16	0.654	15	20	1.000
> 5 cm	1	4		2	3		2	3	
Histological grade									
I&II	13	13	0.101	15	11	0.51	12	14	0.739
III	3	11		6	8		5	9	

[a] Fisher's exact test was used

histological grade, steroid receptor status or HER2 status (data not shown).

Association of CA-IX, CD105 and 8-OHdG Expressions

To determine the association between expression of CA-IX , CD105 and 8-OHdG, CA-IX score values were compared with CD105 and 8-OHdG values with Spearman Rank Test. CA-IX score showed statistically significant, but weak, correlation with 8-OHdG (ρ=0.465, P=0.004, data not shown). Association of high CA-IX expression group with high 8-OHdG group showed marginal statistical significance (P=0.053, data not shown). Expression of CD105 showed association with neither CA-IX nor 8-OHdG.

Relationship of CA-IX, CD105 and 8-OHdG Expressions with Postoperative Recurrence and Survival

Among the 40 patients, 25 (62.5%) had local and/or distant tumor recurrence. Relapse was significantly higher in patients with moderate to severe necrosis (P=0.036), grade III tumors (P=0.040) and positive lymph nodes (P=0.001) (Table 3). Patients with high CA-IX expression had a higher occurrence of relapse (P=0.002) (Table 3). The fraction of patients with high CD105 expression trended toward having tumor recurrence; however, it was of marginal statistical significance (P=0.055) (Table 3). Tumor size and level of 8-OHdG expression were not associated with recurrence (Table 3).

Table 3 Relationship of markers' expression and other clinicopathological variables to disease recurrence

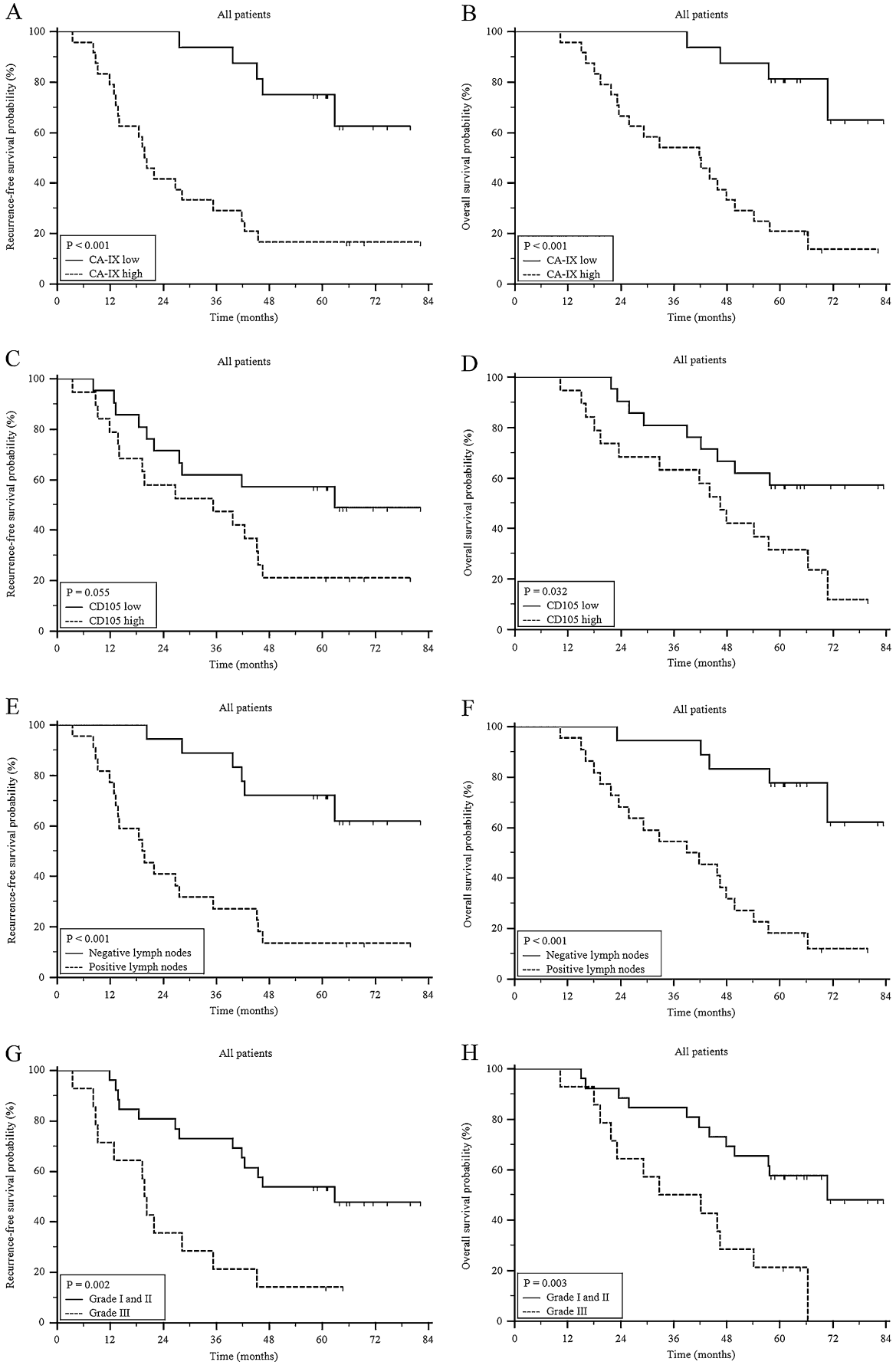
Variables	Non-recurrence	Recurrence	P-value[a]
Necrosis			
Minimal	6	2	0.036
Moderate to severe	9	23	
Lymph node status			
Negative	12	6	0.001
Positive	3	19	
Tumor size			
<= 5 cm	15	20	0.137
>5 cm	0	5	
Histological grade			
I&II	13	13	0.04
III	2	12	
CA-IX expression			
LOW (<=52.5)	11	5	0.002
HIGH (>52.5)	4	20	
CD105 expression			
LOW (<=11)	11	10	0.055
HIGH (>11)	4	15	
8-OHdG expression			
LOW (<=20)	8	9	0.336
HIGH (>20)	7	16	

[a] Fisher's exact test was used

Twenty-four (60%) patients died during follow-up period. The cause of death was primary tumor recurrence. To examine the importance of markers to recurrence-free survival (RFS) and overall survival (OS), univariate analysis was performed. Patients with high CA-IX expression showed shorter RFS and OS (P<0.001 for both, hazard ratio (HR) 0.21 and 0.19, respectively) (Fig. 2a and b). High CD105 expression was associated with shorter RFS with marginal statistical significance (P=0.055, HR 0.46) and with shorter OS (P=0.032, HR 0.41) (Fig. 2c and d). Level of 8-OHdG expression showed no impact on RFS or OS (data not shown). Among the clinicopathological variables, positive lymph nodes were connected with both shorter RFS and OS (P<0.001 for both, HR 0.19 for both) (Fig. 2e and f). Moderate to severe necrosis was also connected with both shorter RFS (P=0.027, HR 0.37) and OS (P=0.006, HR 0.28) (data not shown). Patients with tumors larger than 5 cm showed both shorter RFS (P=0.015, HR 0.15) and OS (P=0.011, HR 0.13) (data not shown). Grade III tumors showed both shorter RFS (P=0.002, HR 0.23) and OS (P=0.003, HR 0.23) compared to grade I&II tumors (Fig. 2g and h).

Statistically significant difference in survival was seen among lymph node-negative patients with elevated levels of CA-IX expression. In this study, patients without positive lymph nodes, but with high CA-IX expression had both shorter RFS (P=0.031, HR 0.14) and OS (P=0.005, HR 0.05) (Fig. 3a and b). Similarly, CA-IX expression correlated with worse prognosis in patients with grade I&II tumors. Those patients with high CA-IX expression showed shorter RFS (P=0.028, HR 0.28) and OS (P=0.008, HR 0.20) (Fig. 3c and d). In the same subgroup of patients, statistically significant difference in OS (P=0.017, HR 0.23) could be seen based on the level of CD105 expression (Fig. 3f). RFS in those who had high CD105 expression trended toward being shorter; however, it was of marginal statistical significance (P=0.055, HR 0.33) (Fig. 3e). Within the subgroup of patients with only grade II tumors, significant difference in OS could only be seen based on the extent of CA-IX expression (P=0.046, HR 0.28) (Fig. 3g). The level of 8-OHdG expression did not correlate with prognosis in any subgroup of patients (data not shown).

To examine the independent prognostic significance of clinicopathological variables and markers expressions, multivariate analysis was performed. Only variables that had significant (P<0.05) univariate impact were used in multivariate analysis (Table 4). Marker expression, extent of necrosis, tumor size, lymph node status and histological grade were used as binary categories. Only CA-IX score


A
All patients
Recurrence-free survival probability (%)
P < 0.001
CA-IX low
CA-IX high
Time (months)
B
All patients
Overall survival probability (%)
P < 0.001
CA-IX low
CA-IX high
Time (months)
C
All patients
Recurrence-free survival probability (%)
P = 0.055
CD105 low
CD105 high
Time (months)
D
All patients
Overall survival probability (%)
P = 0.032
CD105 low
CD105 high
Time (months)
E
All patients
Recurrence-free survival probability (%)
P < 0.001
Negative lymph nodes
Positive lymph nodes
Time (months)
F
All patients
Overall survival probability (%)
P < 0.001
Negative lymph nodes
Positive lymph nodes
Time (months)
G
All patients
Recurrence-free survival probability (%)
P = 0.002
Grade I and II
Grade III
Time (months)
H
All patients
Overall survival probability (%)
P = 0.003
Grade I and II
Grade III
Time (months)

◀ **Fig. 2** Kaplan-Meier survival curves illustrating recurrence-free survival (RFS) and overall survival (OS) for 40 female patients with breast cancer. (**a** and **b**) RFS and OS for all patients according the CA-IX expression; (**c** and **d**) RFS and OS for all patients according the CD105 expression; (**e** and **f**) RFS and OS for all patients according the lymph node status; (**g** and **h**) RFS and OS for all patients according the histological grade. Tick marks indicate censored data

(HR 4.14, 95% CI 1.28–13.35, P=0.018) and lymph node status (HR 3.63, 95% CI 1.24–10.60, P=0.019) were independent prognostic indicators for OS. CA-IX score (HR 3.99, 95% CI 1.38–11.59, P=0.011), lymph node status (HR 3.25, 95% CI 1.22–8.61, P=0.019) and histopathological grade (HR 3.16, 95% CI 1.32–7.57, P=0.010) were significant independent predictors for RFS.

Discussion

It is of great clinical importance to determine reliable and verifiable diagnostic, prognostic, and predictive factors for the purpose of providing individualized treatment strategies and improving treatment outcome for patients with breast cancer. Some of these prognostic factors have already been defined; however, new research has placed greater emphasis on association between various cellular biomarkers and disease recurrence and overall survival. Although most of them still remain unknown there has been a lot of progress in their discovery, such as CA-IX that represents the most promising endogenous hypoxia marker.

This is the first study that evaluates together CA-IX, CD105 and 8-OHdG as potential prognostic factors for overall survival and relapse-free survival in breast cancer patients. Each has been evaluated previously, alone, or in combination with other markers, and only CA-IX and CD105 have previously been shown to correlate to worse prognosis [18–28, 33, 34]. The most uncertain data regarding predictive potential, has been obtained with 8-OHdG. This oxidative stress marker has only been shown to be more expressed in cancer tissue as opposed to normal tissue [36].

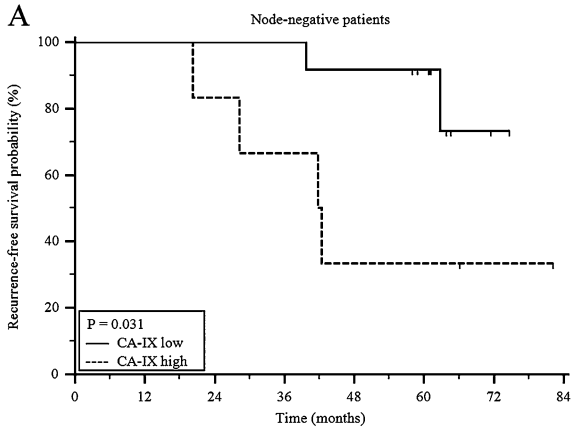
For defining cut-off point for dichotomizing continuous variables into “low” and “high” categories, ROC curve analysis proved to be a better method, rather than using median values of markers expression. The best statistical significance we obtained with 52.5, 11 and 20 as cut-off values to dichotomize CA-IX, CD105 and 8-OHdG expressions, respectively.

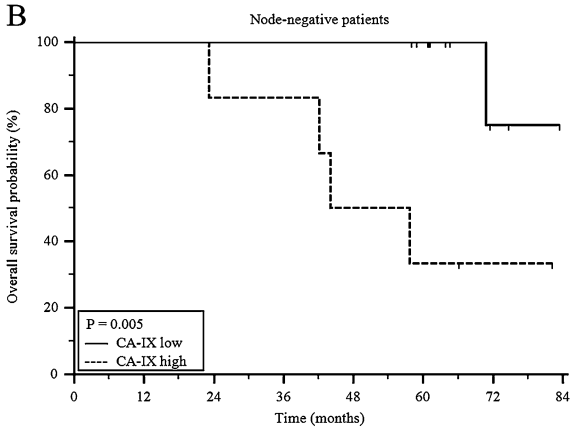
Although, these markers are expressed in normal tissue, their expression is significantly higher in tumor tissue as a consequence of tumor growth and lack of oxygen supply, leading to oxidative stress and hypoxia. In this study, all three markers had higher expression in tumor areas with moderate to severe necrosis, suggesting that necrosis is associated with hypoxia [17], angiogenesis [6] and oxidative stress [42]. Liao et al. [43] investigated the relationship between CA-IX expression and clinical characteristics. The authors found no association between CA-IX expression and lymph node positive status; however, in this study, positive lymph nodes showed higher levels of CA-IX and CD105.

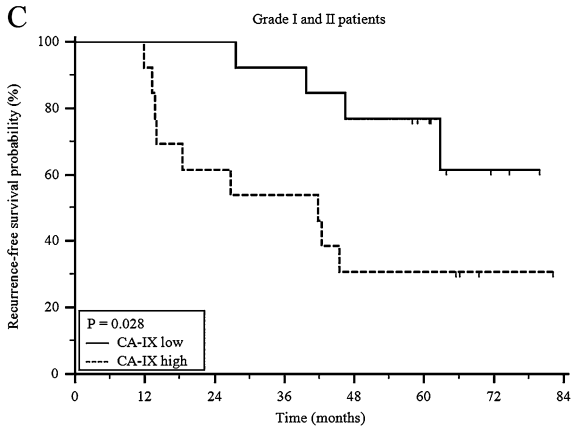
Weak, but statistically significant correlation between 8-OHdG and CA-IX lends support to the hypothesis that reactive oxygen species participate in stabilization of hypoxia-inducible factor-1alpha (HIF-1α). HIF regulates more than 40 genes involved in metabolic adaptation to hypoxia [44, 45]. It would be interesting to see whether there is a stronger correlation between HIF-1α and 8-OHdG in breast cancer tissue. This study showed no association between CD105 and CA-IX expression, which supports previous research [43]. Lack of association suggests that these markers develop independently during the process of tumor hypoxia and have no impact on each other.

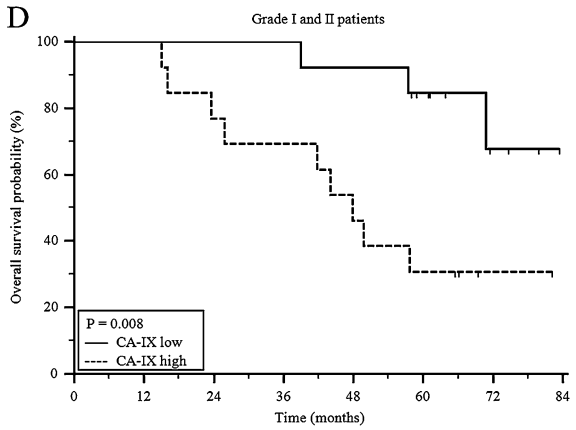
The most important prognostic factors in breast carcinoma still remain the traditional histopathological features of tumor size, lymph node stage and histological grade [46], all of which are incorporated into the Nottingham Prognostic Index (NPI). It is well known that patients with grade I or II tumors have better survival prognosis than those with grade III [47]. However, patients with well-differentiated tumors may often have worse prognosis. We have shown that immunohistochemical staining for CA-IX and CD105 markers could be used to differentiate relapse-free survival in patients with grade I and II tumors. The CA-IX expression could be used to differentiate subgroup of patients with grade II tumors. Prognostic assessment for patients with grade II tumors is often difficult to ascertain as it is unclear whether individual tumors categorized as grade II more closely resemble grade I, which are usually associated with good outcome, or grade III tumors, which are associated with poor outcome [47].

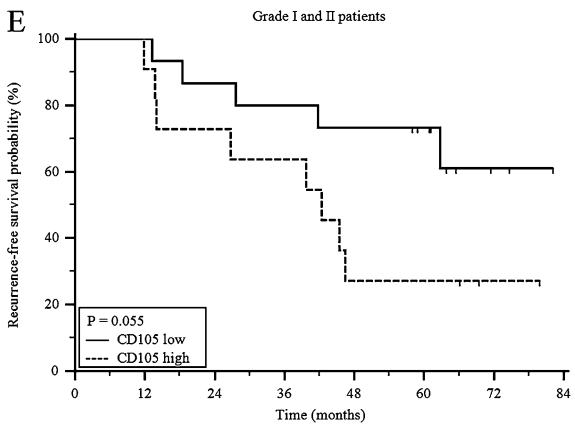
All node-positive breast cancer patients should receive adjuvant systemic therapy because the 10-year recurrence rate in this group reaches 70% [48]. However, for node-negative patients, recommendations for systemic therapy are not as straightforward. The long-term prognosis for clinically node-negative women with very small tumors (<1 cm) is excellent, with a 10-year disease-free survival rate of 88% [49] and 75% of patients showing no evidence of disease at 30 years [50]. Routine adjuvant therapy in this group would be difficult to justify without better prognostic indicators for risk of recurrence. In this study, we have shown that the extent of CA-IX expression is a strong prognostic factor among node-negative breast cancer patients and could be used as a selection tool for patients who require additional treatment after primary therapy. We couldn’t confirm the findings from Dales et al. [34] that CD105 is a marker of high metastatic risk and poor

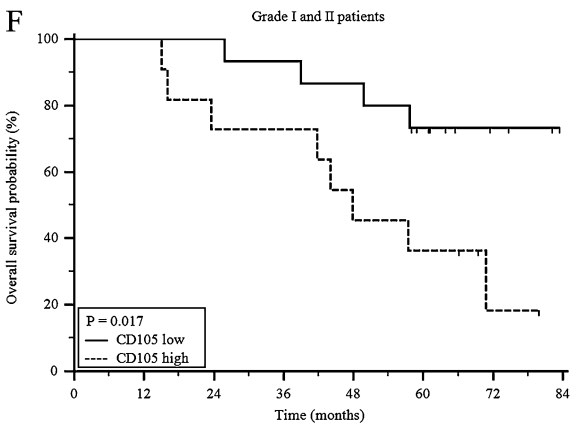

A
Node-negative patients
Recurrence-free survival probability (%)
Time (months)
P = 0.031
CA-IX low
CA-IX high

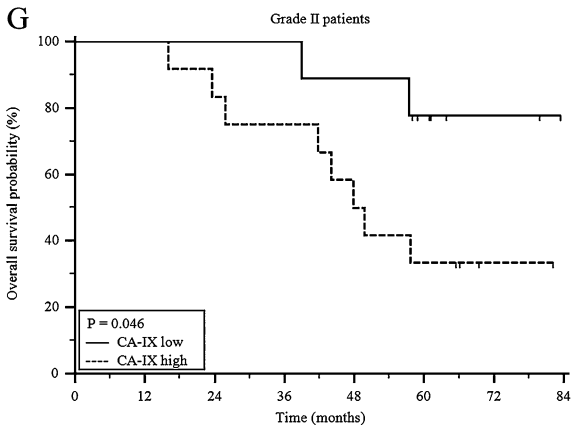

B
Node-negative patients
Overall survival probability (%)
Time (months)
P = 0.005
CA-IX low
CA-IX high


C
Grade I and II patients
Recurrence-free survival probability (%)
Time (months)
P = 0.028
CA-IX low
CA-IX high


D
Grade I and II patients
Overall survival probability (%)
Time (months)
P = 0.008
CA-IX low
CA-IX high


E
Grade I and II patients
Recurrence-free survival probability (%)
Time (months)
P = 0.055
CD105 low
CD105 high


F
Grade I and II patients
Overall survival probability (%)
Time (months)
P = 0.017
CD105 low
CD105 high


G
Grade II patients
Overall survival probability (%)
Time (months)
P = 0.046
CA-IX low
CA-IX high

◀ **Fig. 3** Kaplan-Meier survival curves illustrating recurrence-free survival (RFS) and overall survival (OS) for different subgroups of 40 female patients with breast cancer. (**a** and **b**) RFS and OS for node-negative patients according the CA-IX expression (n=18); (**c** and **d**) RFS and OS for grade I and II patients according the CA-IX expression (n=26); (**e** and **f**) RFS and OS for grade I and II patients according the CD105 expression (n=26); (**g**) OS for grade II patients according the CA-IX expression (n=21). Tick marks indicate censored data

outcome in node-negative patients. However, Dales et al. [34] used 15 microvessels as a cut-off, a much larger series of tumor sections (n=925) and a median follow-up period of 11.3 years.

Among these three markers, we have shown that CA-IX expression still remains the only significant independent prognostic factor for both RFS and OS. A recent study on early-stage cervical cancer has also confirmed the independent prognostic relevance of CA-IX for OS and PFS (progression free survival) [43]. CD105 expression was predictive of RFS and OS values in breast cancer patients; however, CD105 was determined to be of less relevance than CA-IX because the results were of marginal statistical significance. Nevertheless, previous studies have shown that CD105 is an independent prognostic indicator for breast cancer patient prognosis [33, 34].

In recent years, 8-OHdG has been used widely not only as a biomarker for the measurement of endogenous oxidative DNA damage, but also as a risk factor for many diseases including cancer [36, 39, 50]. A pilot study of urinary 8-OHdG suggested that 8-OHdG may be a good prognostic indicator in lymphoma patients [50]. However, in this study no predictive potential was observed for 8-OHdG. These findings confirmed the previous results obtained from Karihtala et al. [41] in which the authors concluded that 8-OHdG cannot be used as a marker for survival prognosis in breast cancer patients.

In conclusion, elevated expression of CA-IX was an independent prognostic marker for decreased relapse-free survival, overall survival and a significant marker for tumor aggressiveness in breast cancer patients. Among lymph node negative patients and those with grade II tumors it was possible to diferentiate patients with better and worse prognosis, based on CA-IX expression. If confirmed in subsequent studies with a larger patient population, these finding could potentially be used as a selection criterion for therapy and follow-up protocols adjustments. CD105 expression may also serve as a prognostic factor, but it's usefulness as a selection criterion should be verified after further investigation and confirmation in larger cohort of patients after a longer follow-up period. 8-OHdG was not a reliable marker for predicting risk of recurrence and survival outcome in breast cancer patients.

Table 4 Cox proportional hazards regression model analysis of recurrence-free survival and overall survival

Covariate	Categories	Recurrence-free survival				Overall survival			
		Univariate		Multivariate		Univariate		Multivariate	
		HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P	HR (95%CI)	P
CA-IX score	low vs. high	5.68 (2.11–15.31)	<0.001	3.99 (1.38–11.59)	0.011	6.74 (2.27–20.03)	<0.001	4.14 (1.28–13.35)	0.018
CD105	low vs. high	2.16 (0.97–4.80)	0.061	–(–)	–	2.41 (1.06–5.50)	0.038	–(–)	–
8-OHdG	low vs. high	1.66 (0.73–3.75)	0.226	–(–)	–	1.74 (0.75–4.08)	0.202	–(–)	–
Necrosis	minimal vs. moderate to severe	4.48 (1.05–19.06)	0.043	–(–)	–	10.24 (1.37–76.65)	0.024	–(–)	–
Lymph Node Status	negative vs. positive	5.35 (2.12–13.50)	<0.001	3.25 (1.22–8.61)	0.019	6.08 (2.23–16.53)	<0.001	3.63 (1.24–10.60)	0.019
Tumor Size	<=5 cm vs. >5 cm	3.27 (1.20–8.92)	0.022	–(–)	–	3.58 (1.26–10.18)	0.018	–(–)	–
Histological grade	I&II vs. III	3.28 (1.47–7.29)	0.004	3.16 (1.32–7.57)	0.010	3.32 (1.45–7.61)	0.005	–(–)	–

HR hazard ratio, *CI* confidence interval

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References

1. Jemal A, Siegel R, Ward E et al (2008) Cancer statistics. CA Cancer J Clin 58:71–96
2. Fisher B, Bauer M, Wickerham DL et al (1983) Relation of number of positive axillary nodes to the prognosis of patients with primary breast cancer. An NSABP update. Cancer 52:1551–1557
3. Davis BW, Gelber RD, Goldhirsch A (1986) Prognostic significance of tumor grade in clinical trials of adjuvant therapy for breast cancer with axillary lymph node metastasis. Cancer 58:2662–2670
4. Koscielny S, Tubiana M, Lê MG et al (1984) Breast cancer: relationship between the size of the primary tumour and the probability of metastatic dissemination. Br J Cancer 49:709–715
5. Clark GM, McGuire WL (1988) Steroid receptors and other prognostic factors in primary breast cancer. Semin Oncol 15:20–25
6. Leek RD, Landers RJ, Harris AL et al (1999) Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. Br J Cancer 79:991–995
7. Mueller-Klieser W, Freyer JP, Sutherland RM (1983) Evidence for a major role of glucose in controlling development of necrosis in EMT6/Ro multicell tumor spheroids. Adv Exp Med Biol 159:487–495
8. Parliament MB, Franko AJ, Allalunis-Turner MJ et al (1997) Anomalous patterns of nitroimidazole binding adjacent to necrosis in human glioma xenografts: possible role of decreased oxygen consumption. Br J Cancer 75:311–318
9. Ramanujan S, Koenig GC, Padera TP et al (2000) Local imbalance of proangiogenic and antiangiogenic factors: a potential mechanism of focal necrosis and dormancy in tumors. Cancer Res 60:1442–1448
10. Ruan K, Song G, Ouyang G (2009) Role of hypoxia in the hallmarks of human cancer. J Cell Biochem 107:1053–1062
11. Vaupel P, Schlenger K, Knoop C et al (1991) Oxygenation of human tumors: evaluation of tissue oxygen distribution in breast cancers by computerized O_2 tension measurements. Cancer Res 51:3316–3322
12. Höckel M, Vaupel P (2001) Tumor hypoxia: definitions and current clinical, biologic, and molecular aspects. J Natl Cancer Inst 93:266–276
13. Harris AL (2002) Hypoxia–a key regulatory factor in tumour growth. Nat Rev Cancer 2:38–47
14. Shweiki D, Itin A, Soffer D et al (1992) Vascular endothelial growth factor induced by hypoxia may mediate hypoxia-initiated angiogenesis. Nature 359:843–845
15. Maxwell PH, Dachs GU, Gleadle JM et al (1997) Hypoxia-inducible factor-1 modulates gene expression in solid tumors and influences both angiogenesis and tumor growth. Proc Natl Acad Sci USA 94:8104–8109
16. Zhong H, De Marzo AM, Laughner E et al (1999) Overexpression of hypoxia-inducible factor 1alpha in common human cancers and their metastases. Cancer Res 59:5830–5835
17. Wykoff CC, Beasley NJ, Watson PH et al (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075–7083
18. Chia SK, Wykoff CC, Watson PH et al (2001) Prognostic significance of a novel hypoxia-regulated marker, carbonic anhydrase IX, in invasive breast carcinoma. J Clin Oncol 19:3660–3668
19. Span PN, Bussink J, Manders P et al (2003) Carbonic anhydrase-9 expression levels and prognosis in human breast cancer: association with treatment outcome. Br J Cancer 89:271–276
20. Hussain SA, Ganesan R, Reynolds G et al (2007) Hypoxia-regulated carbonic anhydrase IX expression is associated with poor survival in patients with invasive breast cancer. Br J Cancer 96:104–109
21. Loncaster JA, Harris AL, Davidson SE et al (2001) Carbonic anhydrase (CA IX) expression, a potential new intrinsic marker of hypoxia: correlations with tumor oxygen measurements and prognosis in locally advanced carcinoma of the cervix. Cancer Res 61:6394–6399
22. Olive PL, Aquino-Parsons C, MacPhail SH et al (2001) Carbonic anhydrase 9 as an endogenous marker for hypoxic cells in cervical cancer. Cancer Res 61:8924–8929
23. Giatromanolaki A, Koukourakis MI, Sivridis E et al (2001) Expression of hypoxia-inducible carbonic anhydrase-9 relates to angiogenic pathways and independently to poor outcome in non-small cell lung cancer. Cancer Res 61:7992–7998
24. Kim SJ, Rabbani ZN, Vollmer RT et al (2004) Carbonic anhydrase IX in early-stage non-small cell lung cancer. Clin Cancer Res 10:7925–7933
25. Kim SJ, Rabbani ZN, Dewhirst MW et al (2005) Expression of HIF-1alpha, CA IX, VEGF, and MMP-9 in surgically resected non-small cell lung cancer. Lung Cancer 49:325–335
26. Hui EP, Chan AT, Pezzella F et al (2002) Coexpression of hypoxia-inducible factors 1alpha and 2alpha, carbonic anhydrase IX, and vascular endothelial growth factor in nasopharyngeal carcinoma and relationship to survival. Clin Cancer Res 8:2595–2604
27. Liao SY, Aurelio ON, Jan K et al (1997) Identification of the MN/CA9 protein as a reliable diagnostic biomarker of clear cell carcinoma of the kidney. Cancer Res 57:2827–2831
28. McKiernan JM, Buttyan R, Bander NH et al (1997) Expression of the tumor-associated gene MN: a potential biomarker for human renal cell carcinoma. Cancer Res 57:2362–2365
29. Colpaert CG, Vermeulen PB, Fox SB et al (2003) The presence of a fibrotic focus in invasive breast carcinoma correlates with the expression of carbonic anhydrase IX and is a marker of hypoxia and poor prognosis. Breast Cancer Res Treat 81:137–147
30. Vleugel MM, Greijer AE, Shvarts A et al (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in invasive breast cancer. J Clin Pathol 58:172–177
31. Kumar P, Wang JM, Bernabeu C (1996) CD105 and angiogenesis. J Pathol 178:363–366
32. Fonsatti E, Del Vecchio L, Altomonte M et al (2001) Endoglin: an accessory component of the TGF-beta-binding receptor-complex with diagnostic, prognostic, and bioimmunotherapeutic potential in human malignancies. J Cell Physiol 188:1–7
33. Kumar S, Ghellal A, Li C et al (1999) Breast carcinoma: vascular density determined using CD105 antibody correlates with tumor prognosis. Cancer Res 59:856–861
34. Dales JP, Garcia S, Bonnier P et al (2003) CD105 expression is a marker of high metastatic risk and poor outcome in breast carcinomas. Correlations between immunohistochemical analysis and long-term follow-up in a series of 929 patients. Am J Clin Pathol 119:374–380
35. Marnett LJ (2000) Oxyradicals and DNA damage. Carcinogenesis 21:361–370
36. Wu LL, Chiou CC, Chang PY et al (2004) Urinary 8-OHdG: a marker of oxidative stress to DNA and a risk factor for cancer, atherosclerosis and diabetics. Clin Chim Acta 339:1–9
37. Nagashima M, Tsuda H, Takenoshita S et al (1995) 8-hydroxydeoxyguanosine levels in DNA of human breast cancer are not significantly different from those of non-cancerous breast tissues by the HPLC-ECD method. Cancer Lett 90:157–162

38. Musarrat J, Arezina-Wilson J, Wani AA (1996) Prognostic and aetiological relevance of 8-hydroxyguanosine in human breast carcinogenesis. Eur J Cancer 32A:1209–1214
39. Kuo HW, Chou SY, Hu TW et al (2007) Urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) and genetic polymorphisms in breast cancer patients. Mutat Res 631:62–68
40. Himmetoglu S, Dincer Y, Ersoy YE et al (2009) DNA oxidation and antioxidant status in breast cancer. J Investig Med 57:720–723
41. Karihtala P, Winqvist R, Syväoja JE et al (2006) Increasing oxidative damage and loss of mismatch repair enzymes during breast carcinogenesis. Eur J Cancer 42:2653–2659
42. Wang X, Ryter SW, Dai C et al (2003) Necrotic cell death in response to oxidant stress involves the activation of the apoptogenic caspase-8/bid pathway. J Biol Chem 278:29184–29191
43. Liao SY, Darcy KM, Randall LM et al (2010) Prognostic relevance of carbonic anhydrase-IX in high-risk, early-stage cervical cancer: a Gynecologic Oncology Group study. Gynecol Oncol 116:452–458
44. Chandel NS, McClintock DS, Feliciano CE et al (2000) Reactive oxygen species generated at mitochondrial complex III stabilize hypoxia-inducible factor-1alpha during hypoxia: a mechanism of O_2 sensing. J Biol Chem 275:25130–25138
45. Klimova T, Chandel NS (2008) Mitochondrial complex III regulates hypoxic activation of HIF. Cell Death Differ 15:660–666
46. Fitzgibbons PL, Page DL, Weaver D et al (2000) Prognostic factors in breast cancer. College of American Pathologists Consensus Statement 1999. Arch Pathol Lab Med 124:966–978
47. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19:403–410
48. Elledge RM, McGuire WL (1993) Prognostic factors and therapeutic decisions in axillary node-negative breast cancer. Annu Rev Med 44:201–210
49. Rosen PR, Groshen S, Saigo PE et al (1989) A long-term follow-up study of survival in stage I (T1N0M0) and stage II (T1N1M0) breast carcinoma. J Clin Oncol 7:355–366
50. Honda M, Yamada Y, Tomonaga M et al (2000) Correlation of urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, and clinical features of hematological disorders: a pilot study. Leuk Res 24:461–468