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ABSTRACT

Aims The aim of this study was to determine the role of hypoxia in male breast carcinogenesis by evaluating the expression of the hypoxia-related proteins, hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1), in ductal carcinoma in situ (DCIS) of the male breast in relation to invasive cancer (IC).

Methods Tumour tissue blocks of 18 cases of pure DCIS, 58 DCIS cases adjacent to IC (DCIS-AIC) and the 58 IC cases were stained by immunohistochemistry for HIF-1α, CAIX and Glut-1, and expression frequencies and patterns (diffuse and/or perinecrotic) were noted.

Results HIF-1α overexpression was observed in 61.1% (11/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 36.2% (21/58) of IC cases (not significant (n.s.)). CAIX overexpression was observed in 16.7% (3/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 24.1% (14/58) of IC cases (n.s.). Glut-1 overexpression was observed in 61.1% (11/18) of pure DCIS, in 75.9% (44/58) of DCIS-AIC and in 62.1% (36/58) of IC cases (n.s.). Expression of hypoxia-related proteins was seen around necrosis in a little over one-third of DCIS cases, and often coincided with expression in adjacent IC when present. All these observations indicate that the hypoxia response is already at its maximum in the preinvasive DCIS stage.

Conclusions In conclusion, male DCIS frequently shows activated hypoxia response, comparable to male IC. This indicates that the activated hypoxia response previously seen in male IC is not a late bystander but likely a genuine carcinogenetic event.

INTRODUCTION

Hypoxia is a condition that occurs when there is a mismatch between oxygen supply and oxygen consumption and this condition has been described in several solid tumours, such as head and neck cancer, cervical cancer as well as breast cancer (BC).[1 2] Tumour cells need to adapt to hypoxia to survive, and are capable of doing this through several different signalling pathways.[3] The key regulator of the hypoxia response is hypoxia-inducible factor-1 (HIF-1), a heterodimeric protein that consists of the HIF-1α and HIF-1β subunits, the latter being constitutively expressed. Under normoxic circumstances, HIF-1α is rapidly degraded, but when hypoxia occurs, HIF-1α is stabilised, resulting in overexpression.[4] The overexpression of HIF-1α leads to upregulation of established downstream targets, such as carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1).[3] CAIX is a member of the family of zinc metalloenzymes and its function is to regulate intracellular and extracellular pH.[5] Glut-1 is a membrane-bound protein involved in glucose transport.[6] In female BC, expression of hypoxia-related markers have been described in IC and expression has been correlated with a decreased overall survival, high risk of metastases and a higher histologic grade in IC.[7–9] This indicates that triggering the hypoxia response contributes to the formation of a more aggressive form of IC.

Male BC is a rare disease, accounting for approximately 1% of all breast carcinomas. In the USA, approximately 2670 men are estimated to develop BC in 2019.[10] Of these men, approximately 5% (range 1%–17%) will be diagnosed with pure ductal carcinoma in situ (DCIS), the final precursor stage before invasion takes place.[11] Because male IC has a low prevalence and male DCIS even more so, studies are much less abundant than studies concerning female BC. Although evidence has shown that male BC and female BC differ on several levels, therapy strategies are still largely extrapolated from female BC clinical trials. Differences between male and female BC patients are, for example, a higher age at diagnosis in male patients, a more advanced disease at the time of diagnosis in men, a different distribution in histologic subtypes and differences at molecular level.[12–16]

In male BC, only one study has been performed in which HIF-1α, CAIX and Glut-1 have been correlated to clinicopathological features and prognosis in a cohort of 134 IC patients. HIF-1α expression was significantly correlated with a high histologic grade, human epidermal growth factor receptor 2 (*HER2)* amplification and poor survival. Glut-1 expression was correlated with a high histologic grade.[17] No data on male DCIS were yet available. Since triggering of the hypoxia response may already take place in the stage of DCIS, like has been described for female IC, we focused, in the present study, on the expression of HIF-1α, CAIX and Glut-1 in male DCIS to determine the role of hypoxia in male breast carcinogenesis, and to assess whether changes in hypoxia-related proteins seen earlier in male IC are carcinogenetic events or late bystanders.[8 18]

Table 1 Overview of the hypoxia-related antibodies and tissue processing methods used

Antibody	Type	Source	Dilution	Antigen retrieval	Antibody incubation time
Glut-1	Polyclonal	DAKO	1:200	EDTA	32 min
CAIX	Polyclonal	Abcam	1:1000	Citrate	60 min
HIF-1α	Monoclonal	BD Biosciences	1:50	EDTA	Overnight

CAIX, carbonic anhydrase IX; Glut-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor-1α.

MATERIALS AND METHODS

Patient material

Patients with DCIS and adjacent IC and patients with pure DCIS were enrolled from a previously selected large male BC cohort (the EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program).[19 20] A (Dutch) subgroup of this initial population was selected based on availability of a representative tumour tissue block for central pathology review, resulting in a total of 18 cases of pure DCIS and 58 cases with DCIS adjacent to IC (DCIS-AIC). Of these 58 cases, the IC component was also analysed. Patient and tumour characteristics including age at diagnosis were recorded. H&E stained slides were reviewed by an experienced pathologist to confirm the diagnosis and to type and grade the IC according to the WHO and modified Bloom and Richardson score.[21] DCIS was graded according to the classification by Holland *et al*.[22] Estrogen receptor alpha (ERα), progesterone receptor (PgR) and HER2 were evaluated using immunohistochemistry and scored according to the Allred score and American Society of Clinical Oncology and the College of American Pathologists (ASCO-CAP) guidelines.[23 24]

The EORTC 10085/TBCRC/BIG/NABCG International Male Breast Cancer Program was conducted as a global effort to retrospectively assess tumour tissue of men diagnosed with BC between 1989 and 2009. Male patients in the Netherlands were identified through the Dutch Cancer Registry. Paraffin-embedded male BC tissue was retrospectively collected by the Dutch Breast Cancer Research Group.

Immunohistochemistry

Immunohistochemistry (table 1) was performed on 4 µm thick slides after silane coating. Slides from one representative tumour tissue block were available. EDTA buffer (pH=9.0 for 20 min at 100°C) was used for antigen retrieval for HIF-1α. Slides were incubated with the primary HIF-1α antibody overnight at 4°C and detected by Novolink Polymer (Novocastra Laboratories, Newcastle upon Tyne, UK). For CAIX, antigen retrieval was carried out in citrate buffer (pH=6.0 for 20 min at 100°C). Incubation with the primary CAIX antibody was 60 min at 20°C followed by detection using Powervision ready to use (poly-HRP-anti Ms/Rb/RtlgG biotin free; immunologic, ImmunoVision Technologies, Brisbane, California, USA). Antigen retrieval for Glut-1 was performed in EDTA buffer and the slides were incubated with the primary Glut-1 antibody for 32 min. Glut-1 staining was performed using a Ventana BenchMark ULTRA automated immunostainer (Ventana Medical Systems, Tucson, Arizona, USA) and staining for HIF-1α and CAIX was carried out manually. All slides were developed with diaminobenzidine. For CAIX and HIF-1α, formalin-fixed and paraffin-embedded clear cell renal cell carcinoma was taken along as positive control and for Glut-1, erythrocytes were used as the internal positive control. Appropriate negative control steps were used throughout the procedures. Stainings were scored by consensus of two experienced observers.

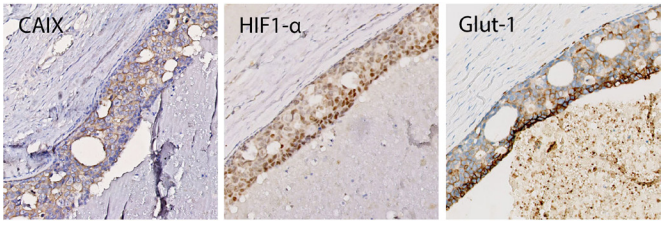

Figure 1 Immunohistochemical stainings for hypoxia-inducible factor-1α (HIF-1α), carbonic anhydrase IX (CAIX) and glucose transporter-1 (Glut-1), all scored positive in this same ductal carcinoma in situ case.

Quantification of immunohistochemical staining

For HIF-1α, mean nuclear staining percentages were used, regarding ≥5% nuclear staining of all tumour cells in the representative slide positive as before.[25] Any clear membranous staining in Glut-1 and CAIX was scored positive. For all markers, two staining patterns were noted as before: a diffuse pattern throughout the tumour cells and a perinecrotic staining

Table 2 Clinicopathological characteristics and expression of ERα, PgR, HER2, HIF-1α, CAIX and Glut-1 in pure ductal carcinoma in situ (DCIS), DCIS adjacent to invasive cancer (DCIS-AIC) and invasive cancer (IC) of the male breast (missing data excluded)

		Pure DCIS	DCIS-AIC	IC	
	N	18	58	58	P value
Age, years	<45	1 (5.6%)	4 (6.9%)	4 (6.9%)	n.s.
	≥45	17 (94.4%)	54 (93.1%)	54 (93.1%)	
Grade*	1	3 (16.7%)	13 (22.4%)	16 (27.6%)	n.s.
	2	14 (77.8%)	35 (60.3%)	27 (46.6%)	
	3	1 (5.6%)	10 (17.2%)	15 (25.9%)	
Mitoses/2 mm^2	<7			35 (60.3%)	
	7–12			14 (24.1%)	
	≥13			9 (15.5%)	
ERα	neg	0 (0%)	0 (0%)	0 (0%)	
	pos	18 (100%)	58 (100%)	58 (100%)	
PgR	neg	0 (0%)	3 (5.2%)	4 (6.9%)	n.s.
	pos	18 (100%)	55 (94.8%)	54 (93.1%)	
HER2†	neg	16 (94.1%)	55 (94.8%)	55 (94.8%)	n.s.
	pos	1 (5.9%)	3 (5.2%)	3 (5.2%)	
HIF-1α	neg	7 (38.9%)	36 (62.1%)	37 (63.8%)	n.s.
	pos	11 (61.1%)	22 (37.9%)	21 (36.2%)	
CAIX	neg	15 (83.3%)	36 (62.1%)	44 (75.9%)	n.s.
	pos	3 (16.7%)	22 (37.9%)	14 (24.1%)	
Glut-1‡	neg	6 (35.3%)	14 (24.1%)	22 (37.9%)	n.s.
	pos	11 (64.7%)	44 (75.9%)	36 (62.1%)	

*Grading for DCIS is according to the classification by Holland *et al*[22] and for IC is according to the modified Bloom and Richardson score.
†One pure DCIS case missing HER2 data.
‡One pure DCIS case missing Glut-1 data.
CAIX, carbonic anhydrase IX; Glut-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor-1α; neg, negative; n.s., not significant; pos, positive.

Table 3 Correlation between HIF-1α staining pattern and overexpression of CAIX or Glut-1 in male DCIS

HIF-1α positive DCIS (n=33)	CAIX and Glut-1 negative	CAIX and/or Glut-1 positive	P value
Diffuse HIF-1α positive	8	12	0.012
Perinecrotic HIF-1α positive	0	13	

CAIX, carbonic anhydrase IX; DCIS, ductal carcinoma in situ; Glut-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor-1α.

pattern, in which membranous staining was restricted to the perinecrotic tumour areas.[8 25] Figure 1 shows an example of the immunohistochemical stainings.

Statistics

Statistical calculations were performed using SPSS for Windows V.24. To evaluate the correlation of clinicopathological features between the three groups (pure DCIS, DCIS-AIC and IC) and for analysis of the expression of the hypoxia-related proteins in the three groups, the χ^2 test, and if appropriate, the Fisher's exact test, was used. Paired analysis, testing the similarity of protein expression between DCIS-AIC and IC, was done using Cohen's kappa test. P values below 0.05 were considered significant .

Table 4 Correlation of HIF-1α overexpression in pure ductal carcinoma in situ (DCIS), DCIS adjacent to invasive cancer (DCIS-AIC) and invasive cancer (IC) of the male breast with clinicopathological features and CAIX and Glut-1 expression

	Pure DCIS			DCIS-AIC			IC		
	HIF-1α			HIF-1α			HIF-1α		
	neg	pos	P value	neg	pos	P value	neg	pos	P value
Age, years									
<45	0	1	n.s.	1	3	n.s.	1	3	n.s.
≥45	7	10		35	19		36	18	
Grade									
1/2	7	10	n.s.	31	17	n.s.	32	11	**0.025**
3	0	1		5	5		5	10	
MAI									
<7							27	8	**0.013**
≥7							10	13	
ERα									
neg	0	0		0	0		0	0	
pos	7	11		36	22		37	21	
PgR									
neg	0	0		2	1	n.s.	2	2	n.s.
pos	7	11		34	21		35	19	
HER2									
neg	6	10	n.s.	35	20	n.s.	36	19	n.s.
pos	0	1		1	2		1	2	
CAIX									
neg	7	8	n.s.	25	11	n.s.	32	12	**0.007**
pos	0	3		11	11		5	9	
Glut-1									
neg	2	4	n.s.	9	5	n.s.	17	5	n.s.
pos	4	7		27	17		20	16	

CAIX, carbonic anhydrase IX; Glut-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor-1α; MAI, mitotic activity index; neg, negative; n.s., not significant; pos, positive.

RESULTS

The mean age was 62 years (range 38–77 years) for patients with pure DCIS and 64 years (range 38–86 years) for patients with DCIS-AIC/IC (p=0.509). The majority of the IC cases were classified as invasive ductal carcinoma (51/58, 88%). The other cases were classified as mixed (ductal/micropapillary, n=2, and ductal/mucinous, n=1), micropapillary (n=1), mucinous (n=1), encapsulated papillary carcinoma (n=1) or tubular carcinoma (n=1).

The clinicopathological characteristics and expression of ERα, PgR and HER2 in pure DCIS, DCIS-AIC and IC are presented in table 2. No significant differences in these clinicopathological features were found between the three groups.

Expression of hypoxia-induced proteins in DCIS and IC

HIF-1α overexpression was observed in 61.1% (11/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 36.2% (21/58) of IC cases (not significant (n.s.)). CAIX overexpression was observed in 16.7% (3/18) of pure DCIS, in 37.9% (22/58) of DCIS-AIC and in 24.1% (14/58) of IC cases (n.s.). Glut-1 overexpression was observed in 61.1% (11/18) of pure DCIS, in 75.9% (44/58) of DCIS-AIC and in 62.1% (36/58) of IC cases (n.s.).

In pure DCIS, a diffuse staining pattern of HIF-1α was seen in 6/11 positive cases (54.5%). Glut-1 showed a diffuse staining pattern in 7/11 positive cases (63.6%) and CAIX in all of the 3 positive cases (100%). All other positive cases showed a perinecrotic staining pattern. In DCIS (pure DCIS and DCIS-AIC as a group), perinecrotic staining of HIF-1α was significantly correlated with overexpression of CAIX or Glut-1, compared with DCIS cases with a diffuse staining pattern of HIF-1α (p=0.012, table 3).

In DCIS-AIC, a diffuse staining pattern was seen in 14/22 (63.6%) of HIF-1α positive cases, in 28/44 (63.6%) of Glut-1 positive cases and in 13/22 (59.0%) of CAIX positive cases. In IC, a diffuse staining pattern was seen in 18/21 (85.7%) of HIF-1α positive cases, in 32/36 (88.9%) of Glut-1 positive cases and in 12/14 (85.7%) of CAIX positive cases.

HIF-1α overexpression was more common in tumours with a high mitotic activity index (>7 mitoses/2 mm^2) and high grade (grade 3) IC cases (table 4, p=0.013 and p=0.025, respectively). No significant differences were found in DCIS.

Table 5 presents that the expression of HIF-1α, CAIX and Glut-1 in paired DCIS and adjacent IC lesions often coincided (p<0.001 for all three proteins).

DISCUSSION

The aim of this study was to determine the role of hypoxia in male breast carcinogenesis by evaluating the expression of the hypoxia-related proteins, HIF-1α, CAIX and Glut-1, in DCIS of the male breast in relation to IC. In previous female BC studies,

Table 5 Correlation of HIF-1α, CAIX and Glut-1 overexpression in paired ductal carcinoma in situ (DCIS) and invasive cancer of the male breast

	Invasive breast cancer								
	HIF-1α			CAIX			Glut-1		
	neg	pos	P value	neg	pos	P value	neg	pos	P value
DCIS									
neg	32	4		33	3		11	3	
pos	5	17	<0.0001	11	11	<0.0001	11	33	<0.0001

CAIX, carbonic anhydrase IX; Glut-1, glucose transporter-1; HIF-1α, hypoxia-inducible factor-1α; neg, negative; pos, positive.

HIF-1α expression was similar between DCIS and IC, but no HIF-1α expression was seen in normal breast tissue or benign lesions, such as ductal hyperplasia and fibroadenomas, indicating that HIF-1α expression is an early payer in female breast carcinogenesis.[8 18] In line with these findings in female BC, we found similar levels of HIF-1α expression in male DCIS and IC, paralleled by expression of CAIX and Glut-1. Expression of HIF-1α, CAIX and Glut-1 often coincided in paired DCIS and IC lesions. This indicates that the activated hypoxia response is already at its maximum in the preinvasive male DCIS stage and is not a late bystander but likely a genuine carcinogenetic event.

There were no significant correlations between grade and HIF-1α, CAIX or Glut-1 expression in DCIS in contrast to IC that we reported before[17]. In female BC, however, it has been described that high-grade DCIS shows significantly higher HIF-1α expression. This was shown in 40 DCIS samples in which HIF-1α expression was seen in 85% of 20 poorly differentiated lesions compared with 55% of 20 well-differentiated lesions.[8] Our lack of significance could be due to our smaller DCIS sample size with only 11/76 (14.5%) grade 3 lesions, compared with 24/44 (54.5%) grade 3 lesions in the female DCIS study, with only 5 high-grade male DCIS samples showing HIF-1α expression. Indeed, expression of hypoxia-related proteins in DCIS was generally lower in male DCIS compared with female DCIS: 43.4% vs 82.1% for HIF-1α, 32.8% vs 56.7% for CAIX and 73.3% vs 25% for Glut-1.[26] Furthermore, quantification of HIF-1α was performed differently; >5% nuclear staining was considered positive by us, compared with >1% nuclear staining in the female BC study.[26] Expression of HIF-1α, CAIX and Glut-1 was not previously examined in male DCIS, to the best of our knowledge, so our present results cannot be compared with the literature.

In our DCIS cases showing HIF-1α expression, the staining was perinecrotic in 39.4%. This staining pattern in DCIS was significantly more correlated to co-expression of CAIX and Glut-1 than a diffuse staining pattern of HIF-1α, like in female BC.[27] This again shows that the hypoxia response is most outspoken around necrosis.

In conclusion, male DCIS frequently shows activated hypoxia response, comparable to male IC. This indicates that the activated hypoxia response previously seen in male IC is not a late bystander but likely a genuine carcinogenetic event.

Take home messages

- Male breast cancer (BC), and especially male ductal carcinoma in situ (DCIS), is a rare disease showing similarities as well as differences with female BC.
- The role of hypoxia in male breast carcinogenesis was evaluated by the expression of the hypoxia-related proteins, hypoxia-inducible factor-1α, carbonic anhydrase IX and glucose transporter-1, in DCIS of the male breast in relation to invasive cancer.
- Results of the present study show an activated hypoxia response in male DCIS which is comparable to male invasive cancer, indicating that this hypoxia response is not a late bystander but likely a genuine carcinogenetic event.

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