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PLOS ONE

HIF-1α Overexpression in Ductal Carcinoma In Situ of the Breast in *BRCA1* and *BRCA2* Mutation Carriers

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Abstract

Recent studies have revealed that *BRCA1* and *BRCA2* germline mutation-related breast cancers show frequent overexpression of hypoxia inducible factor-1α (HIF-1α), the key regulator of the hypoxia response. However, the question remained whether hypoxia is a late stage bystander or a true carcinogenetic event in patients with hereditary predisposition. We therefore studied HIF-1α overexpression in ductal carcinoma *in situ* (DCIS), an established precursor of invasive breast cancer. We used immunohistochemistry to examine the expression of the hypoxia markers HIF-1α, CAIX and Glut-1 in DCIS and available invasive carcinoma lesions of 32 *BRCA1*, 16 *BRCA2* and 77 non-*BRCA* mutation-related cases. HIF-1α expression was detected in 63% of *BRCA1* and 62% of *BRCA2* as compared to 34% of non-*BRCA* mutation-related DCIS cases ($p = 0.005$). CAIX overexpression was present in 56% of *BRCA1* and 44% of *BRCA2* as compared to 6% of non-*BRCA* mutation-related DCIS cases ($p = 0.000$). Glut-1 overexpression was observed in 59% of *BRCA1*, 75% of *BRCA2* and 67% of non-*BRCA* mutation-related DCIS cases ($p = 0.527$). Overall, HIF-1α, CAIX and Glut-1 expression in *BRCA* mutation-related DCIS matched the expression in the accompanying invasive cancers in 60% or more of cases. In non-*BRCA* mutation-related cases the expression of the hypoxia markers in DCIS matched the expression in the invasive part in 46% or more of the cases. Although *BRCA1* and *BRCA2* germline mutation-related invasive breast cancers are different in many ways, the hypoxia-related proteins HIF-1α, CAIX and Glut-1 are expressed in both DCIS and invasive lesions of *BRCA1* and *BRCA2* mutation carriers. This suggests that hypoxia may already play a role in the DCIS stage of *BRCA1* and *BRCA2* germline mutation related breast carcinogenesis, and may also drive cancer progression. Hypoxia-related proteins are therefore putative targets for therapy and molecular imaging for early detection and monitoring therapy response in *BRCA* mutation patients.

Citation: van der Groep P, van Diest PJ, Smolders YHCM, Ausems MGEM, van der Luijt RB, et al. (2013) HIF-1α Overexpression in Ductal Carcinoma In Situ of the Breast in *BRCA1* and *BRCA2* Mutation Carriers. PLoS ONE 8(2): e56055. doi:10.1371/journal.pone.0056055

Editor: Amanda Ewart Toland, Ohio State University Medical Center, United States of America

Received July 23, 2012; **Accepted** January 5, 2013; **Published** February 8, 2013

Funding: This study has been supported by unrestricted educational grants from Aegon Inc and Pink Ribbon The Netherlands. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors received an unrestricted educational grant from Aegon. This does not alter the authors' adherence to all the PLOS ONE policies on sharing data and materials.

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Introduction

Hereditary breast cancer accounts for about 5% of all breast cancers in women and is primarily caused by a germline mutation in one of the *BRCA* genes. Several studies have indicated that the genetic makeup of *BRCA1* and *BRCA2* mutation-related breast cancer is different from that of non-*BRCA* mutation-related breast cancer. These differences comprise gains and losses of specific parts of chromosomes, as well as differences in protein expression [1–7]. Consistent with this, the morphological and immunohistochemical phenotype of *BRCA1* mutation-related breast cancer is also different from that of non-*BRCA* mutation-related breast [8–13]. However, the phenotype of *BRCA2* mutation-related breast cancer is still difficult to distinguish from non-*BRCA* mutation-related breast cancers [14,15].

Hypoxia is a hallmark of many non-*BRCA* mutation-related breast cancer types [16]. Hypoxia inducible factor-1 (HIF-1) is the key regulator of the hypoxia response. HIF-1 consists of 2 subunits, HIF-1α and HIF-1β. While HIF-1β is constitutively expressed, the HIF-1α protein is continuously degraded under normoxia by the ubiquitin-proteasome pathway [17,18]. Under hypoxia, HIF-1α protein degradation is inhibited resulting in its overexpression, subsequent binding to HIF-1β [19] and downstream signalling [20]. In non-*BRCA* mutation-related breast cancer, HIF-1α overexpression plays a role in carcinogenesis [21–26] and correlates with poor prognosis [27,28]. When HIF-1α is overexpressed, established downstream targets like Carbonic anhydrase IX (CAIX) and Glucose transporter-1 (Glut-1) are also up regulated [29,30]. *BRCA1* seems to play a role in the hypoxic response by regulating HIF-1α stability and by modulating expression of vascular endothelial growth factor, a major downstream target of HIF-1α [31]. Furthermore, functional HIF-1α overexpression (mostly hypoxia induced) is seen at a much higher frequency in *BRCA1* mutation-related invasive breast cancer than in sporadic breast cancer [32,33]. In contrast, *BRCA2*

mutation-related invasive cancers express HIF-1α less frequently [33].

However, studies in pre-invasive lesions are required to address the question whether hypoxia is a late stage bystander or a true carcinogenetic event.

There is both clinical and experimental evidence to suggest that ductal carcinoma *in situ* (DCIS) is a precursor lesion to most, if not all, non-*BRCA* mutation-related invasive breast cancers [34–38]. DCIS and other premalignant lesions such as lobular neoplasia, fibroadenoma, and ductal hyperplasia seems to be more common in prophylactic mastectomy (PM) specimens of *BRCA1* and *BRCA2* mutation carriers than in control mammoplasty specimens [10,39–42]. Furthermore, DCIS lesions adjacent to invasive cancers in *BRCA* mutation carriers have been described [43,44]. DCIS in *BRCA* mutation carriers is often high grade [43] and shows a similar morphology and immunophenotype as the accompanying invasive cancer [45]. High grade DCIS of non-*BRCA*-related cases often shows central necrosis [46] indicative of hypoxia. Indeed, overexpression of hypoxia-related proteins HIF-1α, CAIX and Glut-1 DCIS of non-*BRCA* mutation carriers has been described [22]. To find clues whether changes in hypoxia related proteins also is an early event in *BRCA* mutation-related carcinogenesis, we evaluated HIF-1α expression in *BRCA1* and *BRCA2* mutation-related DCIS in relation with the accompanying invasive cancers.

Materials and Methods

Patients

The study group comprised DCIS lesions of 32 patients with pathogenic germline *BRCA1* mutations, 16 patients with pathogenic germline *BRCA2* mutations and 77 patients unselected for family history (further denoted "non-*BRCA* mutation-related"). A synchronous invasive tumor was also present in 28 *BRCA1*, 17 *BRCA2* and 50 non-*BRCA* mutation-related cases. Tissue from these patients was available from our own archives, and from different pathology laboratories in The Netherlands (St Antonius Hospital Nieuwegein, Diakonessenhuis Utrecht, Gelre Ziekenhuizen Apeldoorn, Rijnstate Arnhem, Stichting Pathologisch en Cytologisch laboratorium West Brabant Bergen op Zoom, Ziekenhuis Gelderse Vallei Ede, Deventer Ziekenhuis Deventer, Meander medisch centrum Amersfoort, Onze Lieve Vrouwe Gasthuis Amsterdam, the VU University Medical Center, Amsterdam and the University Medical Center Groningen). Since we used archival pathology material which does not interfere with patient care and does not involve the physical involvement of the patient, no ethical approval is required according to Dutch legislation [the Medical Research Involving Human Subjects Act (Wet medisch-wetenschappelijk onderzoek met mensen, WMO [47])]. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients and therefore informed consent procedure was not required according to our institutional medical ethical review board. This has also been described by van Diest et al. [48].

Histopathology

Tumor size was measured in the fresh resection specimens, and tumor samples were subsequently fixed in neutral buffered formaldehyde, and processed to paraffin blocks according to standard procedures. Four μm thick sections were cut and stained with H&E for histopathology. Tumor type was assessed according to the WHO 2003, and tumors were graded according to the Nottingham grading system. Mitoses counting was performed as previously described [49]. Scoring was performed by one observer (PJvD) who was blinded to the origin of the tumors.

Immunohistochemistry

After deparaffinization and rehydration, antigen retrieval was performed using EDTA buffer at boiling temperature for 20 minutes for ER, HER2 and HIF-1α. A cooling period of 30 minutes preceded the incubation of the slides for HIF-1α with protein block (Novolink Max Polymer detection system, ready to use, Novocastra Laboratories Ltd, Newcastle Upon Tyne, UK) for 5 minutes at room temperature. Incubation of the slides with the HIF-1α mouse monoclonal (BD Biosciences, Pharmingen, Lexington, MA, USA), was done at a dilution of 1:50 overnight at 4°C. For detection, a polymer (Novolink Max Polymer detection system, ready to use) was used. For ER and HER2, the slides were incubated with primary antibodies for ER (1:100, Dako) and HER2 (1:100, Neomarkers) 60 minutes at room temperature.

For PR, Glut-1 and CAIX, antigen retrieval was performed in citrate buffer, pH = 6.0, for 20 minutes at 100°C. A cooling period of 30 minutes preceded the incubation (60 minutes at room temperature) with the primary antibodies. Polyclonal primary antibodies used were: PR (1:100, Dako), Glut-1 (1:200, DAKO) and CAIX (1:1000, Abcam, Cambridge Science Park, Cambridge, UK). For detection of the primary antibodies against ER, PR, HER2, CAIX and Glut-1, a poly HRP anti- Mouse/Rabbit/Rat IgG (ready to use, ImmunoLogic, Duiven, Netherlands) was used. All slides were developed with diaminobenzidine (10 minutes) followed by hematoxylin counterstaining. Before the slides were mounted all sections were dehydrated in alcohol and xylene. Positive controls were used throughout, negative controls were obtained by omission of the primary antibodies from the staining procedure. Representative pictures of positive and negative controls for HIF-1α, CAIX and Glut-1 have been provided as Figure S1.

Scoring of immunohistochemistry was performed by one observer (PJvD). HIF-1α was regarded overexpressed when >1% of nuclei were positive as described before [26]. ER and PR expression was regarded positive when 10% or more of the tumor nuclei stained positive. HER2 was scored positive when a 3+ membrane staining was observed according to the Dako system. CAIX and Glut-1 stainings were scored positive when a clear membrane staining pattern was seen. Associations between stainings were tested by Chi-square analysis. P-values$<$0.05 were considered to be statistically significant.

Results

The clinicopathological characteristics and expression of ER, PR, HER2, HIF-1α, CAIX and Glut-1 of *BRCA1*, *BRCA2 and non-BRCA* mutation-related DCIS cases are described in Table 1. The age of onset is lower in *BRCA* compared to non-*BRCA* mutation carriers (p = 0.000). *BRCA1* mutation-related DCIS cases often are ER, PR and HER2-negative as compared to the *BRCA2* and non-*BRCA* mutation-related DCIS (see Table 1 for correlations).

Expression of hypoxia-induced proteins in BRCA1, BRCA2 and non-BRCA mutation-related DCIS

HIF-1α overexpression was observed in 63% (20/32) of the *BRCA1*, in 62% (10/16) of the *BRCA2* and in 34% (26/77) of the non-*BRCA* mutation-related DCIS cases (p = 0.005;Table 1).

CAIX overexpression was observed in 56% (18/32) of *BRCA1* mutation-related DCIS cases, with accompanying HIF-1α overexpression in 31% (10/32) of the cases (p = 0.358;Table 2). Glut-1 was overexpressed in 59% (19/32) of the *BRCA1* mutation-related DCIS cases and HIF-1α was co-overexpression in 41% (13/32) of these cases (p = 0.403).

Table 1. Clinicopathological characteristics and expression of ER, PR, HER2, HIF-1α, CAIX and Glut-1 in DCIS lesions of *BRCA1*, *BRCA2* and non-*BRCA* mutation carriers.

		BRCA1	*BRCA2*	non-*BRCA*	p-value
	N	**32**	**16**	**77**	
Age	<45	25(78%)	9(56%)	14(18%)	
	>45	7(22%)	7(44%)	63(82%)	0.000
Grade	1	0(0%)	1(6%)	11(14%)	
	2	9(28%)	8(50%)	30(39%)	
	3	23(72%)	7(44%)	36(47%)	0.035
ER	neg	22(69%)	4(25%)	19(25%)	
	pos	10(31%)	12(75%)	58(75%)	0.000
PR	neg	27(84%)	9(56%)	36(47%)	
	pos	5(16%)	7(44%)	41(53%)	0.002
HER2	neg	31(97%)	11(69%)	55(71%)	
	pos	1(3%)	5(31%)	22(29%)	0.014
HIF-1α	neg	12(38%)	6(38%)	51(66%)	
	pos	20(63%)	10(62%)	26(34%)	0.005
CAIX	neg	14(44%)	9(56%)	72(94%)	
	pos	18(56%)	7(44%)	5(6%)	0.000
Glut-1	neg	13(41%)	4(25%)	25(33%)	
	pos	19(59%)	12(75%)	52(67%)	0.527

doi:10.1371/journal.pone.0056055.t001

CAIX was expressed in 44% (7/16) of *BRCA2* mutation-related DCIS cases with accompanying HIF-1α overexpression in 38% (6/16) of the cases (p = 0.091). Glut-1 overexpression was observed in 75% (12/16) of *BRCA2* mutation-related DCIS cases, with HIF-1α co-overexpression in 56% (9/16) of the cases (p = 0.074).

In the non-*BRCA* mutation-related DCIS cases, CAIX expression was seen in 6% (5/77) of the cases which were negative for HIF-1α. Glut-1 was overexpressed in 67% (52/77) of non-*BRCA* mutation-related DCIS cases, with concomitant HIF-1α overexpression in 29% (22/77) of the cases (p = 0.022).

Furthermore, in the *BRCA1* and *BRCA2* mutation-related DCIS no correlations between HIF-1α expression and grade, ER, PR and HER2 expression were found. For the non-*BRCA* mutation-related DCIS cases, a positive trend was observed with grade, and a negative trend with ER (Table 2).

Expression of hypoxia-induced proteins in BRCA1, BRCA2 and non-BRCA mutation-related DCIS and invasive cancer

In the *BRCA1* mutation-related cases with DCIS and concomitant invasive cancer (N = 29), the frequency of HIF-1α overexpression was high in both lesions: 62% (18/29) and 83% (24/29), respectively (p = 0.264;Table 3.). The frequency of CAIX expression was 52% (15/29) and 79% (23/29), respectively, in DCIS and invasive carcinoma (p = 0.311). Further, 59% (17/29) of the DCIS and 83% (24/29) (p = 0.945) of the invasive lesions were positive for Glut-1 expression. Examples of these IHC results are shown in Figure 1.

In the *BRCA2* mutation-related cases with invasive counterparts (N = 16), 63% (10/16) of DCIS lesions were HIF-1α positive as compared to 38% (6/16) if invasive lesions (p = 0.016). The same expression of CAIX was observed in *BRCA2* mutation-related DCIS lesions and the invasive counterpart lesions, 44% (7/16)

Table 2. Correlation of HIF-1α expression in DCIS lesions of *BRCA1*, *BRCA2* and non-*BRCA* mutation carriers with age, grade, ER, PR, HER2, CAIX and Glut-1 expression in these lesions.

		BRCA1			*BRCA2*			non-*BRCA*		
	N	**32**			**16**			**77**		
		HIF-1α		**p-value**	**HIF-1α**		**p-value**	**HIF-1α**		**p-value**
		neg	**pos**		**neg**	**pos**		**neg**	**pos**	
Age	<45	9	16		3	6		8	6	
	>45	3	4	0.740	3	4	0.696	43	20	0.427
Grade	1	0	0		1	0		10	1	
	2	2	7		4	4		21	9	
	3	10	13	0.264	1	6	0.149	20	16	0.081
ER	neg	8	14		1	3		9	10	
	pos	4	6	0.844	5	7	0.551	42	16	0.045
PR	neg	10	17		4	5		22	14	
	pos	2	3	0.900	2	5	0.515	29	12	0.373
HER2	neg	12	19		5	6		39	16	
	pos	0	1	0.431	1	4	0.330	12	10	0.170
CAIX	neg	4	10		5	4		46	26	
	pos	8	10	0.358	1	6	0.091	5	0	0.099
Glut-1	neg	6	7		3	1		21	4	
	pos	6	13	0.403	3	9	0.074	30	22	0.022

doi:10.1371/journal.pone.0056055.t002

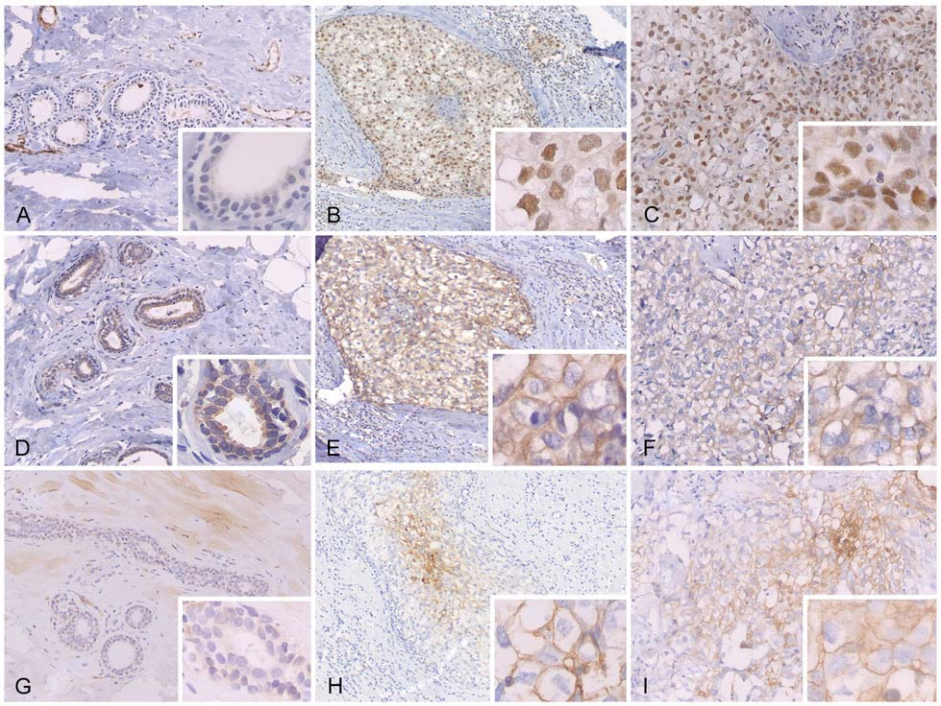

Figure 1. Immunohistochemical staining of HIF-1α, CAIX and Glut-1 in normal breast tissue (A, D and G), DCIS (B, E and H) and concomitant invasive cancer (C, F and I) of a *BRCA1* mutation carrier.
doi:10.1371/journal.pone.0056055.g001

(p = 0.049). Glut-1 was overexpressed in 75% (12/16) of DCIS cases in and in 56% (9/16) (p = 0.146) of the invasive *BRCA2* mutation-related lesions (Table 3).

The frequency of HIF-1α expression in non-*BRCA* mutation-related DCIS and concomitant invasive cancer (N = 50) was 38% (19/50) and 34% (17/50), respectively (p = 0.029). Similar CAIX expression was observed in both lesions, 8% (4/50) and 12% (6/50), respectively (p = 0.015). Glut-1 overexpression was seen in 70% (35/50) of DCIS cases and in 36% (18/50) (p = 0.797) of the invasive non-*BRCA* mutation-related lesions.

In summary, these non-significant differences indicate that HIF-1α positivity was similar in DCIS and the accompanying invasive lesions. Differences in HIF-1α expression between *BRCA1* and *BRCA2* and non-*BRCA* mutation related DCIS were borderline significant (p = 0.062). A significant difference in HIF-1α expression was seen between *BRCA1* and *BRCA2* as compared to non-*BRCA* mutation-related invasive cancer (p = 0.000).

Expression of hypoxia-induced proteins in BRCA non-BRCA mutation-related DCIS vs invasive cancer

Table 4 shows the expression of HIF-1α, CAIX and Glut-1 in paired, DCIS and concomitant invasive cancer, for *BRCA* mutation and non-*BRCA* mutation carriers.

HIF-1α expression was expressed in both lesions in 55% (16/29) of the *BRCA1* mutation-related cases, whereas both lesions were negative for HIF-1α expression in 10% (3/29) of cases. Overall, in 66% (19/29) of the *BRCA1* mutation carrier cases both lesions showed similar expression levels of HIF-1α. In 28% (8/29) of the *BRCA1* mutation-related cases only the invasive part, and in 7% (2/29) only the DCIS lesion showed HIF-1α expression. CAIX and Glut1 were expressed in both lesions in 45% (13/29) and 48% (14/29) of the *BRCA1* mutation carrier cases, respectively, and both lesions lacked expression of these markers in 14% (4/29) and 7%(2/29) of the cases. Thereby, CAIX was concomitantly expressed in both lesions 59% (17/29) of the cases, and the Glut-1 in 55% (16/29). Only the invasive lesion of *BRCA1* mutation carriers expressed both CAIX and Glut-1 in 34% (10/29) of cases. Expression of CAIX and Glut-1 exclusively in *BRCA1* mutation-related DCIS lesions was observed in 7% (2/29) and 10% (3/29) of cases, respectively.

In the *BRCA2* mutation-related cases with DCIS and concomitant invasive cancer, 38% (6/16) of the cases HIF-1α expression was observed and was absent in 38% (6/16) of the cases (Table 4). Thus, in 75% (12/16) of the *BRCA2* mutation-related cases, the DCIS and invasive lesions of the same patient showed similar expression levels of HIF-1α. Expression of HIF-1α in only the DCIS lesion was seen in 25% (4/16) of the *BRCA2* mutation-related cases. CAIX was expressed in both lesions in 31% (5/16) of *BRCA2* mutation-related cases and in 44% (7/16) of the cases both lesions lacked expression (total match 75%). CAIX was expressed in the invasive, but not in the DCIS part in 13% (2/16) of the cases, and CAIX was expressed in the DCIS, but not in the invasive part of 13% (2/16) of the cases. Glut-1 was expressed or absent in both lesions in 50% (8/16) and 19% (3/16) of cases, respectively (total match 69%). Further, Glut-1 expression was confined to the invasive part in 6% (1/16) of cases and the DCIS part in 25% (4/16) of the cases.

HIF-1α was expressed in both lesions in 20% (10/50) of the non-*BRCA* mutation-related cases and both lesions lacked HIF-1α expression in 48% (24/50) of cases. Thus, in total, 68% (34/50) of the non-*BRCA* mutation carrier cases showed similar expression levels of HIF-1α in both lesions. In 14% (7/50) of the non-*BRCA* mutation-related cases only the invasive part, and in 18% (9/50) only the DCIS lesion showed HIF-1α expression. CAIX and Glut-1 were expressed in both lesions in 4% (2/50) and 26% (13/50), respectively, of the non-*BRCA* mutation carrier cases. Conversely, both lesions lacked CAIX expression in 84% (42/50) and Glut-1

Table 3. Clinicopathological characteristics and expression of ER, PR, HER2, HIF-1α, CAIX and Glut-1 in DCIS and accompanying invasive lesions of *BRCA1*, *BRCA2* and non-*BRCA* mutation carriers.

		Invasive			**DCIS**		
		BRCA1	***BRCA2***	**non-*BRCA***	***BRCA1***	***BRCA2***	**non-*BRCA***
	N	**29**	**16**	**50**	**29**	**16**	**50**
Age	<45	21	8	8			
	>45	8	8	42			
Type	ductal	27	15	45			
	lobular	1	0	3			
	other	1	1	2			
Grade	1	0	1	8	0	1	7
	2	4	7	20	8	8	19
	3	25	8	22	21	7	24
ER	neg	22	4	13	21	4	13
	pos	7	12	37	8	12	37
PR	neg	25	5	19	25	9	26
	pos	4	11	31	4	7	24
HER2	neg	26	13	39	28	11	33
	pos	3	3	11	1	5	17
HIF-1α	neg	5	10	33	11	6	31
	pos	24	6	17	18	10	19
CAIX	neg	6	9	44	14	9	46
	pos	23	7	6	15	7	4
Glut-1	neg	5	7	32	12	4	15
	pos	24	9	18	17	12	35

doi:10.1371/journal.pone.0056055.t003

expresssion in 20%(10/50) of these cases. Thereby, CAIX expression in both lesions matched in 88% (44/50) and Glut-1 expression in 46% (23/50) of cases. Expression of CAIX and Glut-1 in only the invasive lesion of non-*BRCA* mutation carriers occurred in 8% (4/50) and 10% (5/50) of cases, respectively, whereas these markers were expressed only in DCIS lesions in 4% (2/50) and 44% (22/50) of cases.

When *BRCA1* and *BRCA2* mutation-related cases were examined together, HIF-1α expression in DCIS matched the expression in the accompanying invasive cancers in 68% (31/45) of cases, as compared to in 68% (34/50) of the non-*BRCA* mutation carrier cases. The expression of CAIX matched in 64% (29/45) of *BRCA1* and *BRCA2* mutation-related cases, as compared to in 88% (44/50) of non-*BRCA* mutation carrier cases. For Glut-1, the expression in DCIS matched the expression in the accompanying invasive cancers in 60% (27/45) of *BRCA1* and *BRCA2* mutation-related cases as compared to 46% (23/50) for non-*BRCA* mutation carrier cases.

Discussion

Non-*BRCA* mutation-related DCIS lesions, especially high grade ones, are known to become centrally deprived of oxygen resulting in activation of the hypoxia pathway, as shown in several studies by the presence of HIF-1α and its downstream targets. The aim of the present study was to examine the expression of HIF-1α in DCIS lesions of *BRCA1* and *BRCA2* mutation carriers in comparison with their invasive counterparts. Activation of HIF-1α in the DCIS stage of *BRCA1* or *BRCA2* germline mutated patients would indicate that hypoxia is an early driver of *BRCA* mutation-related carcinogenesis. HIF-1α overexpression was indeed frequently observed in *BRCA1* and *BRCA2* mutation-related DCIS cases, in association with expression of its downstream genes, indicating that HIF-1α is active.

Overall, 63% (30/48) of *BRCA* mutation-related DCIS lesions were HIF-1α-positive, which was significantly different compared to non-*BRCA* mutation carriers (34%, 26/77). The latter figure is

Table 4. Correlation of HIF-1α, CAIX and Glut-1 between invasive and DCIS lesions of *BRCA1*, *BRCA2* and non-*BRCA* mutation carriers.

BRCA1		**Invasive**								
		HIF-1alpha			**CAIX**			**Glut-1**		
		neg	**pos**	**p-value**	**neg**	**pos**	**p-value**	**neg**	**pos**	**p-value**
DCIS	neg	3	8		4	10		2	10	
	pos	2	16	0.264	2	13	0.311	3	14	0.945
BRCA2		**Invasive**								
		HIF-1alpha			**CAIX**			**Glut-1**		
		neg	**pos**	**p-value**	**neg**	**pos**	**p-value**	**neg**	**pos**	**p-value**
DCIS	neg	6	0		7	2		3	1	
	pos	4	6	0.016	2	5	0.049	4	8	0.146
non-*BRCA*		**Invasive**								
		HIF-1alpha			**CAIX**			**Glut-1**		
		neg	**pos**	**p-value**	**neg**	**pos**	**p-value**	**neg**	**pos**	**p-value**
DCIS	neg	24	7		42	4		10	5	
	pos	9	10	0.029	2	2	0.015	22	13	0.797

doi:10.1371/journal.pone.0056055.t004

lower compared to our earlier observations where 67% of sporadic DCIS lesions were HIF-1α positive [22]. Nevertheless, the current study suggests that hypoxia and HIF-1α already play a similar role in the DCIS stage of *BRCA* mutation-related carcinogenesis as in non-*BRCA* mutation-related DCIS.

BRCA mutation-related invasive cancers (especially *BRCA1* mutation-related ones) more frequently show HIF-1α overexpression than non-*BRCA* mutation-related ones [33,34]. This suggests that hypoxia plays a more important role in cancer progression in *BRCA* mutation carriers than in non-*BRCA* mutation carriers. HIF-1α, CAIX and Glut-1 expression in *BRCA* mutation-related DCIS was usually similar in the accompanying invasive lesions. This implies that next to being involved in early *BRCA* mutation-related carcinogenesis, hypoxia and HIF-1α overexpression may also be a driver of cancer progression, especially in *BRCA1* mutation carriers. Although the number of *BRCA2* mutation-related cases with DCIS and invasive lesions was small, there was a trend towards higher expression of the hypoxia-related markers in *BRCA2* mutation-related DCIS as compared to the invasive lesions. We can speculate that progression to the invasive state in these *BRCA2* mutation carriers might be due to the switch of the HIF-1α to HIF-2α expression under prolonged hypoxia [50]. HIF-2α expression has been observed in sporadic breast cancer [51] and should be analysed in *BRCA* mutation-related breast cancer and pre-invasive lesions. As HIF-1α already plays a role in the pre-invasive lesions of *BRCA* mutation carriers, hypoxia proteins would therefore be putative therapeutic targets for prevention of invasive disease. HIF-1α signalling inhibitors like PX-478 [52], farnesyltransferase inhibitor R115777 or trans-farnesylthiosalicyclic acid [53,54], Cetuximab [55] and other antibodies with the same structural motif [56], 2-methoxyestradiol (2ME2) [57,58], and inhibitors of the RNA binding protein Hur [59,60] are some of the therapeutics currently available.

We conclude that *BRCA1* and *BRCA2* germline mutation-related DCIS show a high frequency of overexpression of HIF-1α and its downstream proteins CAIX and Glut-1, as compared to non-*BRCA* mutation-related DCIS. This suggests that hypoxia may already play a role at the DCIS stage of *BRCA1* and *BRCA2* germline mutation-related breast carcinogenesis, and may also drive cancer progression. The current findings could be clinically relevant for *BRCA* mutation- related breast cancer treatment in several ways. First, HIF-1α and its downstream effectors may be used as molecular imaging targets for early detection and monitoring of therapy response. Second, HIF-1α is an interesting therapeutic target at the pre-invasive stage of *BRCA* mutation-related breast disease to prevent invasive disease.

Supporting Information

Figure S1 Positive controls: Immunohistochemical staining of HIF-1α and CAIX in renal clear cell carcinoma (B and D) and for Glut-1 in placental tissue (F). In A, C and E the primary antibody was omitted to provide negative controls.
(TIF)

Acknowledgments

We thank Dr. I. Ivanova, PhD, for carefully proofreading the manuscript.

Author Contributions

Conceived and designed the experiments: PvdG PJvD FM EvdW. Performed the experiments: PvdG YHCMS. Analyzed the data: PvdG PJvD YHCMS EvdW. Contributed reagents/materials/analysis tools: PvdG PJvD YHCMS MGEMM RBvdL FHM JB EvdW. Wrote the paper: PvdG PJvD MGEMM RBvdL FHM EGEdV EvdW.

References

1. Sørlie T, Tibshirani R, Parker J, Hastie T, Marron JS, et al. (2003) Repeated observation of breast tumor subtypes in independent gene expression data sets. Proc Natl Acad Sci USA 100:8418–23
2. Tirkkonen M, Johannsson O, Agnarsson BA, Olsson H, Ingvarsson S, et al. (1997) Distinct somatic genetic changes associated with tumor progression in carriers of BRCA1 and BRCA2 germ-line mutations. Cancer Res 57:1222–7
3. Jönsson G, Naylor TL, Vallon-Christersson J, Staaf J, Huang J, et al. (2005) Distinct genomic profiles in hereditary breast tumors identified by array-based comparative genomic hybridization. Cancer Res 65:7612–21
4. van Beers EH, van Welsem T, Wessels LFA, Li Y, Oldenburg RA, et al. (2005) Comparative genomic hybridization profiles in human BRCA1 and BRCA2 breast tumors highlight differential sets of genomic aberrations. Cancer Res 65:822–7
5. Alvarez S, Diaz-Uriarte R, Osorio A, Barroso A, Melchor L, et al. (2005) A predictor based on the somatic genomic changes of the BRCA1/BRCA2 breast cancer tumors identifies the non-BRCA1/BRCA2 tumors with BRCA1 promoter hypermethylation. Clin Cancer Res 11:1146–53
6. Hedenfalk I, Ringner M, Ben-Dor A, Yakhini Z, Chen Y, et al. (2003) Molecular classification of familial non-BRCA1/BRCA2 breast cancer. Proc Natl Acad Sci U S A 100:2532–7
7. Wessels LF, van Welsem T, Hart AA, van't Veer LJ, Reinders MJ, et al. (2002) Molecular classification of breast carcinomas by comparative genomic hybridization: a specific somatic genetic profile for BRCA1 tumors. Cancer Res 62:7110–7
8. Lakhani SR, van de Vijver MJ, Jacquemier J, Anderson TJ, Osin PP, et al. (2002) The pathology of familial breast cancer: predictive value of immunohistochemical markers estrogen receptor, progesterone receptor, HER-2, and p53 in patients with mutations in BRCA1 and BRCA2. J Clin Oncol 20:2310–8.
9. Marcus JN, Watson P, Page DL, Narod SA, Lenoir GM, et al. (1996) Hereditary breast cancer: pathobiology, prognosis, and BRCA1 and BRCA2 gene linkage. Cancer 77:697–709
10. Breast Cancer Linkage Consortium (1997) Pathology of familial breast cancer: differences between breast cancers in carriers of BRCA1 or BRCA2 mutations and sporadic cases. Lancet 349:1505–10.
11. Chappuis PO, Nethercot V, Foulkes WD. (2000) Clinico-pathological characteristics of BRCA1- and BRCA2-related breast cancer. Semin Surg Oncol 18:287–95.
12. Foulkes WD, Stefansson IM, Chappuis PO, Begin LR, Goffin JR, et al. (2003) Germline BRCA1 mutations and a basal epithelialphenotype in breast cancer. J Natl Cancer Inst 95:1482–5
13. Van der Groep P, Bouter A, van der Zanden R, Menko FH, Buerger H, et al. (2004) Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 96:712–3
14. Van der Groep P, Bouter A, van der Zanden R, Siccema I, Menko FH, et al. (2006) Distinction between hereditary and sporadic breast cancer on the basis of clinicopathological data. J Clin Pathol 59:611–7
15. Bane AL, Pinnaduwage D, Colby S, Reedijk M, Egan SE, et al. (2009) Expression profiling of familial breast cancers demonstrates higher expression of FGFR2 in BRCA2-associated tumors. Breast Cancer Res Treat 117:183–91
16. Vaupel P, Thews O, Hoeckel M (2001) Treatment resistance of solid tumors: role of hypoxia and anemia. Med Oncol 18:243–59
17. Salceda S, Caro J (1997) Hypoxia-inducible factor 1alpha (HIF-1alpha) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. J Biol Chem 272:22642–7.
18. Huang LE, Gu J, Schau M, Bunn HF (1998) Regulation of hypoxia-inducible factor 1alpha is mediated by an O_2-dependent degradation domain via the ubiquitin-proteasome pathway. Proc Natl Acad Sci 95:7987–92
19. Semenza GL (1999) Regulation of mammalian O2 homeostasis by hypoxia-inducible factor 1. Annu Rev Cell Dev Biol 15:551–78.
20. Semenza GL, Wang GL (1992) A nuclear factor induced by hypoxia via de novo synthesis binds to the human erythropoeitin gene enhancer at a site required for transcriptational activation. Mol Cell Biol 12:5447–54
21. Bos R, van der Groep P, Greijer AE, Shvarts A, Meijer S, et al. (2003) Levels of hypoxia-inducible factor-1α independently predict prognosis in patients with lymph node negative breast cancer. Cancer 97:1573–81
22. Bos R, Zhong H, Hanrahan CF, Mommers EC, Semenza GL, et al. (2001) Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 93:309–14
23. Bos R, van der Hoeven JJM, van der Wall E, van der Groep P, van Diest PJ, et al. (2002) Biologic correlates of 18fluorodeoxyglucose uptake in human breast cancer measured by positron emission tomography. J Clin Oncol 20:379–87.
24. Bos R, van Diest PJ, van der Groep P, Greijer AE, Hermsen MAJA, et al. (2003) Protein expression of B-cell lymphoma gene 6 (BCL-6) in invasive breast cancer

is associated with cyclin D1 and hypoxia-inducible factor-1α (HIF-1α). Oncogene 22:8948–51.
25. Bos R, van Diest PJ, van der Groep P, Shvarts A, Greijer AE, et al. (2004) Expression of hypoxia-inducible factor-1 alpha and cell cycle proteins in invasive breast cancer are estrogen receptor related. Breast Cancer Res 6:R450–R9.
26. Bos R, Van Diest PJ, De Jong JS, Van der Groep P, Van der Valk P, et al. (2005) Hypoxia-inducible factor-1α is associated with angiogenesis and expression of bFGF, PDGF-BB, and EGFR in invasive breast cancer. Histopathology 46:31–6.
27. Vleugel MM, Greijer AE, Shvarts A, van der Groep P, van Berkel M, et al. (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1α expression in invasive breast cancer. J Clin Pathol 58:172–7
28. Trastour C, Benizri E, Ettore F, Ramaioli A, Chamorey E, et al. (2007) HIF-1α and CA IX staining in invasive breast carcinomas: Prognosis and treatment outcome. Int J Cancer 120:1451–8
29. Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, et al. (2000) Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075–83
30. Chen C, Pore N, Behrooz A, Ismail-Beigi F, Maity A (2001) Regulation of glut1 mRNA by hypoxia-inducible factor-1. Interaction between H-ras and hypoxia. J Biol Chem 276:9519–25
31. Kang HJ, Kim HJ, Rih JK, Mattson TL, Kim KW, et al. (2006) BRCA1 plays a role in the hypoxic response by regulating HIF-1α stability and by modulating Vascular endothelial growth factor expression. J Biol Chem 281:13047–56
32. van der Groep P, Bouter A, Menko FH, van der Wall E, van Diest PJ (2008) High frequency of HIF-1α overexpression in BRCA1 related breast cancer. Breast Cancer Res Treat 111:475–80.
33. Yan M, Rayoo M, Takano EA; KConFab Investigators, Fox SB (2009) BRCA1 tumours correlate with a HIF-1α phenotype and have a poor prognosis through modulation of hydroxylase enzyme profile expression. Br J Cancer 101:1168–74
34. Boecker W (2006) Preneoplasia of the breast. A New Conceptual Approach to Proliferative Breast Disease. Elsevier, Gmbh, Munich, p407–p467
35. Sgroi DC (2010) Preinvasive breast cancer. Annu Rev Pathol 5:193–221.
36. Claus EB, Chu P, Howe CL, Davison TL, Stern DF, et al. (2001) Pathobiologic findings in DCIS of the breast: morphologic features, angiogenesis, HER-2/neu and hormone receptors. Exp Mol Pathol 70:303–16.
37. Hu M, Yao J, Carroll DK, Weremowicz S, Chen H, et al. (2008) Regulation of in situ to invasive breast carcinoma transition. Cancer Cell 13:394–406.
38. van Diest PJ (1999) Ductal carcinoma in situ in breast carcinogenesis. J Pathol 187:383–4.
39. Claus EB, Petruzella S, Matloff E, Carter D (2005) Prevalence of BRCA1 and BRCA2 mutations in women diagnosed with ductal carcinoma in situ. JAMA 293:964–9.
40. Hoogerbrugge N, Bult P, de Widt-Levert LM, Beex LV, Kiemeney LA, et al. (2003) High prevalence of premalignant lesions in prophylactically removed breasts from women at hereditary risk for breast cancer. J Clin Oncol 21:41–5
41. Hoogerbrugge N, Bult P, Bonenkamp JJ, Ligtenberg MJ, Kiemeney LA, et al. (2006) Numerous high-risk epithelial lesions in familial breast cancer. Eur J Cancer 42:2492–8.
42. Kauff ND, Brogi E, Scheuer L, Pathak DR, Borgen PI, et al. (2003) Epithelial lesions in prophylactic mastectomy specimens from women with BRCA mutations. Cancer 97:1601–8
43. Hwang ES, McLennan JL, Moore DH, Crawford BB, Esserman LJ, et al. (2007) Ductal carcinoma in situ in BRCA mutation carriers. J Clin Oncol 25:642–7.
44. Arun B, Vogel KJ, Lopez A, Hernandez M, Atchley D, et al. (2009) High prevalence of preinvasive lesions adjacent to BRCA1/2-associated breast cancers. Cancer Prev Res (Phila) 2:122–7
45. van der Groep P, van Diest PJ, Menko FH, Bart J, de Vries EG, et al. (2009) Molecular profile of ductal carcinoma in situ of the breast in BRCA1 and BRCA2 germline mutation carriers. J Clin Pathol 62;926–30
46. Bussolati G, Bongiovanni M, Cassoni P, Sapino A (2000) Assessment of necrosis and hypoxia in ductal carcinoma in situ of the breast: basis for a new classification. Virchows Arch 437(4):360–4.
47. Central Committee on Research involving Human Subjects (Centrale Commissie Mensgebonden Onderzoek) (text in Dutch): http://www.ccmo-online.nl/main.asp?pid = 10&sid = 30&ssid = 51
48. van Diest PJ (2002) No consent should be needed for using leftover body material for scientific purposes. BMJ 325:648–51.
49. Van Diest PJ, Baak JP, Matze-Cok P, Wisse-Brekelmans EC, van Galen CM, et al. (1992) Reproducibility of mitosis counting in 2469 breast cancer specimens: results from the multicenter morphometric mammary carcinoma project. Hum Pathol 23:603–7.
50. Koh MY, Lemos R Jr, Liu X, Powis G (2011) The hypoxia-associated factor switches cells from HIF-1α- to HIF-2α-dependent signaling promoting stem cell characteristics, aggressive tumor growth and invasion. Cancer Res 71:4015–27.
51. Helczynska K, Larsson AM, Holmquist Mengelbier L, Bridges E, Fredlund E, et al. (2008) Hypoxia-inducible factor-2alpha correlates to distant recurrence and poor outcome in invasive breast cancer. Cancer Res 68:9212–20.
52. Welsh SJ, Williams R, Kirkpatrick L, Paine-Murrieta G, Powis G (2004) Antitumor activity and pharmacodynamic properties of PX-478, an inhibitor of hypoxia inducible factor-1a. Mol Cancer Ther 3:233–44.
53. Delmas C, End D, Rochaix P, Favre G, Toulas C, et al. (2003) The farnesyltransferase inhibitor R115777 reduces hypoxia and matrix metalloproteinase 2 expression in human glioma xenograft. Clin Cancer Res 9:6062–8.
54. Blum R, Jacob-Hirsch J, Amariglio N, Rechavi G, Kloog Y (2005) Ras inhibition in glioblastoma down-regulates hypoxia-inducible factor-1α, causing glycolysis shutdown and cell death. Cancer Res 65:999–1006.
55. Luwor RB, Lu Y, Li X, Mendelsohn J, Fan Z (2005) The antiepidermal growth factor receptor monoclonal antibody cetuximab/C225 reduces hypoxia-inducible factor-1 alpha, leading to transcriptional inhibition of vascular endothelial growth factor expression. Oncogene 24:4433–41.
56. Tan C, de Noronha RG, Roecker AJ, Pyrzynska B, Khwaja F, et al. (2005) Identification of a novel small-molecule inhibitor of the hypoxia-inducible factor 1 pathway. Cancer Res 65:605–12.
57. Mabjeesh NJ, Escuin D, LaVallee TM, Pribluda VS, Swartz GM, et al. (2003) 2ME2 inhibits tumor growth and angiogenesis by disrupting microtubules and dysregulating HIF. Cancer Cell 3:363–75.
58. Ricker JL, Chen Z, Yang XP, Pribluda VS, Swartz GM, et al. (2004) 2-methoxyestradiol inhibits hypoxia-inducible factor 1alpha, tumor growth, and angiogenesis and augments paclitaxel efficacy in head and neck squamous cell carcinoma. Clin Cancer Res 10:8665–73.
59. Galbán S, Kuwano Y, Pullmann R Jr, Martindale JL, Kim HH, et al. (2008) RNA-binding proteins HuR and PTB promote the translation of hypoxia-inducible factor 1α. Mol Cell Biol 28:93–107.
60. Abdelmohsen K, Srikantan S, Kuwano Y, Gorospe M (2008) miR-519 reduces cell proliferation by lowering RNA-binding protein HuR levels. Proc Natl Acad Sci U S A 105:20297–302.