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Introduction

In the Western world, one in eight women will develop breast cancer during their life and breast cancer is causing about 458,000 deaths worldwide per year [1,2]. Aggressive forms of breast cancer are frequently refractory to treatment [3], even to established targeted therapy, and thus have a high risk of relapse and formation of distant metastases [4]. Identification of molecular pathways involved in aggressive forms of breast cancer is therefore important to design novel targeted therapeutic agents to counteract tumor progression and metastasis.

DDX3, also known as DDX3X because of its location on the X chromosome, is a member of the DEAD-box RNA helicase family which is involved in transcription, RNA splicing, nuclear export of mRNA and translation initiation [5,6]. Initially, DDX3 was studied because of its manipulation by viruses like hepatitis C (HCV) and human immunodeficiency virus (HIV) [7,8]. Recently, novel compounds were developed which could potentially inhibit DDX3 activity [15-20]. A recent in vitro study [21] showed that DDX3 is a direct downstream target of HIF-1α, the predominant factor in the mammalian hypoxia response [22]. Hypoxia is an important event in breast carcinogenesis [23-26], causing a more aggressive phenotype with increased invasiveness and proliferation, formation of metastases, resistance to therapy [27] and poorer survival [28,29].

However, no data are yet available on the relation between DDX3 and hypoxia in human breast cancer, or any other human tumors specimens. Therefore, we set out to correlate expression of DDX3 and hypoxia related proteins, pointing to a distinct role for DDX3 under hypoxic conditions and supporting the oncogenic role of DDX3 which could have clinical implication for current development of DDX3 inhibitors.

Abstract

Aims: DDX3 is an RNA helicase that has antiapoptotic properties, and promotes proliferation and transformation. In addition, DDX3 was shown to be a direct downstream target of HIF-1α (the master regulatory of the hypoxia response) in breast cancer cell lines. However, the relation between DDX3 and hypoxia has not been addressed in human tumors. In this paper, we studied the relation between DDX3 and the hypoxic responsive proteins in human breast cancer.

Methods and Results: DDX3 expression was investigated by immunohistochemistry in breast cancer in comparison with hypoxia related proteins HIF-1α, GLUT1, CAIX, EGFR, HER2, Akt1, FOXO4, p53, ERα, COMMD1, FER kinase, PIN1, E-cadherin, p21, p27, Transferrin receptor, FOXO3A, c-Met and Notch1. DDX3 was overexpressed in 127 of 366 breast cancer patients, and was correlated with overexpression of HIF-1α and its downstream genes CAIX and GLUT1. Moreover, DDX3 expression correlated with hypoxia-related proteins EGFR, HER2, FOXO4, ERα and c-Met in a HIF-1α dependent fashion, and with COMMD1, FER kinase, Akt1, E-cadherin, TIR and FOXO3A independent of HIF-1α.

Conclusions: In invasive breast cancer, expression of DDX3 was correlated with overexpression of HIF-1α and many other hypoxia related proteins, pointing to a distinct role for DDX3 under hypoxic conditions and supporting the oncogenic role of DDX3 which could have clinical implication for current development of DDX3 inhibitors.

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with HIF-1α without clear functional relationship like E-cadherin [51], p21 [52], c-Met [53,54] and p27 [55].

**Materials and Methods**

**Patients**

Representative paraffin embedded tissue blocks of 422 breast cancer patients collected between 2004 and 2007 were taken from the archive of the Department of Pathology of the University Medical Centre in Utrecht and routinely processed to four tissue microarrays (TMA) as described before [56,57].

Clinicopathological data including tumor stage, histological data (type, grade, mitotic index (MAI), estrogen receptor alpha (ERα) and human epidermal growth factor receptor 2 (HER2)) status was collected from patient files (Table 1). Protein expression data by immunohistochemistry of HIF-1α, FOXO3A, FOXO4, PIN1, Akt1, COMMD1, p33, p21, p27, EGFR, E-cadherin, GLUT1 and CAIX was derived from previous studies[34,40,58–62]. Use of anonymous or coded left over material for scientific purposes is part of the standard treatment contract with patients in the UMCU [63].

**Immunohistochemistry**

Sections of 4 μm were cut, mounted on SuperFrost slides (Menzel&Glaeser, Brunswick, Germany), deparaffinized and rehydrated. Endogenous peroxidase was then blocked for 15 min with a buffer solution containing 0.3% hydrogen peroxide. Antigens were retrieved by boiling for 20 min in 10 mM citrate buffer (pH 6.0) (for DDX3, c-Met, TIR, FER kinase and Notch1), cooled and washed in PBS. Nonspecific binding sites were blocked with a 2% normal goat serum, 1% BSA in PBS (pH 7.4) (Notch1). TMAs were subsequently incubated in a humidified chamber for 1 hour with polyclonal rabbit anti-DDX3 R648 [64] diluted 1:1000, TIR 1:300 (13-6800, Invitrogen, Breda, The Netherlands) and FER kinase 1:300 (clone 5D2, Cell Signaling Technologies, USA). Primary antibodies against c-Met 1:100 (18-2257, Zymed, Invitrogen) and Notch 1:100 (Cell Signaling Technologies, USA) were incubated overnight at 4°C. Subsequently, sections were washed in PBS and incubated for 30 min with secondary antibodies (Brightvision, Immunologic, Duiven, The Netherlands) washed with PBS and developed with diaminobenzidine. Slides were counterstained with hematoxylin, dehydrated and cover-slipped. Appropriate positive and negative controls were used throughout.

**Table 1.** Patient characteristics.

|                      | N (422) | missing |
|----------------------|---------|---------|
| Mean age (range)     | 61.0    | (28–88) |
| Tumor size           |         |         |
| ≤20 mm               | 212     | 50%     | 3 |
| ≤50 mm               | 181     | 43%     | |
| >50 mm               | 26      | 6%      | |
| Lymph node status    |         |         |
| Positive*            | 193     | 48%     | 18 |
| Negative**           | 211     | 52%     | |
| Histological type    |         |         |
| ductal               | 343     | 82%     | 1 |
| lobular              | 42      | 10%     | |
| other                | 36      | 9%      | |
| Grade                |         |         |
| I                    | 80      | 20%     | 26 |
| II                   | 145     | 37%     | |
| III                  | 171     | 43%     | |
| Mitotic index (range)| 17.2    | (0–196) | 0 |
| Estrogen receptorα   |         |         |
| Positive             | 335     | 79%     | 0 |
| Negative             | 87      | 21%     | |
| Progesterone receptor|         |         |
| Positive             | 247     | 59%     | 1 |
| Negative             | 174     | 41%     | |
| HER2 receptor        |         |         |
| Positive             | 44      | 10%     | 0 |
| Negative             | 378     | 90%     | |

*Positive = >N1mi.
**Negative = N0 or N0(i+ (according to TNM 7th edition, 2010).

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Scoring of Immunohistochemistry

Scoring was done by a single experienced pathologist (PJvD). Intensity of cytoplasmic DDX3, FER kinase and membranous E-cadherin, Tir and c-Met was scored semi-quantitatively from 0–3 and percentages of cells with nuclear DDX3 and Notch1 expression were estimated. Out of three cores from the same patient, the maximum cytoplasmic DDX3 score was used for further analysis.

DDX3 scores 1 and 2 were grouped as low DDX3 expression and evaluated against high DDX3 expression (scores 3). For E-cadherin, Tir, c-Met and FER kinase scores 0 and 1 were defined as low expression versus score 2 and 3 as high expression. For HIF-1α, the 1% threshold was used as before [59].

Statistics

Expression levels of DDX3 and the other proteins were compared by chi-square test or t-test whenever applicable. Logistic regression or ANCOVA was used for multivariate analysis to determine dependence of these relations on HIF-1α.

Since EGFR and HER2 are upstream regulators of HIF-1α via PI-3K/AKT, we also assessed the relation of EGFR and HER2 with DDX3 independent of Akt1 and HIF-1α. In lobular breast cancer there is very little or no expression of E-cadherin, so the lobular cancers were excluded in analysis with respect to E-cadherin.

Pearson correlation coefficient was determined for correlation analysis.

All statistical analyses were carried out with SPSS 17.0 for Windows. (SPSS Inc., Chicago, IL, USA), regarding two-sided p-values below 0.05 as significant.

Figure 1. Examples of DDX3 and HIF-1α staining. Breast cancer photomicrographs are taken at 20X. A. low HIF-1α expression (0%); B. low DDX3 expression (1), same patient as in A; C. high HIF-1α expression (90%); D. high DDX3 expression (3), same patient as in C.
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**Results**

DDX3 staining could be evaluated in 366 of the 422 breast cancer cases. The drop outs were caused by damaged or detached cores during cutting, mounting, or staining, or did not contain tumor. All breast cancer cases showed some expression of DDX3 of which 127 (35%) showed strong cytoplasmic DDX3 expression. (table 2).

HIF-1α overexpression correlated with expression of CAIX, GLUT1, EGFR, HER2, Akt1, FER kinase, ERα, FOXO4, TfR, c-Met as expected (data not shown). Strong cytoplasmic DDX3 expression was associated with overexpression of the master regulator of the hypoxia response HIF-1α (OR = 2.83; p < 0.001) (figure 1) and its downstream proteins GLUT1 (OR = 2.36; p = 0.001) and CAIX (2.39; p = 0.012). In logistic regression, HIF-1α (OR = 2.52; p = 0.001), GLUT1 (OR = 1.94; p = 0.021), CAIX (OR = 2.23; p = 0.042) predicted cytoplasmic DDX3 levels independently. (table 2).

The HIF-1α transcription regulators HER2 (OR = 3.04; p = 0.001), EGFR (OR = 2.01; p = 0.013) and Akt1 (83% vs. 95%; p = 0.001) were correlated with DDX3 expression. Association of EGFR and HER2 with DDX3 was dependent on HIF-1α. (table 3).

**Table 2.** Expression of DDX3 in relation to oxygen sensing proteins.

|            | Cytoplasmic DDX3 | Multivariate |
|------------|------------------|--------------|
|            | N (%) | Low (%) | High (%) | OR  | p value | OR  | p value |
| HIF-1α ≤1% | 366   | 239     | 127      | 2.83 | < 0.001 | 2.52 | 0.001 |
| >1%        | 108   | 52      | 40       | 2.36 | 0.001   | 1.94 | 0.021 |
| GLUT1 negative | 123 | 54      | 44       | 2.36 | 0.001   | 1.94 | 0.021 |
|            | 190   | 105     | 85       | 2.39 | 0.012   | 2.23 | 0.042 |
| GLUT1 positive | 190 | 105     | 85       | 2.39 | 0.012   | 2.23 | 0.042 |
| CAIX negative | 62  | 37      | 25       | 2.39 | 0.012   | 2.23 | 0.042 |
|            | 260   | 159     | 101      | 2.39 | 0.012   | 2.23 | 0.042 |

*p-chi-square test.  
 logistic regression.
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**Table 3.** Expression of DDX3 in relation to regulators of HIF-1α.

|            | Cytoplasmic DDX3 | Correction for HIF-1α |
|------------|------------------|-----------------------|
|            | N (%) | Low (%) | High (%) | OR  | p value | OR  | p value |
| EGFR negative | 366 | 239     | 127      | 2.01 | 0.013   | 1.61 | 0.134 |
| positive     | 62   | 32      | 30       | 3.04 | 0.001   | 1.88 | 0.092 |
| HER2 negative | 325 | 223     | 102      | 3.04 | 0.001   | 1.88 | 0.092 |
| positive     | 41   | 17      | 24       | 1.16 | 0.802   | 1.42 | 0.571 |
| p53 negative | 80   | 45      | 35       | 3.90 | 0.013   | 5.45 | 0.006 |
| positive     | 19   | 10      | 9        | 3.90 | 0.013   | 5.45 | 0.006 |
| COMMD1 low   | 23   | 17      | 6        | 3.90 | 0.013   | 5.45 | 0.006 |
| high         | 57   | 24      | 33       | 3.90 | 0.013   | 5.45 | 0.006 |
| FER kinase low | 203 | 161     | 42      | 4.49 | < 0.001 | 4.10 | < 0.001 |
| high         | 152  | 70      | 82       | 4.49 | < 0.001 | 4.10 | < 0.001 |
| PIN1 low     | 58   | 32      | 26       | 2.05 | 0.225   | 1.73 | 0.305 |
| high         | 24   | 9       | 15       | 2.05 | 0.225   | 1.73 | 0.305 |

*p-chi-square test.  
 logistic regression.
*cstudent's t-test.  
*dANOVA.
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Proteins known to regulate HIF-1α in a PI-3K/AKT independent fashion were associated with DDX3 as well; COMMD1 (OR = 3.90; p = 0.013), FER kinase (OR = 4.49; p<0.001), FOXO4 (30% vs. 16%; p = 0.035), but not p53 (OR = 1.16; p = 0.802) and PIN1 (OR = 2.05; p = 0.225). Logistic regression indicated a HIF-1α independent relation between cytoplasmic DDX3 on the one hand and COMMD1 (OR = 5.45; p = 0.006) and FER kinase (OR = 4.10; p<0.001) on the other (table 3).

DDX3 was further associated with ERα (OR = 0.48; p = 0.005), E-cadherin (OR = 2.84; p = 0.005), TIR (OR = 2.77; p<0.001), c-Met (OR = 1.72; p = 0.042) and FOXO3A (83% vs. 94%; p = 0.021). After correction for HIF-1α expression, E-cadherin (OR = 2.91; p = 0.009), TIR (OR = 2.01; p = 0.007) and FOXO3A (78% vs. 95%; p = 0.007) were still associated with DDX3 (table 4). Table 5 shows the Pearson correlation analysis results.

**Discussion**

The aim of this study was to investigate the relation between DDX3 and the hypoxic response in human breast cancer in the light of *in vitro* results pointing to regulation of DDX3 by HIF-1α. We indeed show a positive correlation between HIF-1α and DDX3 overexpression in a large series of human breast cancer cases, as well as an association between DDX3 overexpression and various other hypoxia related proteins.

However, we have established a correlation between DDX3 overexpression and nuclear HIF-1α overexpression which supports the direct regulation of DDX3 by HIF-1α found *in vitro* [21], but this is obviously no more than an association at this point no proof for a causal relationship. Immunohistochemistry has some limitations like being inherently a more qualitative than quantitative method, and semiquantitative scoring and dichotomization with non-optimal reproducibility. To compensate for these issues we standardized the IHC procedure, used control tissue throughout, scored three samples per patient, studied a large cohort of breast cancer patients and results obtained from dichotomized parameters were confirmed by correlation analysis for the most important parameters with the DAKO score of DDX3 (table 5). Patient features in this study corresponded with known clinico-pathological characteristics in breast cancer (table 1) [65].

Furthermore, DDX3 correlated with EGFR, HER2, FOXO4, ERα and c-Met in a HIF-1α dependent way. Also, we found a positive correlation with COMMD1, FER kinase, Akt1, E-cadherin, TIR and FOXO3A independent of HIF-1α. COMMD1 down regulates HIF-1α by competition with HSP90β [41], or

### Table 4. Expression of DDX3 in relation to various other hypoxia induced proteins.

|          | N (%) | Low (%) | High (%) | OR   | p valuea | OR   | p valueb |
|----------|-------|---------|----------|------|----------|------|----------|
| ERα      | 366   | 239     | 127      | 0.48 | 0.005    | 0.67 | 0.166    |
|          |       |         |          |      |          |      |          |
| E-cadherin* | 81 (22) | 42 (18) | 39 (31) | 0.84 | 0.005    | 2.91 | 0.009    |
|          | 285 (78) | 197 (82) | 88 (69) |      |          |      |          |
| p21      | 46 (46) | 28 (51) | 18 (41) | 1.50 | 0.418    | 1.26 | 0.625    |
|          | 53 (54) | 27 (49) | 26 (59) |      |          |      |          |
| TIR      | 221 (63) | 161 (72) | 60 (48) | 2.77 | <0.001   | 2.01 | 0.007    |
|          | 128 (37) | 63 (28) | 65 (52) |      |          |      |          |
| c-Met    | 264 (77) | 181 (81) | 83 (71) | 1.72 | 0.042    | 1.62 | 0.096    |
|          | 77 (23) | 43 (19) | 34 (29) |      |          |      |          |
| p27      | 99     | 42%     | 44%      |      |          |      |          |
| FOXO3A   | 86     | 83%     | 94%      | 0.021 | 0.007    |      |          |
| Notch1   | 305    | 63%     | 55%      |      |          |      |          |

*chi-square test.

### Table 5. DDX3 correlations with the most important hypoxia related proteins.

|          | N | r* | p value |
|----------|---|----|---------|
| HIF-1α   | 322 | 0.276 | <0.001 |
| GLUT1    | 313 | 0.186 | 0.001 |
| CAX      | 322 | 0.136 | 0.015 |
| HER2     | 366 | 0.185 | <0.001 |
| Efr      | 366 | -0.132 | 0.011 |

*Pearson correlation coefficient.
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Table 5. DDX3 correlations with the most important hypoxia related proteins.

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| HIF-1α   | 322 | 0.276 | <0.001 |
| GLUT1    | 313 | 0.186 | 0.001 |
| CAX      | 322 | 0.136 | 0.015 |
| HER2     | 366 | 0.185 | <0.001 |
| Efr      | 366 | -0.132 | 0.011 |

*Pearson correlation coefficient.
doi:10.1371/journal.pone.0063548.t005

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down regulates the transcriptional activity of HIF-1α [40]. However, we could not detect an association with COMMD1 and HIF-1α expression or its downstream targets: E-cadherin, TIR, p21, p27 or c-Met. Nonetheless, COMMD1 correlates with DDX3 independent of HIF-1α.

FER kinase helps cells to withstand stress, including hypoxia, via up regulation of HIF-1α [42]. We found a strong relation with FER kinase with both HIF-1α and DDX3. After correction for the effect FER kinase has on HIF-1α, a strong relation between FER kinase and DDX3 remained, implying a HIF-1α dependent and independent relation.

DDX3 was shown to down regulate E-cadherin [12], but in the present study we show a positive correlation, for which we have no obvious explanation. TIR is also under transcriptional control of HIF-1α [47]. TIR is overexpressed in many cancers, which could be attributed to the increased need for iron as a cofactor of the ribonucleotide reductase enzyme involved in DNA synthesis of rapidly dividing cells. Thus, the HIF-1α independent relation between DDX3 and TIR corroborates previous reports on the oncogenic properties of DDX3. Nuclear expression of FOXO3A in breast cancer is associated with anti-apoptotic signaling via Akt1, an aggressive phenotype and poor survival [66]. In response to hypoxia, FOXO3A accumulates in a HIF-1α dependent way to inhibit HIF-1α induced apoptosis [48]. Although we did not find a relation between HIF-1α and FOXO3A we did find a relation between FOXO3A and DDX3, independent of HIF-1α and Akt1. Perhaps DDX3 and FOXO3A function in a concerted survival response after stress stimuli.

**References**

1. Ferlay J, Shin HR, Bray F, Forman D, Mathers C, et al. (2010) Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. Int J Cancer 127: 2893–2917.
2. Albrektsen S, Kosary C, Krapcho M, Neyman N, Aminou R, et al. (2010) SEER Cancer Statistics Review, 1975-2007, National Cancer Institute, Bethesda, MD, based on November 2009 SEER data submission, posted to the SEER web site: http://seercancergov/csr/1975_2007/Accessed 2012 Nov.
3. Liedtke C, Mazouni C, Hess KR, Andre F, Tordai A, et al. (2008) Response to neoadjuvant therapy and long-term survival in patients with triple-negative breast cancer. J Clin Oncol 26: 1275–1281.
4. Dent R, Trudeau M, Pritchard KI, Hanna WM, Kahn HK, et al. (2007) Triple-negative breast cancer: clinical features and patterns of recurrence. Clin Cancer Res 13: 4289-4454.
5. Rocak S, Linder P (2004) DEAD-box proteins: the driving forces behind RNA metabolism. Nat Rev Mol Cell Biol 5: 232–241.
6. Lonsch JK (2002) RNA chaperones exist and DEAD box proteins get a life. Cell 108: 787–790.
7. Yedavalli VS, Neuveteu C, Chi YH, Kleiman L, Jeang KT (2004) Requirement of DDX3 DEAD box RNA helicase for HIV-1 Rev-RRE export function. Cell 119: 301–312.
8. Oosiouka AM, Patel AH (1999) Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology 257: 330–340.
9. Huang JS, Chao CC, Su TL, Yeh SH, Chen DS, et al. (2004) Diverse cellular effects FER kinase has on HIF-1α. J Biol Chem 279: 252–256.
10. Graziano M, Moras D, Maise G, Chiodoni C, Sigurgeirsson O, et al. (2011) Expression of DDX3 is directly modulated by hypoxia inducible factor-1 alpha in breast epithelial cells. PLoS ONE 6: e17563.
11. Majumdar AJ, Wong WJ, Simon MC (2010) Hypoxia-inducible factors and the response to hypoxic stress. Molecular cell 40: 294–309.
12. Bos R, Hennacher CF, Monmors EC, Semenza GL, et al. (2001) Levels of hypoxia-inducible factor-1 alpha during breast carcinogenesis. J Natl Cancer Inst 93: 309–314.
13. Bos R, Dier PJ, Groen Pvd, Greijer AE, Hermans 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-1alpha [HIF-1[alpha]]. Oncogene 22: 8948–8951.
14. Bos R, Greijer AE, Pinter MR, van der Groep P, van der Valk P, et al. (2005) Hypoxia-inducible factor-alpha is associated with angiogenesis, and expression of BCL6, PDGF-BB, and EGF and in invasive breast cancer. Histopathology 46: 306–316.
15. Greijer AE, de Jong JS, Schellff GL, Shivas A, van Diet PJ, et al. (2005) Hypoxia-induced differentiation causes mi1 exonance resistance not mediated by drug transporters in human breast cancer cells. Cellular Oncology 27: 45–50.
16. Semenza GL (2000) Hypoxia, clonal selection, and the role of HIF-1 in tumor progression. Curr Rev Biochem Mol Biol 35: 71–103.
17. Blasi F, Favia G, Tesei M, Bonomi P, et al. (2008) Cross-talk between epithelial growth factor receptor and hypoxia-inducible factor-1 alpha signal...
pathways increases resistance to apoptosis by up-regulating survivin gene expression. J Biol Chem 281: 25905-25914.

31. Laughner E, Taghavi P, Chiles K, Mahon PC, Semenza GL (2001) HER2 (neu) signaling increases the rate of hypoxia-inducible factor alpha (HIF-1alpha) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor factor expression. Mol Biol Cell 21: 3955-4003.

32. Mottet D, Dumont V, Decache Y, Demazy C, Ninane N, et al. (2003) Regulation of hypoxia-inducible factor-1alpha protein level during hypoxic conditions by the phosphatidylinositol 3-kinase/Akt/glycogen synthase kinase 3beta pathway in HepG2 cells. J Biol Chem 278: 31277-31285.

33. Brugarolas J, Kaelin WG (2004) Disregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes. Cancer Cell 6: 7-10.

34. Gort EH, Groot AJ, van de Ven TLD, van der Groep P, Verlaan I, et al. (2006) Hypoxia-inducible factor-1 alpha expression requires PI 3-kinase activity and correlates with Akt1 phosphorylation in invasive breast carcinomas. Oncogene 25: 6123-6127.

35. Blagosklonny MV, An WG, Romanova LY, Trepel J, Fojo T, et al. (1998) p53 inhibits hypoxia-inducible factor-stimulated transcription. J Biol Chem 273: 11995-11998.

36. Yamakuchi M, Lottermann CD, Rao C, Hruban RH, Karim B, et al. (2010) P53-induced microRNA-107 inhibits HIF-1 and tumor angiogenesis. Proc Natl Acad Sci U S A 107: 6334-6339.

37. Ravi R, Mookerjee B, Bhujwalla ZM, Sutter CH, Artemov D, et al. (2000) Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor alpha. Genes Dev 14: 34-44.

38. Schmid T, Zhou J, Kohl R, Brune B (2004) p300 relieves p53-evoked transcriptional repression of hypoxia-inducible factor-1 (HIF-1). Biochem J 380: 289-295.

39. Sano M, Minamino T, Toko H, Miyazaki H, Orimo M, et al. (2007) p53-induced inhibition of Hif-1 causes cardiac dysfunction during pressure overload. Nature 446: 444-448.

40. van de Sluis B, Vermeulen JF, van de Ven RAH, Ercan C, van der Groep P, van der Wall E, et al. (2008) COMMD1 disrupts HIF-1alpha/beta dimerization and inhibits human tumor cell invasion. J Clin Invest 120: 2119-2130.

41. van de Sluis B, Groot AJ, Vermeulen J, van der Wall E, van Diest PJ, et al. (2009) COMMD1 Promotes pVHL and O2-Independent Proteolysis of HIF-1alpha via HSP90/70. PLoS ONE 4: e7332.

42. Saleem Y, Shipunin S, Pased O, Pomp O, Taler M, et al. (2005) Forer kinase protein-independent mechanism. J Biol Chem 278: 30125-30135.

43. Yuan WC, Lee Y, Huang SF, Lin YM, Chen TY, et al. (2011) A Cullin3-dependent probe amplification in comparison with immunohistochemistry and in situ hybridization. Cellular oncology: the official Journal of the International Society for Cellular Oncology 31: 1-10.

44. Qiang L, Wu T, Zhang HW, Lu N, Hu R, et al. (2012) HIF-1 alpha is critical for hypoxia-mediated maintenance of glioblastoma stem cells by activating Notch signaling pathway. Cell Death and Differentiation 19: 284-294.

45. Gustafsson MV, Zheng X, Pereira T, Gradin K, Jin S, et al. (2005) Hypoxia requires notch signaling to maintain the undifferentiated cell state. Dev Cell 9: 617-628.

46. Krishnamachary B, Zaggag D, Nagasawa H, Rainey K, Okuyama H, et al. (2006) Hypoxia-inducible factor-1 dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. Cancer Res 66: 2725-2731.

47. Koshiji M, Kageyama Y, Pete EA, Horikawa I, Barrett JC, et al. (2004) HIF-1 alpha induces cell cycle arrest by functionally counteracting Myc. Embo Journal 23: 1939-1946.

48. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, et al. (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3: 347-361.

49. Hayashi M, Sakata M, Takeda T, Tafara M, Yamamoto T, et al. (2005) Up-regulation of c-met protooncogene product expression through hypoxia-inducible factor-1alpha is involved in trophoblast invasion under low-oxygen tension. Endocrinology 146: 4682-4689.

50. Horre G, Gort EH, van der Groep P, Heintz AP, Vooijs M, et al. (2006) Hypoxia-inducible factor 1 alpha is essential for hypoxic p27 induction in endometrioid endometrial carcinoma. J Pathol 214: 38-45.

51. Krishnamachary B, Zaggag D, Nagasawa H, Rainey K, Okuyama H, et al. (2006) Hypoxia-inducible factor-1 dependent repression of E-cadherin in von Hippel-Lindau tumor suppressor-null renal cell carcinoma mediated by TCF3, ZFHX1A, and ZFHX1B. Cancer Res 66: 2725-2731.

52. Moelans CB, de Jonge P, van den Broek NJ, van der Groep P, van Diest PJ, et al. (2009) HER-2/new amplification testing in breast cancer by multiplex ligation-dependent probe amplification in invasive breast cancer. J Clin Pathol 62: 11995-11998.

53. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, et al. (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3: 347-361.

54. van der Groep P, Bouter A, van der Zanden R, Menko FH, Baerger H, et al. (2004) Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 96: 712-713; author reply 714.

55. Vleugel MM, Greijer AE, Shinvart A, van der Groep P, van Berkel M, et al. (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in breast cancer. J Clin Pathol 58: 638-644.

56. van der Groep P, Bouter A, Menko FH, van der Wall E, van Diest PJ (2008) High frequency of HIF-1alpha overexpression in BRCA1 related breast cancer. Breast Cancer Res Treat 111: 475-480.

57. van der Groep P, Bouter A, van der Zanden R, Menko FH, Baerger H, et al. (2004) Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 96: 712-713; author reply 714.

58. Vermeulen JF, van de Ven RAH, Verlaan I, van der Groep P, van Diest PJ, et al. (2008) The peptidyl-isomerase Pin1 regulates p27kip expression through inhibition of Forkhead box O tumor suppressors. Cancer Res 68: 7597-7605.

59. Gort EH, Sunjerbrink JP, Roobol SM, Raman V, Vooijs M, et al. (2008) Methylation of the TWIST1 promoter, TWIST1 mRNA levels, and immunohistochemical expression of TWIST1 in breast cancer. Cancer Epidemiol Biomarkers Prev 17: 3325-3330.

60. Pennacchietti S, Michieli P, Galluzzo M, Mazzone M, Giordano S, et al. (2003) Hypoxia promotes invasive growth by transcriptional activation of the met protooncogene. Cancer Cell 3: 347-361.

61. Moelans CB, de Jonge P, van den Broek NJ, van der Groep P, van Diest PJ, et al. (2009) HER-2/new amplification testing in breast cancer by multiplex ligation-dependent probe amplification in invasive breast cancer. J Clin Pathol 62: 11995-11998.

62. van der Groep P, Bouter A, van der Zanden R, Menko FH, Baerger H, et al. (2004) Re: Germline BRCA1 mutations and a basal epithelial phenotype in breast cancer. J Natl Cancer Inst 96: 712-713; author reply 714.

63. Vleugel MM, Greijer AE, Shinvart A, van der Groep P, van Berkel M, et al. (2005) Differential prognostic impact of hypoxia induced and diffuse HIF-1alpha expression in breast cancer. J Clin Pathol 58: 638-644.

64. Moelans CB, de Jonge P, van den Broek NJ, van der Groep P, van Diest PJ, et al. (2009) HER-2/new amplification testing in breast cancer by multiplex ligation-dependent probe amplification in invasive breast cancer. J Clin Pathol 62: 11995-11998.

65. Li CI, Uribe DJ, Daling JR (2005) Clinical characteristics of different histologic types of breast cancer. PLoS ONE 7: e37864.

66. Chen J, Gomes AR, Monteiro LJ, Wong SY, Wu LH, et al. (2010) Constitutive FOXO3a localization predicts poor survival and promotes Akt phosphorylation in breast cancer. PLoS ONE 5: e12293.