A generic cycling hypoxia-derived prognostic gene signature: application to breast cancer profiling

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Keywords: hypoxia, breast cancer, biomarker, gene signature, prognosis

Received: June 09, 2014  Accepted: July 31, 2014  Published: July 31, 2014

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ABSTRACT

Background: Temporal and local fluctuations in O2 in tumors require adaptive mechanisms to support cancer cell survival and proliferation. The transcriptome associated with cycling hypoxia (CycHyp) could thus represent a prognostic biomarker of cancer progression.

Methods: We exposed 20 tumor cell lines to repeated periods of hypoxia/reoxygenation to determine a transcriptomic CycHyp signature and used clinical data sets from 2,150 breast cancer patients to estimate a prognostic Cox proportional hazard model to assess its prognostic performance.

Results: The CycHyp prognostic potential was validated in patients independently of the receptor status of the tumors. The discriminating capacity of the CycHyp signature was further increased in the ER+ HER2- patient populations including those with a node negative status under treatment (HR=3.16) or not (HR=5.54). The CycHyp prognostic signature outperformed a signature derived from continuous hypoxia and major prognostic metagenes (P<0.001). The CycHyp signature could also identify ER+HER2 node-negative breast cancer patients at high risk based on clinicopathologic criteria but who could have been spared from chemotherapy and inversely those patients classified at low risk based but who presented a negative outcome.

Conclusions: The CycHyp signature is prognostic of breast cancer and offers a unique decision making tool to complement anatomopathologic evaluation.

INTRODUCTION

Hypoxia is nowadays described as a hallmark of tumors [1, 2]. Tumor angiogenesis and glycolytic metabolism are two extensively studied responses of cancer cells to a deficit in oxygen [1]. The building of new blood vessels to bring O2 and the respiration-independent metabolism to survive under low O2 are actually complementary responses of tumors to hypoxia [1, 2]. These somehow opposite modes of adaptation account for local and temporal heterogeneities in tumor O2 distribution. The terms ‘intermittent hypoxia’ or ‘cycling hypoxia’ were settled to describe this phenomenon of fluctuating hypoxia in tumors [3, 4]. As a corollary, the extent of cycling hypoxia reflects tumor plasticity and thus measures the capacity of tumor cells to survive and proliferate in a hostile environment [3].

Although we and others have contributed to demonstrate the existence of cycles of hypoxia and/or ischemia in mouse, canine and human tumors [see [5, 6] for review], technologies aiming to routinely measure tumor O2 fluctuations in the clinic are not (yet) available despite important progresses in the in vivo imaging of hypoxia [7-11]. In the absence of readily accessible
Table 1: Gene list of the CycHyp signature

| Probe     | Entrez ID | GenBank    | Symbol     | Gene Title                                                                 |
|-----------|-----------|------------|------------|----------------------------------------------------------------------------|
| 1         | 8018860   | NM_001168  | BIRC5      | baculoviral IAP repeat containing 5                                        |
| 2         | 8064156   | NM_032527  | ZGPAT      | zinc finger, CCCH-type with G patch domain                                 |
| 3         | 8138912   | NM_012322  | LSM5†      | LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)              |
| 4         | 7921786   | NM_012394  | PFDN2      | prefoldin subunit 2                                                        |
| 5         | 8165011   | NM_002003  | FCN1       | ficolin (collagen/fibrinogen domain containing) 1                           |
| 6         | 7964262   | NM_001113201| NACA*     | nascent polypeptide-associated complex alpha subunit                       |
| 7         | 7949792   | NM_005608  | PTPRCAP*   | protein tyrosine phosphatase, receptor type, C-associated protein          |
| 8         | 8034101   | NM_006858  | TMED1      | transmembrane emp24 protein transport domain containing 1                  |
| 9         | 8168087   | NM_015551  | IGBP1      | immunoglobulin (CD79A) binding protein 1                                   |
| 10        | 7963575   | NM_001417  | EIF4B§     | eukaryotic translation initiation factor 4B                                |
| 11        | 8124397   | NM_005319  | HIST1H1C*  | histone cluster 1, H1c                                                     |
| 12        | 7975989   | NM_031210  | SLIRP§     | SRA stem-loop interacting RNA binding protein                              |
| 13        | 8127692   | NM_008863  | HTR1B      | 5-hydroxytryptamine (serotonin) receptor 1B                                 |
| 14        | 8127087   | NM_008847  | GSTA3      | glutathione S-transferase alpha 3                                          |
| 15        | 7941122   | NM_013299  | SAC3D1     | SAC3 domain containing 1                                                    |
| 16        | 7998692   | NM_002528  | NTHL1      | nth endonuclease III-like 1 (E. coli)                                      |
| 17        | 8073623   | NM_00144370| MPPED1     | metallophosphoesterase domain containing 1                                 |
| 18        | 8014865   | NM_006160  | NEUROD2*   | neurogenic differentiation 2                                               |
| 19        | 8005726   | NM_021012  | KCNJ12     | potassium inwardly-rectifying channel, subfamily J, member 12              |
| 20        | 7966631   | NM_022363  | LHX5*      | LIM homeobox 5                                                             |
| 21        | 8037853   | NM_017854  | TMEM160    | transmembrane protein 160                                                  |
| 22        | 8104136   | 3166       | NM_018942  | HMX1*                                                                   |
| 23        | 7948606   | 746        | NM_014206  | C11orf10*                                                                |
| 24        | 8044773   | 8685       | NM_006770  | MARCO                                                                     |
| 25        | 7947015   | 7251       | NM_006292  | tumor susceptibility gene 101                                              |
| 26        | 7931553   | 8433       | NM_003577  | undifferentiated embryonic cell transcription factor 1                      |
| 27        | 7956876   | 84298      | LLPH       | LLP homolog, long-term synaptic facilitation (Aplysia)                     |
| 28        | 8117372   | 8334       | NM_03512   | HIST1H2AC*                                                                |
| 29        | 8001329   | 869        | NM_004352  | CBLN1                                                                     |
| 30        | 8027205   | 51079      | NM_015965  | NDUFA13                                                                    |
| 31        | 8042896   | 3196       | NM_016170  | T-cell leukemia homeobox 2                                                 |
| 32        | 7911532   | 54998      | NM_017900  | aurora kinase A interacting protein 1                                      |
| 33        | 8039923   | 54998      | NM_017900  | aurora kinase A interacting protein 1                                      |
| 34        | 7992043   | 65990      | BC001181   | FAM173A                                                                   |
| 35        | 8063074   | 90204      | NM_080603  | ZSWIM1*                                                                   |
| 36        | 7992191   | 23430      | NM_012217  | TPSD1                                                                     |
| 37        | 8108435   | 7322       | NM_181838  | UBE2D2                                                                    |
| 38        | 8165309   | 8721       | NM_003792  | endothelial differentiation-related factor 1                               |
| 39        | 7946267   | 63875      | NM_022061  | mitochondrial ribosomal protein L1                                        |
| 40        | 7945536   | 51286      | NM_016564  | CEND1                                                                     |
| 41        | 8159609   | 8636       | NM_003731  | Sjogren syndrome nuclear autoantigen 1                                    |
| 42        | 8005471   | 6234       | NM_001031  | ribosomal protein S28                                                     |
| 43        | 8025395   | 6234       | NM_001031  | ribosomal protein S28                                                     |
| Gene ID   | Ensembl ID | Symbol   | Description                                |
|-----------|------------|----------|--------------------------------------------|
| 1         | 87942824   | NM_001031 | RPS28                                      |
| 2         | 8170753    | NM_014370 | SRPK3                                      |
| 3         | 8032718    | NM_001348 | ribosomal protein S28                     |
| 4         | 7967067    | NM_001037495 | cofactor of BRCA1                       |
| 5         | 8159654    | NM_015456 | COBRA1 *                                   |
| 6         | 8001121    | NM_003001 | SDHC                                       |
| 7         | 8011968    | NM_016060 | MED31 *                                    |
| 8         | 7977440    | NR_026800 | KIAA0125                                   |
| 9         | 8016508    | NM_002471 | SNF8 *                                     |
| 10        | 8168567    | NM_0003007 | POU3F4 *                                   |
| 11        | 8086317    | NM_031899 | GORASP1                                    |
| 12        | 8052834    | BC005079  | C2orf42                                    |
| 13        | 8073334    | NM_014248 | RBX1 *                                     |
| 14        | 7915846    | NM_003684 | MKNK1                                      |
| 15        | 8071920    | NM_004175 | SNRPD3 §                                   |
| 16        | 8032371    | NM_031213 | FAM108A1 family with sequence similarity 108, member A1 |
| 17        | 7924884    | NM_003493 | HIST3H3                                    |
| 18        | 8006845    | NM_009891 | RPL19 §                                    |
| 19        | 7946812    | NM_001017 | RPS13 §                                   |
| 20        | 7949015    | NM_001144936 | chromosome 11 open reading frame 95 |
| 21        | 8009784    | NM_015971 | MRPS7 §                                   |
| 22        | 8174509    | NM_005274 | GNG5                                       |
| 23        | 7906235    | NM_005973 | PRCC §                                     |
| 24        | 8020179    | NM_020412 | CHMP1B                                     |
| 25        | 7947450    | NM_005574 | LMO2                                       |
| 26        | 8064370    | NM_004609 | TCF15 §                                    |
| 27        | 7955896    | NM_016057 | COPZ1                                      |
| 28        | 8137805    | NM_003550 | MAD1L1 §                                   |
| 29        | 8117334    | NM_003538 | HIST1H4A §                                 |
| 30        | 8117368    | NM_003542 | HIST1H4C §                                 |
| 31        | 7977507    | NM_002312 | RPPH1 §                                    |
| 32        | 7949410    | BC018448  | MALAT1                                     |
| 33        | 8150433    | NM_152568 | NKX6-3 §                                   |
| 34        | 8071168    | NM_024583 | POM121L8P                                  |
| 35        | 7989611    | NM_032231 | FAM96A                                     |
| 36        | 7980859    | NM_001080113 | transmembrane and immunoglobulin domain containing 2 |
| 37        | 8032782    | NM_144615 | TMIGD2                                     |
| 38        | 8110861    | NM_032479 | MRPL36 §                                   |
| 39        | 7901687    | NM_182532 | TMEM61                                     |
| 40        | 7916130    | NM_138417 | KTI12                                      |
| 41        | 8048712    | NM_001080113 | transmembrane and immunoglobulin domain containing 2 |

* indicates a gene with known function in the context of the disease or condition, § indicates a gene with a role in the disease or condition, # indicates a gene with a role in the disease or condition and has been studied in a specific organism.
Table 2: Gene list of the ContHyp signature

| Probe   | Entrez ID | GenBank     | Symbol             | Gene Title                                                                 |
|---------|-----------|-------------|--------------------|----------------------------------------------------------------------------|
| 85      | 8018993   | 146713      | NM_001082575       | RBFOX3 § RNA binding protein, fox-1 homolog (C. elegans) 3                 |
| 86      | 8032601   | 84839       | NM_032753          | RAX2 retina and anterior neural fold homeobox 2                            |
| 87      | 8010719   | 201255      | NM_144999          | LRRC45 leucine rich repeat containing 45                                   |
| 88      | 8036584   | 3963        | NM_002307          | LGALS7 lectin, galactaside-binding, soluble, 7                             |
| 89      | 8133209   | 441251      | NR_003666          | SPDYE7P speedy homolog E7 (Xenopus laevis), pseudogene                     |
| 90      | 815901    | 286256      | NM_175836          | LCN12 lipocalin 12                                                         |
| 91      | 8028546   | 3963        | NM_002307          | LGALS7 lectin, galactaside-binding, soluble, 7                             |
| 92      | 8065013   | ENST00000427835 |                  |                                                                            |
| 93      | 8018502   | 201292      | NM_173547          | TRIM65 * tripartite motif containing 65                                    |
| 94      | 7903294   | 64645       | NM_033055          | HIAT1 hippocampus abundant transcript 1                                    |
| 95      | 7989473   | 388125      | NM_001007595       | C2CD4B C2 calcium-dependent domain containing 4B                          |
| 96      | 8054449   | 644903      | AK095987           | FLJ38668 hypothetical LOC644903                                            |
| 97      | 8081867   | 51300       | NM_016589          | TIMMDC1 translocase of inner mitochondrial membrane domain containing 1    |
| 98      | 7934544   | 118881      | NM_144589          | COMTD1 catechol-O-methyltransferase domain containing 1                    |
| 99      | 7968260   | 219409      | NM_145657          | GSX1 * GS homeobox 1                                                       |
| 100     | 8022952   | 56853       | NM_020180          | CELF4 § CUGBP, Elav-like family member 4                                   |

* common to the ContHyp signature; † regulators of transcription; § involved in RNA processing
|     | Gene ID   | Gene Name                          | Description                                                                                           |
|-----|-----------|------------------------------------|-------------------------------------------------------------------------------------------------------|
| 25  | 7955117   | NM_012404 ANP32D                   | acidic (leucine-rich) nuclear phosphoprotein 32 family, member D                                      |
| 26  | 8098604   | NM_181726 ANKR37                   | ankyrin repeat domain 37                                                                             |
| 27  | 8121076   | NM_006813 PNRC1                    | proline-rich nuclear receptor coactivator 1                                                           |
| 28  | 7921076   | NM_182679 GPATCH4                  | G patch domain containing 4                                                                            |
| 29  | 7908879   | NM_015053 PPFIA4                   | protein tyrosine phosphatase, receptor type, f polypeptide (PTPRF), interacting protein (liprin), alpha 4 |
| 30  | 8103518   | NM_012403 ANP32C                   | acidic (leucine-rich) nuclear phosphoprotein 32 family, member C                                      |
| 31  | 8050591   | NM_174889 NDUFAF2                  | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, assembly factor 2                                  |
| 32  | 8172154   | NM_002952 RPS2                     | ribosomal protein S2                                                                                   |
| 33  | 794846    | NM_001130028 CLK3                  | CDC-like kinase 3                                                                                      |
| 34  | 7946812   | NM_001017 RPS13                    | ribosomal protein S13                                                                                  |
| 35  | 7982531   | NM_006305 ANP32A                   | acidic (leucine-rich) nuclear phosphoprotein 32 family, member A                                      |
| 36  | 8119898   | NM_001025366 VEGFA                 | vascular endothelial growth factor A                                                                  |
| 37  | 8004331   | NM_014716 ACAP1                    | ArfGAP with coiled-coil, ankyrin repeat and PH domains 1                                              |
| 38  | 8159441   | NM_001135861 PHPT1                 | phosphohistidine phosphatase 1                                                                        |
| 39  | 8168500   | NM_000291 PGK1                     | phosphoglycerate kinase 1                                                                             |
| 40  | 793890    | NM_005788 PRMT3                    | protein arginine methyltransferase 3                                                                  |
| 41  | 7930398   | NM_005962 MXII                     | MAX interactor 1                                                                                       |
| 42  | 7997740   | NM_022818 MAP1LC3B                 | microtubule-associated protein 1 light chain 3 beta                                                   |
| 43  | 8004360   | NM_001002914 KCTD11                | potassium channel tetramerisation domain containing 1                                                |
| 44  | 7909782   | NM_016052 RRPI5                    | ribosomal RNA processing 15 homolog (S. cerevisiae)                                                   |
| 45  | 7949792   | NM_005608 PTPRCAp                  | protein tyrosine phosphatase, receptor type, C-associated protein                                      |
| 46  | 8124385   | NM_003544 H3T1H4B                  | histone cluster 1, H4b                                                                                 |
| 47  | 8117368   | NM_003542 H3T1H4C                  | histone cluster 1, H4c                                                                                 |
| 48  | 8081241   | NM_032359 C3orf26                  | chromosome 3 open reading frame 26                                                                     |
| 49  | 8050079   | NM_002936 RNASEH1                  | ribonuclease H1                                                                                        |
| 50  | 8005765   | NM_015626 WSB1                     | WD repeat and SOCS box containing 1                                                                    |
| 51  | 7924491   | NM_022831 AIDA                     | axin interactor, dorsalization associated                                                               |
| 52  | 8133273   | ENST00000455206                   |                                                                                                        |
| 53  | 8124391   | NM_003513 H3T1H2AB                 | histone cluster 1, H2ab                                                                               |
| 54  | 8159609   | NM_003731 SSNA1                    | Sjogren syndrome nuclear autoantigen 1                                                                |
| 55  | 7957890   | NM_014503 UTP20                    | UTP20, small subunit (SSU) processome component, homolog (yeast)                                      |
| 56  | 7933582   | NM_006327 TIMM23                   | translocase of inner mitochondrial membrane 23 homolog (yeast)                                        |
| 57  | 8153002   | NM_001135242 NDRG1                 | N-myc downstream regulated 1                                                                           |
| 58  | 7926037   | NM_004566 PFKFB3                   | 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3                                                 |
| 59  | 8082066   | NM_014367 FAM162A                  | family with sequence similarity 162, member A                                                         |
| 60  | 8042962   | NM_014763 MRPL19                   | mitochondrial ribosomal protein L19                                                                   |
| 61  | 809078    | NM_007208 MRPL3                    | mitochondrial ribosomal protein L3                                                                     |
| 62  | 7977507   | NR_002312 RPPH1                    | ribonuclease P RNA component H1                                                                       |
| 63  | 8007397   | NM_176863 PSME3                    | proteasome (prosome, macropain) activator subunit 3 (PA28 gamma/ Ki)                                 |
| 64  | 7998902   | NM_017885 HCFC1R1                  | host cell factor C1 regulator 1 (XPO1 dependent)                                                      |
|   |   |   |   |   |   |
|---|---|---|---|---|---|
| 65 | 8117372 | 8334 | NM_003512 | HIST1H2AC | histone cluster 1, H2ac |
| 66 | 7997230 | 5713 | NM_002811 | PSMD7 | proteosome (prosome, macropain) 26S subunit, non-ATPase, 7 |
| 67 | 7915485 | 10969 | NM_006824 | EBNA1BP2 | EBNA1 binding protein 2 |
| 68 | 8113873 | 3094 | NM_005340 | HINT1 | histidine triad nucleotide binding protein 1 |
| 69 | 7958152 | 5223 | NM_002629 | PGAM1 | phosphoglycerate mutase 1 (brain) |
| 70 | 7947867 | 5702 | NM_002804 | PSMC3 | proteosome (prosome, macropain) 26S subunit, ATPase, 3 |
| 71 | 7964460 | 1649 | NM_004083 | DDIT3 | DNA-damage-inducible transcript 3 |
| 72 | 7928395 | 170384 | NM_173540 | FUT11 | fucosyltransferase 11 (alpha 1,3 fucosyltransferase) |
| 73 | 8163629 | 944 | NM_001244 | TNFSF8 | tumor necrosis factor (ligand) superfamily, member 8 |
| 74 | 7965486 | 51134 | NM_016122 | CCDC41 | coiled-coil domain containing 41 |
| 75 | 8136179 | 23008 | AF277175 | KLHDC10 | kelch domain containing 10 |
| 76 | 8095870 | 901 | NM_004354 | CCNG2 | cyclin G2 |
| 77 | 8127526 | 6170 | NM_001000 | RPL39 | ribosomal protein L39 |
| 78 | 8174710 | 6170 | NM_001000 | RPL39 | ribosomal protein L39 |
| 79 | 8137517 | 3361 | NM_024012 | HTR5A | 5-hydroxytryptamine (serotonin) receptor 5A |
| 80 | 7929624 | 5223 | NM_002629 | PGAM1 | phosphoglycerate mutase 1 (brain) |
| 81 | 8052331 | 87178 | NM_033109 | PNPT1 | polyribonucleotide nucleotidyltransferase 1 |
| 82 | 8015969 | 7343 | NM_014233 | UBTF | upstream binding transcription factor, RNA polymerase I |
| 83 | 8069168 | 386685 | NM_198699 | KRTAP10-12 | keratin associated protein 10-12 |
| 84 | 7941087 | 5526 | NM_006244 | PPP2R5B | protein phosphatase 2, regulatory subunit B', beta |
| 85 | 8026875 | 26780 | NR_000012 | SNORA68 | small nucleolar RNA, H/ACA box 68 |
| 86 | 8027621 | 2821 | NM_000175 | GPI | glucose-6-phosphate isomerase |
| 87 | 8130539 | 117289 | NM_054114 | TAGAP | T-cell activation RhoGTPase activating protein |
| 88 | 8004691 | 92162 | NM_203411 | TMEM88 | transmembrane protein 88 |
| 89 | 7962183 | 205 | NM_001005353 | AK4 | adenylylate kinase 4 |
| 90 | 8137805 | 8379 | NM_003550 | MAD1L1 | MAD1 mitotic arrest deficient-like 1 (yeast) |
| 91 | 8124388 | 8358 | NM_003537 | HIST1H3B | histone cluster 1, H3b |
| 92 | 8083223 | 205428 | NM_173552 | C3orf58 | chromosome 3 open reading frame 58 |
| 93 | 8113305 | 1105 | NM_001270 | CHD1 | chromodomain helicase DNA binding protein 1 |
| 94 | 8169659 | 4694 | NM_004541 | NDUFA1 | NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1, 7.5kDa |
| 95 | 8046408 | 5163 | NM_002610 | PDK1 | pyruvate dehydrogenase kinase, isozyme 1 |
| 96 | 8053599 | 23559 | NM_012477 | WBP1 | WW domain binding protein 1 |
| 97 | 8043377 | 23559 | NM_012477 | WBP1 | WW domain binding protein 1 |
| 98 | 7960878 | 642559 | GU480887 | POU5F1P3 | POU class 5 homeobox 1 pseudogene 3 |
| 99 | 7959023 | 643246 | NM_001085481 | MAP1LC3B2 | microtubule-associated protein 1 light chain 3 beta 2 |
| 100 | 8073148 | 468 | NM_001675 | ATF4 | activating transcription factor 4 (tax-responsive enhancer element B67) |
monitoring strategies, the analysis of the transcriptome associated with this phenomenon could represent a prognostic biomarker of cancer progression. Indeed, although mutations and defects in tumor suppressor genes directly influence the whole genetic profile of a given tumor cell clone, cycling hypoxia could be envisioned as a supra-oncogenic phenomenon influencing gene expression [3]. In other words, independently of the genetic background of tumor cells, cycling hypoxia has the potential to lead to common alterations in the expression of some transcripts, and thus to a possible clinically exploitable signature.

Clinical data sets derived from breast cancer patients could be used to evaluate the performance of such cycling hypoxia-related gene signature. The clinical and genetic heterogeneities of this disease and the very large panel of data sets available represent indeed good opportunities to evaluate new prognostic gene expression signatures [12]. Whole genome analysis already provided several molecular classifications for breast cancer beyond standard clinicopathologic variables [12-21]. The latter include tumor size, presence of lymph node metastasis and histological grades [22] but also encompass three predictive markers of response, namely expression of oestrogen (ER), progesterone (PR) and HER2 receptors [12]. Treatment guidelines are nowadays still largely based on algorithms integrating these informations such as the Nottingham Prognostic Index [22, 23] or Adjuvant! Online [24]. Accordingly, for early-stage breast cancer, adjuvant chemotherapy is recommended for most patients with ER-negative or HER2-positive tumors [13, 25-27]. The challenge actually resides in selecting patients with

| Table 3: Breast Cancer Patient Demographics and Characteristics |
|---------------------------------------------------------------|
|                                                                 |
|                                                                 |
| Age                                                                 |
|                                                                 |
| ≤50                                                                 |
| 649 30 388 27 218 24 190 32 |
| >50                                                                 |
| 945 44 649 45 367 41 237 40 |
| NA                                                                   |
| 556 26 415 28 314 35 163 28 |
|                                                                 |
| Tumor size                                                          |
|                                                                 |
| ≤2cm                                                                |
| 742 35 537 37 474 53 424 72 |
| >2cm                                                                |
| 473 22 326 22 210 23 158 28 |
| NA                                                                   |
| 935 43 589 41 215 24 8 1   |
|                                                                 |
| Grade                                                               |
|                                                                 |
| 0-1                                                                 |
| 224 10 200 14 148 17 104 18 |
| 2                                                                    |
| 605 28 485 33 346 38 270 46 |
| 3                                                                    |
| 487 23 206 14 162 18 137 23 |
| NA                                                                   |
| 834 39 561 39 243 27 79 13 |
|                                                                 |
| Node status                                                          |
|                                                                 |
| Negative                                                            |
| 1329 62 899 62 899 100 590 100 |
| Positive                                                            |
| 821 38 553 38 0 0 0 0                                              |
| Estrogen receptor                                                    |
|                                                                 |
| Negative                                                            |
| 443 21 0 0 0 0 0 0                                                 |
| Positive                                                            |
| 1607 75 1452 100 899 100 590 100                                   |
| NA                                                                  |
| 100 4 0 0 0 0 0 0                                                 |
| HER2 status                                                          |
|                                                                 |
| Negative                                                            |
| 1835 85 1452 100 899 100 590 100                                   |
| Positive                                                            |
| 315 15 0 0 0 0 0 0                                                |
| Treatment                                                            |
|                                                                 |
| None                                                                |
| 901 42 590 41 590 66 590 100                                      |
| Chemotherapy                                                        |
| 691 32 410 28 73 8 0 0                                            |
| Hormonotherapy                                                      |
| 558 26 452 31 236 26 0 0                                          |

Data obtained from GSE11121 (n=200), GSE17705 (n=298), GSE2034/5327 (n=344), GSE20685 (n=327), GSE21653 (n=253), GSE2990 (n=138), GSE3494 (n=178), GSE6532 (n=214), and GSE7390 (n=198). NA = Not Available.
ER-positive HER2-negative disease who could benefit from chemotherapy.

In this study, we derived a transcriptomic signature of cycling hypoxia (CycHyp) using 20 cell lines derived from various human tumors and characterized by a large variety of distinct genetic anomalies. We then validated the capacity of the CycHyp signature to optimize patient stratification. In particular, we showed how the CycHyp signature could identify ER-positive node-negative breast cancer patients at high risk based on conventional NPI (and who could have been spared from chemotherapy) and inversely those patients classified at low risk but who could have drawn benefits of chemotherapy.

RESULTS

Identification of the CycHyp signature

Tumor cells covering a large diversity of tissues (Suppl. Table 1) were submitted to cycling hypoxia (CycHyp) for 24 hours, maintained under normoxic conditions or exposed to continuous hypoxia (ContHyp) for the same period of time (Figure 1A). Corresponding mRNA samples were analysed by hybridization using Human Gene 1.0 ST Affymetrix microarrays. Gene expression profiles of each cell type under normoxia vs. cycling hypoxia (CycHyp) were produced to identify the most differentially expressed probesets. The CycHyp signature was determined as the top 100 probesets with the lowest FDR-corrected p-values averaged over 200 resamplings (Table 1); a ContHyp signature was also determined in parallel (Table 2). The heatmaps made with the 100 probe sets of the CycHyp signature confirmed its excellent potential of discrimination between cycling hypoxia and either normoxia (Figure 1B) or continuous hypoxia (Figure 1C). Moreover, Gene Set Enrichment Analysis (GSEA) [28] indicated that when considering differentially expressed probesets (after FDR correction), only 2 gene sets were significantly enriched in the CycHyp signature (Suppl. Table 2) whereas we identified 52 gene sets enriched in the ContHyp signature, including 17 directly related to hypoxia (Suppl. Table 3). Also, when using the MSigDB molecular signature database referring to hypoxia or HIF (www.broadinstitute.org), we found 13 hypoxia gene sets sharing, on average, only 1.4 gene with CycHyp (Suppl. Table 4) whereas 44 hypoxia gene sets showed overlap with ContHyp with an average of 6.6 (1-27) common genes (Suppl. Table 5). We also compared the CycHyp signature to 13 other hypoxia-derived signatures described by Seigneuric et al. [29] and Starmans et al. [30]. The CycHyp signature was again far from those signatures with an average of only 1 gene in common. The overlap was larger between ContHyp and those signatures with an average of 6 genes in common (Suppl. Table 6).

Finally, using TFactS [31] to analyse transcription factors regulating expression of genes associated to either signature, HIF-1α was only found as positively associated with the ContHyp signature.

The CycHyp signature predicts clinical outcome in breast cancer patients

To evaluate the prognostic value of the CycHyp signature, we focused on breast cancer because of the very large amounts of well-annotated clinical data sets available and a clearly identified need to discriminate between patients at low and high risks among subgroups determined on the basis of clinicopathologic criteria [12, 13]. Publicly available GEO data sets allowed us to collect information on the survival of 2,150 patients with primary breast cancer (see clinical features in Table 3).

In order to exploit these data sets, we first transferred the Gene 1.0ST datasets in the HU133 platform. We then used the VDX dataset (GSE2034 and GSE5327) as a reference because of its large number of node negative untreated patients [17]. This training dataset was used to estimate a prognostic multivariate Cox proportional hazard model built on the CycHyp signature (see Methods for details). The other eight datasets (see references in Table 3) were used according to the methodology described by Haibe-Kains and colleagues [32], to assess the prognostic performance of the CycHyp signature on independent samples. We first chose to evaluate our signature independently of the clinicopathological data. The prognostic potential of the CycHyp signature to discriminate between patients at low or high risk was confirmed with a HR = 2.39 and a p-value = 1.13e-18 whatever the treatment and the tumor histology (Figure 2A). We then focused on the ER+ HER2- population which is known to be heterogeneous and thus difficult to treat [12, 13]. The discriminating capacity of the CycHyp signature remained strikingly high in the ER+ HER2- patient populations (HR = 2.47, p-value = 3.88e-13, Figure 2B). Finally, among this subpopulation of patients, we considered those with a node negative status (Figure 2C) and among the latter, those who did not receive any treatment (Figure 2D). Hazard ratios rose to 3.16 and 5.54 in these conditions (p-values = 2.85e-9 and 6.44e-10, respectively), further supporting the discriminating potential of the CycHyp signature. In particular, the data presented in Figure 2D allowed to exclude any confounding influence of the potential benefit arising from the treatment administered to these patients and thus clearly identified a population of patients who remained inadequately untreated.

Using the same methodology, we examined the prognostic capacity of the ContHyp signature (discriminating between normoxia and continuous hypoxia). The performance of the ContHyp signature
Figure 1: The CycHyp and ContHyp signatures. (A.) Flowchart of the signature determination from tumor cells exposed either to normoxia, cycling or continuous hypoxia. (B.) Heatmap depicting the transcripts from the CycHyp signature either underexpressed (green) or overexpressed (red) (centered to median values). Each column corresponds to a specific human Gene 1.0 ST probeset; each line represents a specific cell line either maintained under normoxia (black label) or exposed to cycling hypoxia (red label); cells under normoxia and cycling hypoxia are perfectly separated in two distinct clusters, except for one cycling hypoxia sample in the normoxia cluster. (C.) Similarly, a heatmap depicting the relative expression of transcripts from the CycHyp signature in the cell lines maintained under continuous hypoxia (blue) or cycling hypoxia (red); only two cycling hypoxia samples are grouped with the continuous hypoxia samples.
was satisfactory on the ER+ HER2- untreated population (HR = 2.58, p-value = 1.46e-4, see Supplementary Fig. 1) but was significantly lower (p-value = 3.61e-8) than the CycHyp signature.

The CycHyp signature provides significant additional prognostic information to available multigene assays

To evaluate the performance of the CycHyp signature, we compared it with other well-established prognostic multigene assays for breast cancer, namely Gene70 or Mammaprint [14], Gene76 [17] and Oncotype Dx [15]. Using the same set of ER+ HER2- node negative patients as used in Figure 2D, we could determine the low vs. high risk patient stratification according to these signatures. The superior prognostic potential of the CycHyp signature could be captured from the Kaplan Meier curves obtained with the Gene 70, Gene76 and Oncotype DX signatures (compare Figure 3A with Figure 2D). Hazard ratios confirmed the net advantage of the CycHyp signature with a significantly higher value than the three other metagenes (Figure 3B). The concordance index, which is the probability of a high risk patient to relapse before a low risk patient, was also higher with the CycHyp signature (Figure 3B). Finally, the Balanced Classification Rate (BCR), which represents the average between sensitivity and specificity to discriminate between patients with progressing disease vs. disease-free at 5 years, was significantly higher for the CycHyp signature than the three other multigene assays (Figure 3B). The sensitivity of the CycHyp was above 80% and the specificity of the CycHyp signature was well above the level of the others (Figure 3B). Of note, the metrics corresponding to each data set taken separately is depicted in Suppl. Figure 2.

Importantly, to further validate the prognostic significance of the CycHyp signature, a comparison with random gene signatures was performed according to the methodology described by Venet et al. [33] and Beck et al. [34]. Figure 3C shows the distribution of the p-values (logrank test in log 10) for 1000 randomly generated signatures together with the p-values of the CycHyp and ContHyp signatures. The logrank test (or Mantel-Haenszel test) [35] is commonly used to assess whether there is a significant survival difference between risk groups. The discrimination between risk groups was significantly higher (P < 0.001) with the CycHyp signature as compared to each of the random signatures whereas

Figure 2: Kaplan-Meier survival curves of patients with primary breast cancer, as determined by using the CycHyp signature. (A) All patients. (B.) ER+/HER2- patients, (C.) node-negative ER+/HER2-, (D.) node-negative, untreated ER+/HER2- patients (DFS Mantel-Cox comparison); hazard ratio (HR), balanced classification rate (BCR) and concordance index (C-index) for the prediction in high risk vs. low risk groups are reported; HRs are presented with their associated p-values.
the ContHyp signature was not significantly better (vs. random ones; P=0.141). The same analysis was carried out for the three other metrics (HR, CI and BCR) to assess the discrimination capability between risk groups and confirmed the significantly higher value of the CycHyp signature (vs. random signatures) (Suppl. Figure 3).

The CycHyp signature in association with NPI offers a powerful prognostic tool

We then aimed to determine whether the CycHyp signature could improve the Nottingham Prognostic Index (NPI) for better predicting the survival of operable breast cancers. The NPI algorithm combines nodal status, tumour size and histological grade and allows to model a continuum of clinical aggressiveness with 3 subsets of patients divided into good, moderate, and poor prognostic groups with 15-year survival [22, 23, 36]. Since few patients were assigned a poor index, we merged here the moderate and poor indices into a high risk group to facilitate the comparison with the CycHyp signature.

We found that by integrating the CycHyp signature, an important proportion of patients could be reclassified to another risk group (Figure 4). 44.1% of patients classified at high risk using the NPI algorithm were identified at low

Figure 3: Comparison of the prognostic potential of the CycHyp signature vs. Gene 70 (Mammaprint), Gene 76 and Oncotype Dx signatures. (A) Kaplan-Meier survival curves of node-negative, untreated ER+/HER2- patients, as determined by using the indicated signature (DFS Mantel-Cox comparison); hazard ratio (HR), balanced classification rate (BCR) and C-index for the prediction in high risk vs. low risk groups are reported; HR are presented with their associated p-values. (B.) Forest plots of the hazard ratio (HR), Concordance index (CI), balance classification rate (BCR), sensitivity and specificity for the prediction in high risk vs. low risk groups; p-values refer to the comparisons of CycHyp vs. Gene 70 (Mammaprint), Gene 76 and Oncotype Dx. (C.) Graph represents the power of discrimination in high vs. low risk groups (expressed as the logarithm of the p-values of the logrank) of the ContHyp and CycHyp signatures (see red dots) versus 1,000 randomly generated signatures (yellow shapes depicting their distribution).
risk when using the CycHyp signature and were confirmed
to be “false positive” since they actually exhibited a profile
of survival closer to the low risk NPI patient (Figure 4A).
Inversely, using the CycHyp signature, we also identified
in the patients at low risk based on the NPI criteria,
33.1% of patients with a risk profile closer to the patients
with a negative outcome (Figure 4B). This increased
discriminating potential remained highly relevant when
considering all patients or patients with a ER+ HER2-
status (and among the latter, those with a node negative
status or the untreated ones) (see Suppl. Figure 4).

DISCUSSION

This study demonstrates that a gene signature
derived from the transcriptomic adaptation of tumor cells
to cycling hypoxia is prognostic of breast cancer. The
CycHyp signature that we have identified and validated
in this study has not only prognostic value independently
of molecular risk factors but also provides significant
additional prognostic information to clinicopathologic
criteria. Clinical outcome of breast cancer patients is
nowadays largely based on histological grade and the
status of ER, PR, and HER2 receptors [12, 13, 22]. In
eyearly breast cancer, a lack of expression of ER (and PR)
will almost systematically lead to the administration
of adjuvant chemotherapy in addition to locoregional
treatment [12, 25, 26]. Also, for patients with a tumor
expressing HER2, chemotherapy and/or trastuzumab
represents the option the most likely to be beneficial
based on current clinical knowledge [12]. The impact
of chemotherapy is actually more difficult to anticipate
for the rest of early-stage breast cancer patients, i.e.
those diagnosed with a ER-positive and HER2-negative
disease. These patients represent indeed a wide spectrum
different risk profiles: for women with high-risk
disease, if chemotherapy is appropriate, others will derive
little benefit from it. Our study therefore represents a
significant advance for this population of patients, which
consists of two third of all breast cancers. We have indeed
demonstrated that the CycHyp signature outperforms the
existing major prognostic gene expression signatures
and offers a unique decision making tool to complement
the discrimination of breast cancer patients based on
anatomopathologic evaluation.

More generally, the excellent prognostic value
of CycHyp confirms the link between cycling hypoxia
and cancer aggressiveness [4, 5]. This gives credentials
to the phenotypic adaptation of tumors resulting from
heterogeneities in blood flow distribution as a trigger of
cancer progression [3, 4]. Also, with the recent impetus
in the understanding of tumor metabolism [37, 38], it has
become obvious that the capacity of a given tumor cell
to survive in both aerobic and anaerobic environments
represents a critical advantage [39-41]. Interestingly, our
study also documents the higher prognostic value of a
transcriptomic signature derived from cycling hypoxia
vs. continuous hypoxia. This confirms that although
hypoxia is a frequent feature of poor-prognosis tumors
and was reported to drive gene signature associated with
negative outcome [42-45], prognostic markers integrating
fluctuations in the hypoxic status of tumors (this study)
introduce an additional layer of complexity that better fits
the in vivo situation.

Whether the CycHyp signature encompasses genes
that actively drive cancer progression or reflects a context
of metabolic and hypoxic stress favorable to increased
mutagenesis and genetic instability [3], warrants further
studies. A few hints can however be gleaned from the
comparison of the different signatures.

First, the comparison of the CycHyp and ContHyp
signatures indicates that the cycling nature of hypoxia
leads to specific alterations in mRNA expression since
only 11 common transcripts were found in the two gene
lists (see symbols # in Table 1). Furthermore, among
these 11 genes, most encode for proteins involved in
housekeeping functions such as chromatin packaging
(HIST1H1C, 2AC, 4A and 4C) and RNA processing
(RPS13 and 28). The only gene common to the two
signatures with a known function related to hypoxia
is RBX1 or E3 ubiquitin ligase which mediates the

Figure 4: Kaplan-Meier survival curves of node-
negative, untreated ER+/HER2- patients stratified
by using the CycHyp signature to detect. (A.) false
positive patients among those identified at high risk based
on the NPI nomenclature and (B.) false negative patients
among those identified at low risk based on the NPI nomenclature (DFS Mantel-Cox comparison).
ubiquitination and subsequent proteasomal degradation of target proteins [46], including the misfolded proteins known to accumulate under low pO₂. Besides the RBX1 gene, the CycHyp signature does not actually contain genes known to be consistently regulated in response to chronic hypoxia. By contrast, the ContHyp signature contains 14 genes already reported to be overexpressed under low pO₂ and even directly under the control of the transcription factor HIF-1α, including those coding for glucose metabolism enzymes (ALDOA, PFKB3, PFKB4, PGK1, PGAM1, GPI) and the angiogenic growth factor VEGFA. This HIF-dependent gene expression program of the ContHyp signature was actually confirmed in the GSEA and MSigBD analyses and was consistent with previously reported hypoxia-driven gene signatures [42, 44, 45]. More generally, these findings position the CycHyp signature far from the conventional hypoxia-derived signatures [29, 30] but instead as a biomarker of a distinct tumor biology process involving adaptation to fluctuations in the tumor microenvironment.

Second, a large amount of transcripts of the CycHyp signature encode for proteins themselves involved in the regulation of transcription. Data mining revealed that more than 18 transcripts of the CycHyp signature are transcription factors/regulators and 13 others are directly involved in RNA processing (see symbols * and § in Table 1, respectively). This represents one third of the genes comprising the CycHyp signature and reflects a major difference with the ContHyp signature. While hypoxia is usually associated with cell cycle arrest and mTOR inhibition, cycling hypoxia may be compatible with a maintained proliferation potential. This is further supported by the suppression of geroconversion (i.e., the process leading from proliferative arrest to irreversible senescence) observed in response to hypoxia [47, 48] that offers tumor cells the opportunity to re-enter cell cycle when O₂ is again available. Further studies are needed to compare the evolution of mTOR activity and mTOR-dependent genes (including those encoding for ribosomal proteins) during cycling and continuous hypoxia.

Finally, the in vitro conditions at the origin of the establishment of the CycHyp signature may actually have specific bearing on its robustness and applicability. Indeed, we previously documented that fluctuating oxygen levels could also directly impact endothelial cells within a tumor [49, 50] indicating that non-tumor cells may also contribute to the same transcriptomic adaptation as tumor cells, thereby reinforcing the relevance of the CycHyp signature. Also, although we have used the CycHyp signature as a prognostic biomarker for early-stage breast cancer, this signature was identified by integrating the information arising from tumor cells of various origins and characterized by various oncogenic alterations; the prognostic value of the CycHyp signature in other cancers is currently under investigation in our laboratory.

Altogether, the above findings indicate that the CycHyp signature represents a new generation of prognostic biomarker reflecting a generic environmental condition in tumors that differs from the conventional view of a static, continuous hypoxia occurring in tumors. When applied to breast cancer, the CycHyp signature has a powerful prognostic value independently of molecular risk factors but also offers a unique decision making tool to complement the discrimination of patients based on anatopathologic evaluation. The CycHyp signature is distinct from conventional hypoxia-related gene signature but also from existing prognostic metagenes, and the rationale behind its discovery supports a potential broad applicability to evaluate cancer patient outcomes.

MATERIALS AND METHODS.

Tumor cells

Twenty cell lines derived from cancer patients (see Suppl. Table 1 for details) were submitted to cycling hypoxia (CycHyp), i.e. 24 cycles of 30 min incubation under normoxia and 30 min incubation under hypoxic (1% O₂) conditions to reproduce tumor hypoxic fluctuations, as previously reported [5, 51]. We also considered control conditions of 24 h continuous exposure of tumor cells to either 21% O₂ (Normoxia) or 1% O₂ (ContHyp). For each culture condition, cells were immediately snap-frozen at the end of the last incubation period.

Identification of the signatures

mRNA extracts from each tumor cell cultured under the three above conditions (normoxia, cycling hypoxia and continuous hypoxia) were analysed by hybridization on Human Gene 1.0 ST Affymetrix microarrays (GEO accession number: GSE42416):

http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=probzowmylseqxm&acc=GSE42416

The extent of the resulting tumor cell datasets (20 samples in each of the three conditions) led us to resort on a resampling mechanism to increase the robustness of the signatures to be identified. For every resampling experiment, a subset of 90% of the samples was chosen uniformly at random as a training set and the remaining 10% were used as validation set. Differentially expressed probesets (one probeset = a collection of probes designed to interrogate a given sequence) were assessed on each subset according to a t-test and the corresponding FDR corrected p-values were reported. The 100 probesets with the lowest corrected p-values, averaged over 200 resamplings [52-54], formed the CycHyp (Table 1) or ContHyp (Table 2) signatures. All such expression differences were highly significant (p<1e-4) after Benjamini-Hochberg FDR correction for the multiplicity...
of the test [55]. Of note, in each resampling, the 10 % data not used to select probesets allowed one to estimate the discrimination potential between (cycling or continuous) hypoxia versus normoxia conditions. The average classification accuracy over all resamplings amounted to 97.5 % for CycHyp and 94.3% for ContHyp.

The 100 HGU1.0 ST probesets forming the CycHyp signature corresponded to 94 unique Entrez GeneID in the NCBI database, out of which 69 genes were available on the HGU133a platform (i.e., the technology used in most clinical studies considered here). Those 69 genes were represented by 87 HGU133a probesets. The few datasets collected on HGU133plus2 were reduced to the probesets also present on HGU133a.

Patient data sets

All breast cancer expression data were summarized with MAS5 and represented in log2 scale (except for GSE6532 already summarized with RMA). Breast cancer subtypes (ER+/HER2-, ER-/HER2- and HER2+) were identified with the genefu R package [56] (see Supplementary R Package). Disease-free survival at 5 years was used as the survival endpoint. The data from all patients were censored at 10 years to have comparable follow-up times across clinical studies [32].

Prognostic models of the clinical outcome

The VDX dataset (GSE2034 and GSE5327 from the GEO database) was considered as a reference because of its large number of node-negative untreated patients [17]. This dataset formed the training set used to estimate a prognostic model of the clinical outcome. A risk score for each patient was computed from a penalized Cox proportional hazards model [57] implemented in the Penalized R package [58]; the parameters of the elastic net penalty were learned on the training set by cross-validation. Prediction into a high risk vs. low risk group resulted from a predefined threshold value on this risk score. The decision threshold was chosen on the training set to maximize the specificity and sensitivity of the discrimination between patients with progressing disease versus disease-free patients at 5 years. Following the methodology described by Haibe-Kains et al. [32], all other datasets were used as validations to assess the prognostic performances on independent samples, i.e. balanced classification rate (BCR), concordance index (CI) [59] and hazard ratio (HR) [60]. The survcomp R packages were used to test the significance of the HR and CI values [33] while a Z-test allowed to infer p-values for the BCR relying on an approximation by a normal distribution.

Prognostic performances of a penalized Cox model defined on the CycHyp signature were also compared with well-established prognosis models for breast cancer, namely Gene 70 (Mammaprint) [14], Gene 76 [17] and Oncotype DX [15] signatures. Those existing signatures were associated to specific prognostic models implemented in the genefu R package [56]. Comparison of CycHyp and ContHyp signatures was also carried out with random gene signatures of the same sizes, i.e. 87 and 123 probesets, respectively. One thousand signatures of each size were generated and analysed using the methodology described by Venet et al. [11]. The objective of those experiments was to assess to which extent the CycHyp and ContHyp signatures had a better discrimination power between risk groups than random signatures. Gene Set Enrichment Assay (GSEA) analysis was also performed using the molecular signature database (MSigDB) and the CycHyp and ContHyp signatures expanded to 2118 and 2065 differentially expressed genes, respectively (after FDR correction and averaged over all resamplings).

ACKNOWLEDGEMENTS

This work was supported by grants from the Fédération Wallonie-Bruxelles (WB Health program HypoScreen), the Fonds de la Recherche Scientifique (F.R.S-FNRS), the Télévie, the Belgian Foundation against cancer, the J. Maisin Foundation, the interuniversity attraction pole (IUAP) research program #UP7-03 from the Belgian Science Policy Office (Belspo) and an Action de Recherche Concertée (ARC 09/14-020), O. Feron and P. Dupont equally supervised this work.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest relating to this study.

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