Identification of a metabolic and canonical biomarker signature in Mexican HR$^+$/HER2$^-$, triple positive and triple-negative breast cancer patients

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Abstract. Infiltrating ductal breast cancer (IDC) is the principal tumor associated-malignancy in Mexican women. In IDC, the development of intermittent hypoxia leads to an adaptive response coordinated by the transcriptional factor HIF-1α. In the present pilot, retrospective/cross-sectional study, the HIF-1α expression was analyzed in 102 tru-cut biopsies from female patients (51±12 years) without previous clinical treatment and compared to 31 normal breast biopsies. The 102 IDC samples corresponded to 56% of HER2$^-$/HR$^+$; 8% of HER2$^+$/HR$^-$; 22% of triple positive (HER2$^+$/HR$^+$); and 14% of triple negative (TN, HER2$^-$/HR$^-$) subtypes. To assess HIF-1α functionality, proteomic and kinetic analysis of glycolytic as well as mitochondrial enzymes, were determined. Validation of HIF-1α as cancer biomarker was assessed by determining the contents of the commonly used biomarkers c-MYC, Ki67, and H- and K-RAS, as well as metastatic and autophagy proteins. Proteomic analysis revealed that HIF-1α, c-MYC, HER2 and COXIV contents were significantly increased in all IDC subtypes vs. normal tissue. The contents and activities of glycolytic proteins were similar between normal and IDC samples, except for HER2$^-$/HR$^+$ where a substantial increase of HKII was observed. Significant increase in 2OGDH and E-cadherin was detected for TN samples vs. other IDC subtypes and for normal samples. These results clearly indicated that HIF-1α + COXIV + c-MYC (+ HER2 for HER2$^+$ subtype) may be useful to depict a breast cancer metabolic marker pattern for diagnosis, whereas the contents of HIF-1α + c-MYC + 2OGDH + E-cadherin may be an alternative useful and reliable signature for TN subtype cancer prognosis.

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Introduction

Breast cancer involves a wide range of genetic and biochemical alterations and different clinical behavior and outcomes. As a consequence, there is an imperative clinical need to accurately identify the different classes of breast cancer to better define specific therapies. Molecular characterization may allow for a more precise classification of breast cancer by identifying new functional and more specific markers and which may represent potential therapeutic targets and/or indicators of prognosis (1). Currently, routine clinical management of breast cancer involves the use of presumably sensitive and specific molecular markers such as the hormone receptors (HR) for estrogen (ER) and progesterone (PR), and the epidermal growth factor receptor 2 (HER2). Thus, ER, PR and HER2 immunohistochemistry (IHC) has become a routine and standard clinical task for diagnosis and treatment of breast cancer subtypes.

However, some relevant disadvantages have emerged from using these standard clinical procedures for diagnosis such as: i) the elevated number of false negatives (20% vs. total cases) and false positives (7% vs. total cases), i.e., increased levels of the ER, HER2 and PR proteins are also attained in non-tumor but proliferative tissues such as hematopoietic cells (2) and adipocytes of post-menopausal women (3) stimulated by proliferation-related cytokines (4); ii) the undefined and trial/error based treatment for the TN breast cancer patients (which account for approximately 20% of total cases) (4); iii) the acquired tumor resistance to prolonged trastuzumab initial treatments (35% vs. total cases) leading to metastatic progression despite the significant diminution detected in HER2 levels (70% vs. total cases) (5,6); and iv) the development of chemotherapy-resistant tumors after anti-hormonal treatments (7).

These disadvantages prompt the need for developing more reliable biomarkers with lower percentages of false positive and false negative responses.

Cancer is a multi-factorial disease (8). Therefore, the identification of several key proteins constituting different altered pathways (i.e., signaling, metabolism and proliferation), and whose contents and activities are very likely altered in tumor cells vs. normal cells, seems a more rational strategy, than that based on the identification of one single protein or gene (9,10), for precise tumor subtype diagnosis and improvement in the design of treatments for primary, refractory and metastatic tumors.

It has been proposed that the changes observed in several tumor signal transduction and metabolic pathways vs. normal ones may provide a molecular signature (8,10). The abnormal activation of the glycolytic pathway even under high oxygen availability is considered to be one of the most important metabolic hallmarks of cancer (reviewed in refs. 11,12). This observation takes relevance under normoxic and hypoxic conditions as well as normoglycemia and hypoglycemia, because glycolysis provides both ATP and glycolytic intermediaries for DNA, protein and lipid synthesis required for tumor proliferation and survival (12). In this regard, it has been determined that overexpression and secretion of glycolytic enzymes may improve diagnosis of ER^+ breast cancer (13).

The molecular mechanism of the glycolytic activation involves several transcription factors and oncogenes (14,15). In particular, the hypoxia-inducible factor 1α (HIF-1α) increases the transcription of genes coding for specific glycolytic protein isoforms (GLUT1, GLUT 3, HK-I, HK-II, PFK-L, ALDO-A and -C, PGK1, ENO-α, PYK-M2, LDH-A and PFKFB-3) found overexpressed in tumor cells and absent (or in low expression) in their normal counterparts (reviewed in ref. 15). It has been also documented in monolayer cultures of metastatic tumors that elevated HIF-1α contents correlate with accelerated glycolysis (15) which in turn, correlates with high malignancy (16). Notably, the expression of all these particular glycolytic isoforms is linked to other activated tumor pathways such as cellular survival, apoptosis resistance and cellular migration onset, which are indeed also regulated by HIF-1α as well as by other transcription factors (15).

The HIF1α-glycolysis-malignancy interrelationship has not been studied in human tumor biopsies, although a high content in HIF1-α protein has been found in advanced clinical stages (IIIA) and in highly metastatic breast tumor biopsies vs. non-metastatic stage tumors, suggesting that this transcription factor can be a reliable diagnosis/predictive/prognostic biomarker (17). Unfortunately, HIF-1α increased levels can also be associated with other hypoxic diseases (heart attack, preeclampsia, diabetes, inflammation, ischemia and psoriasis) which are not linked to cancer development (18), deterring its potential role as cancer biomarker. Notwithstanding, to examine the possibility of establishing HIF1-α as selective biomarker in Mexican breast cancer patients, its content and functionality (assessing the contents and activities of glycoytic protein isoforms targeted by HIF-1α) were determined in human infiltrating ductal breast carcinoma biopsies. In parallel, canonical diagnosis/prognosis biomarkers involved in altered signaling tumor pathways were also analyzed. Furthermore, the contents of several mitochondrial enzymes were also determined to establish a possible relationship between HIF-1α and mitochondrial metabolism in cancer biopsies. The design of a global biomarker panel, including proteins of the most altered pathways in tumors (metabolism, proliferation and signal transduction) in different breast cancer subtypes may help to improve cancer prognosis and hence its clinical treatment.

Materials and methods

Human breast tumor tissue collection and histopathology. The present study represents a pilot (19), prospective/cross-sectional research approved by the Ethics and Research Committees of the Instituto Nacional de Cancerología (INCAN), México. The study from 2008 to 2013 included the evaluation of 102 women (age from 30-86, mean 51±12 years) diagnosed with infiltrating ductal breast carcinoma (IDC) also called invasive ductal carcinoma at the INCAN, México. According to the Cochran's categorical formula for determining sample sizes in pilot and prospective studies, 100 was the appropriate sample size based on i) the α level = 0.05; and ii) an acceptable margin of error <10% (20).

Normal tissue was surgically withdrawn from selected areas of normal breast tissue of 31 cancer patients (age from 27 to 89 years, mean 58±17 years) and stored in liquid nitrogen in the INCAN Tumor Bank for 12-24 months (21). Internal control of sample stability revealed that the contents of several proteins (HIF-1α, GLUT, LDH and ATPase) from fresh samples were similar to those stored in the INCAN Tumor Bank (data not shown). Normal samples were further validated as non-tumorigenic by assessing immunohistochemistry (IHC) negativity towards HER2 and hormone receptor. Patients diagnosed with IDC and no previous clinical treatments were used as the inclusion criterion for the present study, whereas insufficient material for biochemical assays was used as the exclusion criterion.

Tumor samples were obtained following the medical proceedings for tissue handling and patient care approved by the Institutional Ethics Committee supported by a patient's informed consent according to the Declaration of Helsinki. Patients were punctured with a tru-cut biopsy needle in the absence of local anesthesia. Histopathology was performed on hematoxylin and eosin stained biopsy slides. Each specimen was analyzed by visual inspection using standard light microscope by experimented pathologists as described (22) without knowledge of IHC results.

IHC analysis of biopsies. Human tissue was fixed in 10% neutral buffered formalin for 24 h. The sample was embedded in paraffin, cut with a microtome at 3 μm thick and placed on microscope slide. Immunostaining was performed with a BenchMark Ultra automated immunostainer (Ventana Medical Systems, Tucson, AZ, USA); estrogen receptor, progesterone receptor, HER2 and Ki67 protein antibodies were used at 1:100-250 dilutions. All antibodies were from Ventana Medical Systems, except for Ki67 (Bio SB Inc., Santa Barbara, CA, USA).

For estrogen and progesterone receptor identification, H-SCORE method was employed as described (23). For HER2

identification, negative score indicated <10% of stained cells; positive score indicated >30% of cellular staining.

The IHC analysis revealed that from 102 IDC biopsies, 23 corresponded to triple positive; 57 to $HER2^-/HR^+$; 8 to $HER2^+/HR^-$; and 14 to TN.

Western blot analysis. Samples from biopsies (0.4-1.8 mg total cellular protein, n=102) were placed in liquid nitrogen and kept at -70˚C until used. For western blotting processing, frozen tumor and normal samples were powdered, re-suspended and homogenized in 0.6 ml 25 mM Tris-HCl buffer, pH 7.4, plus 1 mM PMSF (phenylmethanesulfonyl fluoride), 1 mM EDTA and 5 mM DTT, and centrifuged at 2,000 x g for 30 min at 4˚C. Supernatants were recollected and protein content was determined by using the Lowry assay. IDC and normal breast samples (50 μg cellular protein) were re-suspended in loading buffer (10% glycerol; 2% SDS and 5% β-mercaptoethanol) and loaded onto 12.5% SDS-PAGE under denaturalizing conditions. The proteins were blotted to PVDF membranes (Bio-Rad Laboratories, Hercules, CA, USA) and protein identification was performed by overnight incubation with anti-HIF-1α, -GLUT1, -HKI, -HKII, -LDHA, -COXIV, -ATPase, -ANT, -GA, -2OGDH, -HER2, -c-MYC, -Ki67, -α-tubulin and -HRAS (Santa Cruz Biotechnology, Santa Cruz, CA, USA) specific monoclonal antibodies (1:500-1:1,000 dilution). Detection of the hybridization bands was performed with the horseradish peroxidase reaction in photographic plates as previously described. Densitometry analysis was carried out using Scion Image software (Scion Corp., Walkersville, MD, USA). Double normalization of tumor sample signal was first performed against its respective load control (α-tubulin) and then considering the normal tissue as 100% (24).

Enzyme activities. The supernatants from the frozen-thawed biopsy samples, prepared as described above, were stored at -20˚C in the presence of 10% (v/v) glycerol until determination of enzyme activities. Hexokinase (HK) and lactate dehydrogenase (LDH) activities were spectrophotometrically determined at 340 nm and 37˚C as described elsewhere (25). Briefly, HK activity was assayed in 50 mM MOPS buffer, pH 7.0 plus 2 U glucose-6-phosphate dehydrogenase, 1 mM $NADP^+$, 15 mM $MgCl_2$, 10 mM ATP and 20-60 μg cell extract protein/ml. The reaction was started by adding 3 mM glucose after 3-min pre-incubation and generation of NADPH was measured at 340 nm. Lactate dehydrogenase (LDH) was assayed in 50 mM MOPS, pH 7.0, 0.15 mM NADH and 10-20 μg cell extract protein/ml; after 3-min pre-incubation; the reaction was started with 1 mM pyruvate and NADH consumption was registered at 340 nm.

Statistical analyses. To identify significant differences in protein contents between non-tumor and tumor samples as well as among the different tumor subtypes, parametric and non-parametric statistical analyses were performed. The Kolmogorov-Smirnov and Levene tests (26,27) were applied to demonstrate the protein normal distribution and homogeneity of variance of samples. Analysis of Kolmogorov-Smirnov and Levene tests data indicated that non-parametric analysis (NPA) should be used for appropriate statistical assessment between non-tumor and tumor samples. The NPA analysis and graphical data were carried out by using the Microsoft SPSS v.20 (SPSS Inc., Chicago, IL, USA) and Microsoft OriginPro 8 (OriginLab Corp., Northampton, MA, USA) software, respectively. To validate the results obtained with NPA test, samples were re-analyzed by the Mann-Withney U test with a $P<0.01$ (28).

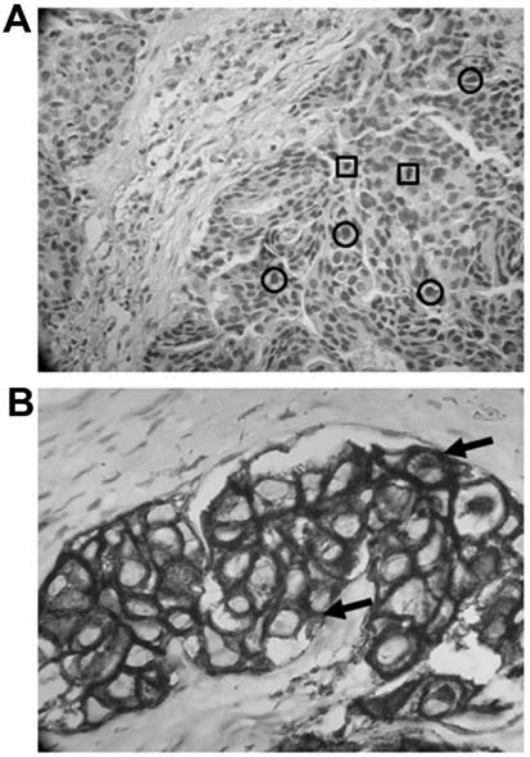

Figure 1. (A) Hematoxylin and eosin stain (H&E) of ductal infiltrating carcinoma. Circles show apparent atypias and squares cellular mitosis (original magnification, x20). (B) Immunohistochemical analysis showing increased HER2 intensity (arrows) in the plasma membrane of $HER2^+$ ductal infiltrating breast carcinoma cells (original magnification, x40).

To assess differences of the analyzed proteins among the tumor subtypes $HER2^+/HR^-$; $HER2^-/HR^+$; TP and TN the NPA Kruskal-Wallis test and the parametric analysis ANOVA were applied. To validate the results of the Kruskal-Wallis and ANOVA tests, the Mann-Whitney U test (28) corrected by the Holm-Bonferroni method and Scheffé post hoc test was used. Receiver operative characteristic (ROC) curves were also built for further validation as well as identification of the cut-off values for each protein. All statistical tests were performed at significance level of at least 0.05 as reported for the majority of human biopsy studies (29).

Results

Patient characteristics. Infiltrating ductal breast cancer (IDC) is the most dominant type of breast carcinoma found in the Mexican female population (30). From a total of 116 cases analyzed, 88% (102 biopsies) corresponded to IDC. IDC incidence was identified by standard IHC analyses inspecting hematoxylin-eosin stains for apparent cellular abnormalities (atypias) and cellular mitosis (Fig. 1A). For HER2 positive subtypes, all samples showed HER2 intensity on tumor cell plasma membrane with 3+ score (i.e., >30% of stained cells)

Table I. Clinical characteristics of infiltrating ductal breast carcinoma (IDC) patients.

Patients	Total n=97 (%)	Ki67 score (%)	TP (%)	Her2$^-$/HR$^+$ (%)	Her2$^+$/HR$^-$ (%)	TN (%)
Age (years)						
≤45	40	22	11	16	4	9
>45	60	20	12	40	3	5
IDC disease stage						
I	4	8	0	4	0	0
II	36	20	8	24	0	4
III	55	21	13	27	5	10
IV	5	20	2	0	3	0
Tumor size						
T1	3	15	1	2	0	0
T2	32	21	5	20	2	5
T3	30	23	8	16	1	5
T4	34	20	9	16	5	4
Histological grade SBR						
3	7	14	1	6	0	0
4	4	15	0	2	2	0
5	11	9	3	8	0	0
6	20	20	3	15	1	1
7	19	13	7	8	2	2
8	22	8	5	7	3	7
9	18	30	4	11	0	4

TN, triple negative; TP, triple positive; SBR, Scarff-Bloom-Richardson scale. Stage I corresponds to small tumors (<2 cm); stage II corresponds to tumors <5 cm which spread to axillary lymph nodes; stage III corresponds to cancer of any size that has spread to axillary lymph nodes, to lymph nodes near the breastbone or to the chest wall and/or skin; stage IV corresponds to metastatic cancer. The tumor size is based on the size of the tumor and the extent to which it has grown into neighboring breast tissue. The rating scale Scarff-Bloom-Richardson used in histopathological analysis considers the formation of breast tubule formation, nuclear pleomorphism and the number of cells entering mitosis.

(Fig. 1B), whereas the score of all HER2 negative samples was 0 and 1+ (i.e., 100% non-stained cells) (31).

The clinical characteristics of the IDC subtypes are described in Table I. The highest number of IDC patients (57 samples or 56%) corresponded to HER2$^-$/HR$^+$ followed by 23 samples (22%) of HER2$^+$/HR$^+$ (triple-positive, TP), 14 samples (14%) of HER2$^-$/HR$^-$ (triple-negative, TN) and 8 samples (8%) of HER2$^+$/HR$^-$. The percentages found in the present study for all IDC subtypes correlated with those reported in other clinical studies (32). The TN patients arguably have survival advantages as a result of the absence of HER2 overexpression. However, they lack the benefit of both routinely available targeted therapy and specific biomarkers. It is worth noting that the present prospective study analyzes a small number of IDC samples, as it has been reported for pilot clinical studies (19), and in contrast to other non-pilot epidemiological studies the IDC sample number is higher (>450 patients) (32); in consequence, in the present study the number of samples analyzed for each IDC subtype was smaller. Therefore, to avoid erroneous interpretations derived from a relative small number of samples, the analyses of the metabolic protein contents and activities were performed without considering the patient's age or menopausal status. Future investigations will be oriented to increase the sample size to further validate the results found in the present study with other analytical methods such as RT-PCR and microarray approaches.

Contents and activity of HIF-1α glycolytic and mitochondrial proteins in the IDC subtypes. The content of the transcription factor HIF-1α and its glycolytic targets GLUT1, HK (I and II) and LDH-A of tumor and non-tumor biopsies were normalized against α-tubulin (Fig. 2). The HIF-1α contents in tumor samples were significantly higher than those in non-tumor biopsies following the Mann-Whitney U test (Table II) and ROC analyses (Fig. 3A). The last test also revealed a cut-off point of 27 (i.e., 27% of the HIF1-α band intensity respect to the α-tubulin signal) for tumor vs. non tumor samples, where the highest sensibility (>80%) and specificity (>90%) were attained (Table II). These data indicated that, at least, a HIF-1α band intensity of a 27% is required to ensure that, in the IDC biopsy, <20% of false negatives and 10% of false positives can be found.

The increased HIF-1α content detected in tumor samples was accompanied by a slight, but non-significant increase in the protein content of its glycolytic target GLUT1. On the contrary, HK (I and II) and LDH-A contents were without change compared to normal breast tissue (Fig. 2). The functional determination of HK and LDH supported the observed western blot analysis. Activity of HK and LDH from tumor biopsies (22±5 mU/mg protein for total HK; n=23; and 307±78 mU/mg protein for total LDH; n=23) was similar to those from breast normal tissue (16±5 mU/mg protein for total HK; n=5; and 474±196 mU/mg protein for total LDH; n=5).

The contents of HIF-1α, GLUT1, HKI and LDH-A were similar among the different IDC subtypes (Fig. 4). However, for HKII a significant change, indicated by ANOVA and Scheffé post hoc tests, was determined in HER2$^+$/HR$^-$ vs. TP, vs. HER2$^-$/HR$^+$ and vs. TN (Fig. 4; Table III). The ROC for HKII indicated a cut-off of 73.5 with sensitivity and specificity of 86 and 31%, respectively.

For mitochondrial proteins, no apparent changes were observed in the contents of 2OGDH, GA isoforms K and L, and ATP synthase between tumor breast biopsies vs. non-tumor tissue (Fig. 2). On the contrary, COXIV and adenine nucleotide translocase (ANT) levels were significantly higher and lower, respectively, in tumor vs. non-tumor samples by using Mann-Withney U test (Fig. 2; Table II). However, the ROC tests (Fig. 3A) revealed that only COXIV, but not ANT, was significantly different vs. non-tumor tissue. For this case, the cut-off point was 15 with 100% specificity but low (38%) sensitivity, indicating that COXIV could be considered as a good tumor biomarker but with high probability (>50%) to detect also false positives (Table III). The content of 2OGDH was similar between TP and HER2$^-$/HR$^+$. On the contrary, in TN samples, the levels of 2OGDH were significantly higher

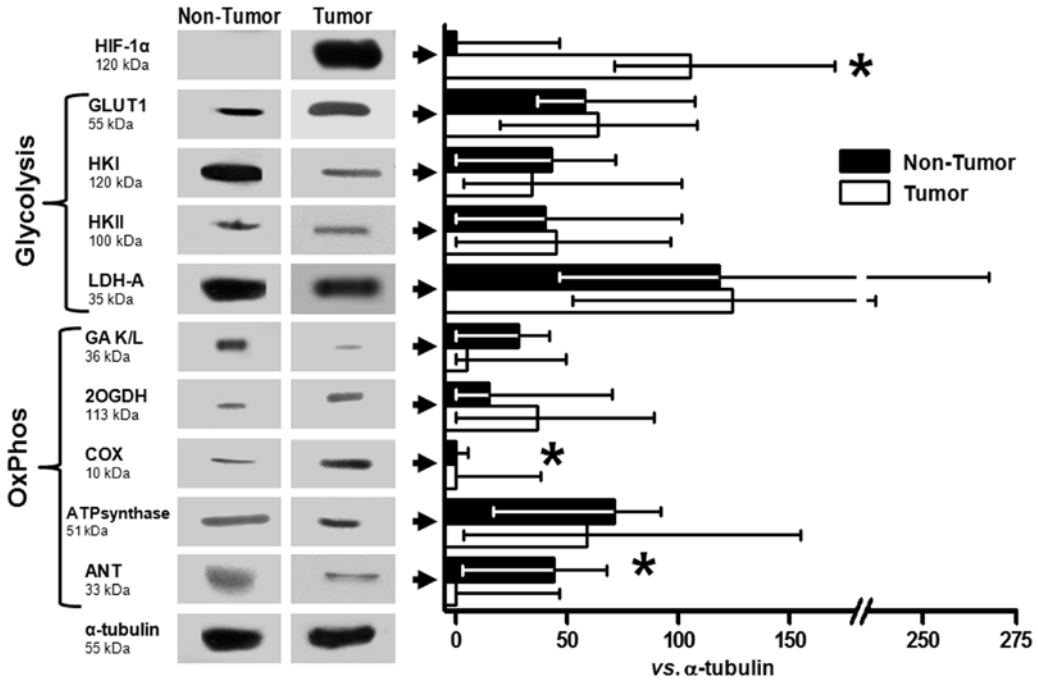

Figure 2. Representative western blotting showing glycolytic and mitochondrial protein contents in tumor and non-tumor breast tissue. The histogram represents the densitometry analysis of the western blotting results, and the values shown are the median ± the interquartile range; n=102 for tumor biopsies and n=31 for non-tumor biopsies. *P<0.05 by Mann-Withney U test. HIF-1α, hypoxia-inducible factor 1α; GLUT-1, glucose transporte-1; HK, hexokinase; LDH-A, lactate dehydrogenase isoform A; GA K/L, glutaminase isoform kidney or liver; 2OGDH, 2 oxoglutarate dehydrogenase; ANT, adenine nucleotide translocator.

Table II. Statistical analysis revealing significant differences (showed in bold letters) in tumor vs. non-tumor breast tissue samples.

	Protein	Mann-Withney U test P-value (P<0.01)	ROC analysis		Result vs. non-tumor samples
			AUC	P-value	
	HIF-1α	**<0.001**	**0.905**	**0.000**	**Statistically different**
Glycolytic	GLUT1	0.793			Non-statistically different
	HKI	0.578			Non-statistically different
	HKII	0.420			Non-statistically different
	LDH-A	0.874			Non-statistically different
OxPhos	GA	0.698			Non-statistically different
	2OGDH	0.193			Non-statistically different
	ANT	0.004	0.42	0.471	Non-statistically different
	COX	**<0.001**	**0.706**	**0.001**	**Statistically different**
	ATPsynthase	0.656			Non-statistically different
Canonical	Ki67	<0.001	0.610	0.321	Non-statistically different
	HER2	**<0.001**	**0.825**	**0.000**	**Statistically different**
	C-MYC	**<0.001**	**0.770**	**0.000**	**Statistically different**
Oncogenes	HRAS	0.593			Non-statistically different
	KRASs	0.224			Non-statistically different
Metastasis	Vimentin	0.283			Non-statistically different
	E-cadherin	0.871			Non-statistically different
Autophagy	BNIP3	0.008	0.701	0.058	Non-statistically different
	LAMP	0.035	0.700	0.071	Non-statistically different

vs. the pooled subpopulation of the other IDC subtypes (Fig. 4 and Table IV), whereas for $HER2^{+}/HR^{-}$ the content of 2OGDH was lower although non-significantly different vs. TP and $HER2^{-}/HR^{+}$ subtypes, by applying ANOVA and Scheffé post

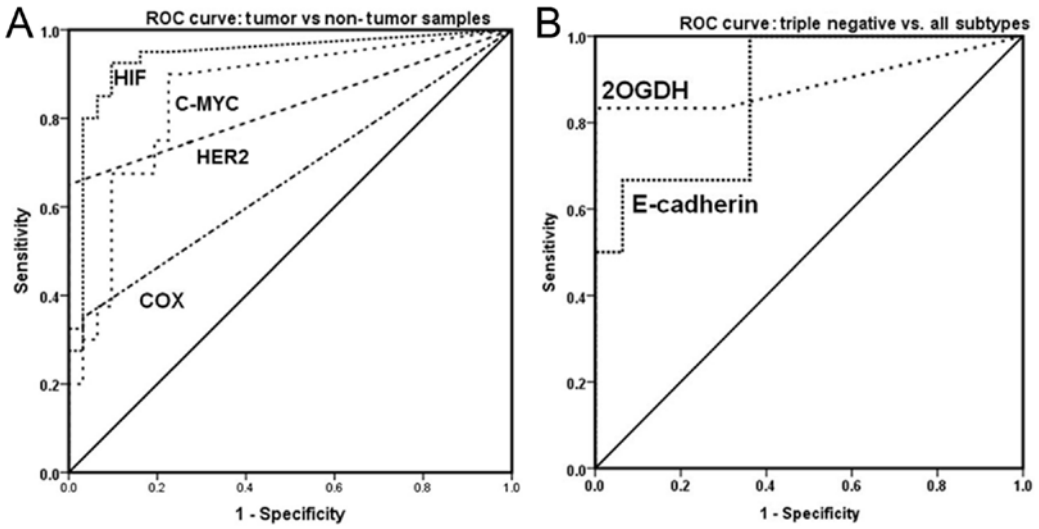

Figure 3. ROC analysis illustrating the performance of several proteins significantly different in (A) tumor vs non-tumor samples and (B) triple negative vs. TP, $HER2^+/HR^-$, and $HER2^-/HR^+$. Sensitivity is related to the true positive samples whereas 1-specificity is related to the false positive samples found in the 102 analyzed IDC samples. This approach reveals that HIF-1α, C-MYC, HER2 (for TP and $HER2^+/HR^-$) and COX could be considered as predictors of IDC cancer. 2OGDH and E-cadherin could be considered as TN predictors. n=102 for tumor and n=31 for non-tumor biopsies.

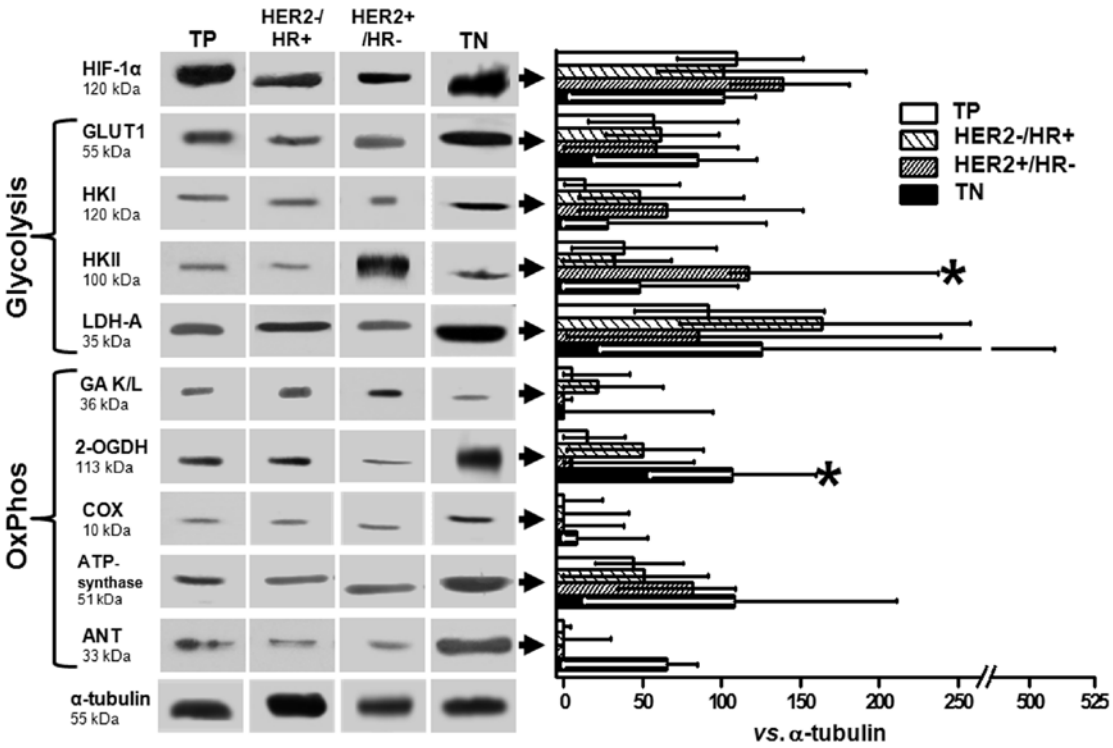

Figure 4. Representative western blotting showing glycolytic and mitochondrial protein contents in different IDC subtypes. The values shown in the histogram represent the median ± the interquartile range. *P<0.05 by Mann-Withney U test. TP, triple positive sample (n=23); TN, triple negative sample (n=14); $HR^+/HER2^-$ (n=57); $HR^-/HER2^+$ (n=8). HIF-1α, hypoxia-inducible factor 1α; GLUT-1, glucose transporte-1; HK, hexokinase; LDH-A, lactate dehydrogenase isoform A; GA K/L, glutaminase isoform kidney or liver; 2OGDH, 2 oxoglutarate dehydrogenase; ANT, adenine nucleotide translocator.

hoc tests (Fig. 4). For 2OGDH, data of cut-off value, sensitivity and specificity (Table V) showed that it is required at least 70% of band intensity (respect to that of tubulin as the loading control) in the biopsy to diminish to 20 and 40% the probability to have false positives or negatives, respectively. Also, a substantial but not significant increase in the ANT content was observed in TN compared to pooled subpopulation of the other IDC subtypes (Fig. 4), in which the ANT content was similar.

Contents of the proliferation, oncogenes, metastasis and autophagy proteins in IDC subtypes. In tumor breast biopsies, the proliferation protein Ki67, the oncogene c-MYC and the growth factor HER2 (in TP and $HER2^+/HR^-$ subtypes) were

Table III. ROC parameters revealing cut-off points, sensitivity and specificity percentages of different biomarkers in tumor vs. non-tumor samples.

Protein	AUC	P-value	Cut-off	Sensitivity %	Specificity %	RR	PPV %	NPV %
HIF1-α	0.905	0.000	0.5	88.7	77.4	2.95	92.5	68.6
			4.5	87.6	83.9	2.88	93.4	67.6
			27.05	85.6	90.3	2.79	95.4	65.9
HER2	0.825	0.000	3.5	65	100	3.21	100	69
			13.8	60	100	2.94	100	66
			26.2	55	100	2.72	100	63.3
C-MYC	0.770	0.000	0.35	76.7	74.2	1.88	89.6	52.27
			2.1	74.4	77.4	1.85	90.5	51.1
			30.78	61.1	80.6	1.59	90.3	43.1
COX	0.706	0.001	0.5	43.3	96.8	1.51	97.7	35.3
			15.04	38.1	100	1.53	100	34.8

AUC, area under ROC; RR, relative risk; PPV, positive predictive value; NPV, negative predictive value.

Table IV. Statistical analysis revealing significant differences among IDC subtypes.

Protein	1st Statistical analysis	P-value	2nd Statistical analysis	P-value	Statistically different between subtypes
HKII	ANOVA test	0.024	Scheffé post hoc test	0.046	Her 2 vs. TP
			Scheffé post hoc test	0.033	Her 2 vs. HR
2OGDH	ANOVA test	0.001	Scheffé post hoc test	0.001	TN vs. TP
			Scheffé post hoc test	0.008	TN vs. HR
			Scheffé post hoc test	0.045	TN vs. Her 2
HER2	ANOVA test	<0.001	Scheffé post hoc test	0.025	Her2 vs. TP
			Scheffé post hoc test	0.00	Her2 vs. HR
			Scheffé post hoc test	0.003	Her2 vs. TN
KRAS	Kruskal-Wallis test	0.023	Mann Whitney U corrected by the Holm-Bonferroni method	No differences	
E-cadherin	Kruskal-Wallis test	0.001	Mann Whitney U corrected by the Holm-Bonferroni method	0.000249	TN vs. HR
				0.009	TN vs. TP

Her2 (Her2 positive), $Her2^{+}/HR^{-}$; HR, $Her2^{-}/HR^{+}$ (hormone receptor positive); TP, triple positive; TN, triple negative.

Table V. ROC parameters revealing cut-off points, sensitivity and specificity percentages of 2OGDH and E-cadherin in triple negative samples.

Protein	AUC	P-value	Cut-off	Sensitivity %	Specificity%
2OGDH	0.869	0.004	7.95	100	23.4
			43.45	100	40.4
			76.23	83.3	64.8
E-cadherin	0.892	0.002	2.8	83.3	70.2
			36.9	83.3	85.1
			102.3	66.7	100

AUC, area under ROC.

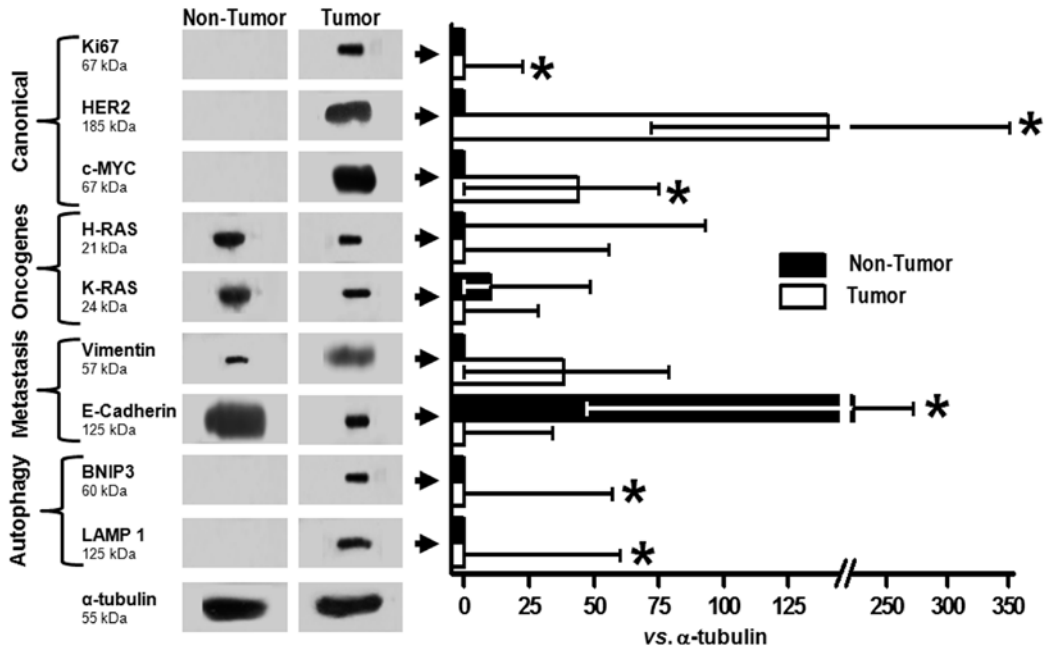

Figure 5. Representative western blotting showing proliferation, oncogenes, metastatic and autophagy proteins in tumor vs. non-tumor cells. The values shown in the histogram represent the median ± the interquartile range. *P<0.05 by ANOVA test. For tumor samples: Ki67 (n=94); HER2 (n=78); c-MYC (n=95); H-RAS (n=94); K-RAS (n=36); vimentin (n=68); E-cadherin (n=61); BNIP3 (n=68); and LAMP1 (n=60). For non-tumor biopsies: Ki67, HER2, c-MYC and K-RAS (n=31); H-RAS (n=15); vimentin and E-cadherin (n=26); BNIP3 (n=13); LAMP1 (n=19).

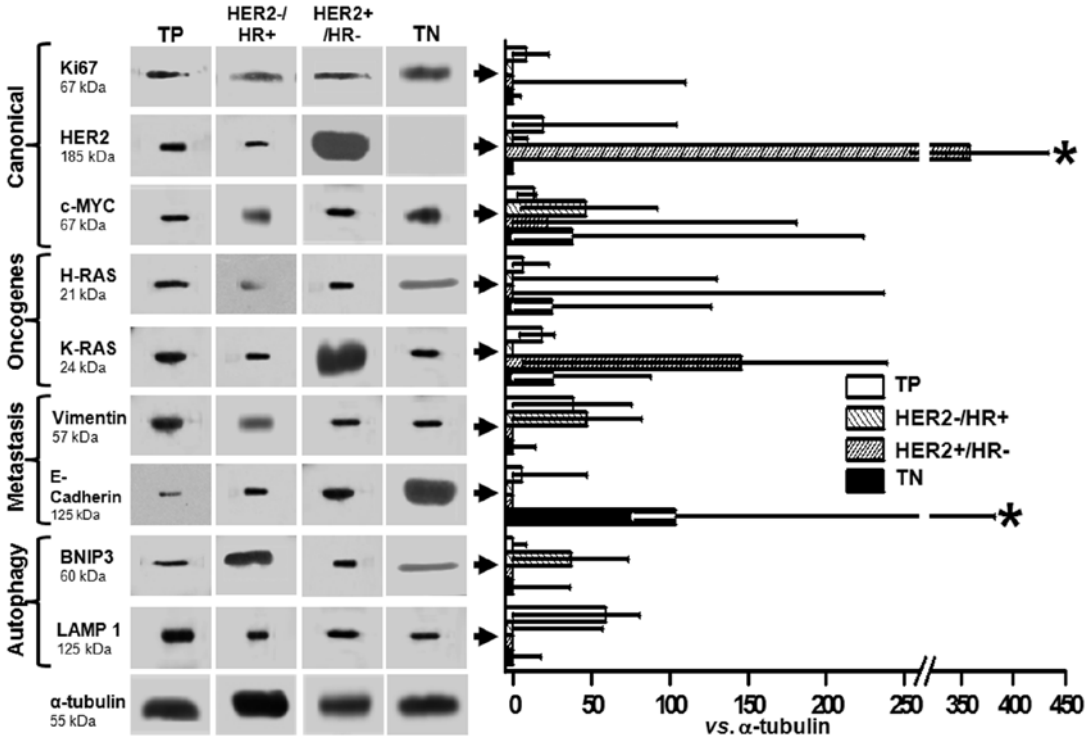

Figure 6. Representative western blotting showing proliferation, oncogenes, metastatic and autophagy proteins in IDC subtypes. The values shown in the histogram represent the median ± the interquartile range. *P<0.05 by ANOVA test. For TP, H-RAS, K-RAS, Ki67 and c-MYC (n=20-23); HER2 (n=19); vimentin, E-cadherin and BNIP3 (n=13); LAMP1 (n=11). For TN, H-RAS, K-RAS and c-MYC, (n=13); Ki67 (n=12); HER2, vimentin, BNIP3 and LAMP1 (n=7-8); E-cadherin (n=6). For $HR^+/HER2^-$, Ki67, c-MYC, H-RAS and K-RAS (n=53-54); HER2 (n=48); vimentin, E-cadherin, BNIP3 and LAMP1 (n=39-43). For $HR^-/HER2^+$, Ki67, c-MYC and LAMP1 (n=8); HER2, vimentin, H-RAS, K-RAS and BNIP3 (n=5); E-cadherin (n=4).

fully apparent, whereas no presence of these proteins was detected in normal breast tissue (Fig. 5), in agreement with previous reports (33). The Mann-Withney U test (Table II) showed that Ki67, c-MYC and HER2 were significantly different in TP and $HER2^+/HR^-$ vs. normal biopsies. ROC analysis showed that HER2 and c-MYC exhibited higher

area under curve (AUC) than Ki67 (Table III) indicating that HER2 and c-MYC were indeed significantly different in TP and $HER2^+/HR^-$ vs. normal biopsies (Fig. 3A). For these two proteins, similar cut-off points, sensitivity and specificity were determined (Table III), indicating that only 26-30% of protein signal (respect to the tubulin content) in TP and $HER2^+/HR^-$ samples is required to achieve: i) high probability (80-100%) to discard negative diagnosis (i.e., values lower than 26-30% indicate the absolute certainty of absence of disease), although ii) moderate probability (55-60%) for true positive identification (vs. false positive, i.e., the sample derived from a non-cancer patient having some other health problem).

HER2 presence (TP, $HER2^+/HR^-$) or absence (TN, $HER2^-/HR^+$), as well as HR positivity or negativity, was confirmed for the majority of the samples by western blotting (Fig. 6), further validating the standard IHC clinical approach (Fig. 1B; Table I) currently used at the Instituto Nacional de Cancerología de México. However, 12 out of 71 $HER2^-$ samples (17%), and 3 out of 22 HR^- samples (14%), as indicated by the IHC assay, yielded positive signal by western blot assay (data not shown). These results clearly indicated that additional support for breast cancer subtype diagnosis such as western blotting should be routinely implemented to decrease the emergence of false negatives and hence to establish the appropriate therapy.

Surprisingly, H- and K-RAS proteins were also found in normal tissue (Fig. 5). This result could not be confirmed by literature data regarding the presence or absence of H- or K-RAS in normal cells. Assessment of Ki67, oncogenes and transcription factor contents among the different IDC subtypes by the Krustal-Wallis test only revealed significant difference for the HER2 content in TP vs. $HER2^+/HR^-$. However, the Holm-Bonferroni/Mann-Whitney U corrected test showed no significant difference for such datasets.

Proteins involved in the metastatic response of tumor cells such as vimentin and E-cadherin were determined in tumor vs. non-tumor samples to assess the migration and invasion profiles of tumor biopsies. Both proteins were not statistically different between assayed groups (Fig. 5; Table II). Although, both BNIP3 (a key regulator of hypoxia-induced autophagy) as well as LAMP1 (lysosome biogenesis-induced autophagy) were significantly higher in IDC samples vs. non-tumor samples (Fig. 5), according to the Mann-Whitney test, ROC analysis showed no significant differences. Among IDC subtypes, metastasis and autophagy protein contents were similar, except for TN whose E-cadherin content was significantly higher (Fig. 3B; Table IV) to those determined for $HER2^-/HR^+$ and TP. In this regard, an E-cadherin cut-off value of 3-37 was determined (Table V), indicating that this is the required protein expression (respect to the tubulin signal) to reach a high probability (80-100%) to identify true positive samples and discard false positive and false negative results.

Identification of new metabolic biomarkers in IDC subtypes. Identification of significant differences among the contents of all proteins assayed (metabolic proteins, oncogenes, transcription factors, as well as metastatic and autophagic proteins) between tumor and non-tumor tissue was determined by using the stringent non-parametric statistical Mann-Whitney U test validated by the ROC graphs (Fig. 3A; Tables II, III and V). With these rigorous statistical analyses, significant differences between non-tumor and tumor samples were observed for HIF-1α > HER2 > c-MYC > COXIV (Fig. 3A; Table II), whereas non-significant changes were detected for the rest of the mitochondrial and glycolytic proteins analyzed. In contrast, for the identification of significant differences on the proteins assayed among the different IDC subtypes, non-parametric statistical Kruskal-Wallis test and the parametric ANOVA validated by the ROC graphs (Figs. 1B and 3B; Tables IV and V) were used. These last analyses showed significant changes in 2OGDH and E-cadherin protein contents in TN vs. TP, $HER2^+/HR^-$ and $HER2^-/HR^+$, thus, providing a differentiated and selective panel of biomarkers constituted by HIF-1α > c-MYC > 2OGDH > E-cadherin for TN subtype IDC biopsies.

Discussion

Accurate and early detection, and successful treatment of cancer are at present still unsolved challenging clinical problems. This situation most likely derives from the variety of genetic and biochemical strategies a tumor is able to develop to survive under a wide range of environmental stresses such as hypoxia and normoxia, hypoglucemia and normoglucemia, and immune host response. This cellular robustness allows a tumor to efficiently deal with the inhibition of a single kinase, transcription factor or oncogene, as usually thought, designed, and applied in the treatment of cancer in experimental animal models (9,34). Under this context, it is clearly understandable and expected the eventual and frequent emergence of tumor chemo-resistance from these mono-therapy regimes.

Tumor protective mechanisms include the activation of multiple and redundant kinases, transcription factors and oncogenes that readily circumvent the initial therapy directed to a single target (34). Therefore, as a suitable alternative strategy, multisite or combinatory therapy should be considered (9,34,35). Some clinical treatments empirically have employed multisite therapy against well-known overexpressed proteins in tumor cells with moderately higher success rates.

In breast tumor biopsies two overexpressed proteins (HER2 and HR) have frequently been used as biomarkers (36,37). Therefore, a clinical combination treatment of breast cancer with tamoxifen against HR plus trastuzumab against HER2 is commonly used. However, i) positive responses against HER2 or $HER2^+/HR^+$ treatment are not always obtained. For instance, trastuzumab alone or combined with taxol or cisplatin or 5-fluorouracil plus surgery plus hormone therapy showed no effect in 50-75% of cases with metastatic breast cancer (reviewed in ref. 9); ii) the content of this marker is low (15-20%) compared with other highly expressed proteins such as mTOR (40%) and cyclin D1 (50%) in HER2-positive cancer patients (36,38); and iii) HER2 and HR predictive power severely diminishes when they are individually analyzed (39).

It has been demonstrated that the HER2 and HR protein contents change over time affecting the selected clinical treatment (40). One study revealed a severe cancer recurrence after anti-HER2 or anti-HR treatment in more than 1000 women diagnosed with early-stage breast cancer. A puzzling observa-

tion is that tumor recurrence correlates with a diminution in the levels of HER2 (33%) and HR (15%) contents in all biopsies analyzed compared with the initial diagnoses validated by a high HER2 and HR overexpression (40). In other breast cancer patients the status of the HER2 receptor changes from positive to negative or vice versa (40). The inconsistencies described for these canonical markers have led to the search for other molecular biomarkers which may reliably improve cancer diagnosis and prognosis.

HIF-1α as a suitable cancer biomarker in breast cancer. High HIF-1α content and glycolysis are indicative of increased malignancy and poor prognosis (41). Therefore, HIF-1α has been proposed as a biomarker for different metastasic carcinomas (colon, breast, gastric, lung, skin, prostate, ovarian, pancreatic, brain, glioblastoma and renal) (reviewed in refs. 42,43). In all these studies, the HIF-1α content in cancer samples, solely evaluated by IHC, showed an increase of 30-83% vs. normal tissue (43). Our present data using western blot analyses revealed a substantial increment of HIF-1α in all cancer subtypes compared with their respective normal tissue (Fig. 2), indicating that this transcription factor can be a reliable biomarker of breast cancer biopsies. The high sensitivity and specificity of the HIF-1α detection by western blotting (i.e., the antibody selectively detects a target protein in a mixture of several thousand different proteins coming from heterogeneous tissue) compared to the IHC assay makes the former the method of choice for routine clinical use, despite the longer processing time for western blotting (days) than for IHC.

On the other hand, HIF-1α functional status has not been systematically evaluated to establish a correlation between protein content and transcriptional activity (42,44). In the present study the transcriptional functionality of HIF-1α was assessed in all IDC biopsies. The data indicated that although a substantial increment in the HIF-1α levels was observed compared to normal tissue (Fig. 1), all IDC subtypes maintain similar GLUT1, HKII and LDH-A contents and activities to those determined in non-tumor samples. Unfortunately, there are no studies in which the content and activity of these glycolytic proteins have been determined, except for LDH whose activity, determined here, was within the range reported for breast cancer patients (45). Nevertheless, the present data indicated that HIF-1α, but not the glycolytic proteins can be a striking reliable marker of Mexican breast carcinoma as has been already suggested for human squamous cancer cervix epithelium (46). In other studies performed in breast tumor perinecrotic area and cervical cancer a strong correlation between HIF-1α and GLUT1 has been found (44,46).

Regarding mitochondrial OxPhos proteins, only the ANT content was detected to be significantly decreased in the cancer biopsies. In PC12 tumor cells, short-term hypoxia (30 min) downregulates the transcription of genes encoding mitochondrial complex I/NADH dehydrogenase as an adaptive response mechanism for adjusting the OxPhos rate to the low O_2 availability (47). A putative regulatory site in the ANT gene for HIF-1α has not been reported.

Clinical implications in the search of new biomarkers for triple negative breast cancer. Epidemiological studies of breast cancer in the Mexican female population revealed that approximately 20% of patients develop the TN phenotype (36). Unfortunately until now, the treatment with anti-hormone drugs (tamoxifen) or monoclonal anti-HER2 (trastuzumab) has been ineffective in the majority of these diagnosed cases (48). Therefore, it appears relevant to develop research focused on the identification of specific TN biomarkers. Recently it has been documented that the MAG13-AKT3 protein may fulfill such as role (49). However, its content is only overexpressed in a scarce number of TN patients (7% or 5/72), disabling its use for all TN patients (49). In the present study, significant changes in some mitochondrial (2OGDH) and metastatic (E-cadherin) proteins were observed in TN samples vs. other IDC subtypes (Figs. 4 and 6). Therefore, an alternative therapeutic strategy may be the use of combined treatment including the usual first line of treatment (tamoxifen or 5-fluorouracil or doxorubicin or cyclophosphamide or trastuzumab) plus some mitochondrially-targeted inhibitor (casiopeina II-gly and vitamin E analogues) (50,51).

In conclusion, all analyzed breast cancer subtypes exhibited high HIF-1α levels. Therefore, anti-HIF therapy (i.e., echinomycin and bortezomib) combined with canonical drugs (and/or energy metabolism drugs for TN cases) could be a promising alternative treatment against breast cancer. In this regard, it has been documented that HIF-1α overexpression in cancer cells is linked to a substantial augment in EGFR levels. However, mono-therapy with cetuximab, a monoclonal anti-EGFR antibody or gefitinib yields low efficacy (52), suggesting that combinatory therapy with canonical drugs plus anti-HIF1α therapy (plus energy inhibitors for TN cases) may be required for effective tumor abolishment.

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