Multi–Biomarker Pattern for Tumor Identification and Prognosis

Sara Rodríguez-Enríquez,[1,2]* Silvia Cecilia Pacheco-Velázquez,[1]
Juan Carlos Gallardo-Pérez,[1] Alvaro Marín-Hernández,[1] José Luis Aguilar-Ponce,[2]
Erika Ruiz-García,[2] Luz María RuizGodoy-Rivera,[2,3] Abelardo Meneses-García,[2,3] and
Rafael Moreno-Sánchez[1]

[1]*Department of Biochemistry, Instituto Nacional de Cardiología, Tlalpan, México*
[2]*Laboratory of Translational Medicine, Instituto Nacional de Cancerología, Tlalpan, México*
[3]*Department of Tumor Bank, Instituto Nacional de Cancerología, Tlalpan, México*

ABSTRACT

In last decades, the basic, clinical, and translational research efforts have been directed to the identification of standard biomarkers associated with the degree of malignancy. There is an increasingly public health concern for earlier detection of cancer development at stages in which successful treatments can be achieved. To meet this urgent clinical demand, early stage cancer biomarkers supported by reliable and robust experimental data that can be readily applicable in the clinical practice, are required. In the current standard protocols, when one or two of the canonical proliferating index biomarkers are analyzed, contradictory results are frequently reached leading to incorrect cancer diagnostic and unsuccessful therapies. Therefore, the identification of other cellular characteristics or signatures present in the tumor cells either alone or in combination with the well-established proliferation markers emerge as an alternative strategy in the improvement of cancer diagnosis and treatment. Because it is well known that several pathways and processes are altered in tumor cells, the concept of "single marker" in cancer results incorrect. Therefore, this review aims to analyze and discuss the proposal that the molecular profile of different genes or proteins in different altered tumor pathways must be established to provide a better global clinical pattern for cancer detection and prognosis. J. Cell. Biochem. 112: 2703–2715, 2011. © 2011 Wiley-Liss, Inc.

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I
n cancer cells, the uncontrolled growth, apoptotic resistance, and stimulated anabolism are characteristics associated with the accentuated changes in their genetic, histology, and metabolic profiles. Cancer onset and development derives primarily from the accumulation of many genetic alterations which have functional consequences.

In this regard, it has been described that the tumor altered metabolism is a consequence of a permanent interaction between some activated and/or mutated oncogenes and/or tumor suppressor with genes of metabolic and signaling enzymes [DeBerardinis et al., 2007; DeBerardinis et al., 2008; Markowitz and Bertagnolli, 2009]. In particular, tumor metabolism is reprogrammed to sustain tumor survival when oxygen and essential metabolite supplies become limited. Although *all* human cancers undergo this metabolic reprogramming, the degree of response toward specific insults will depend on the individual properties of each tumor, that is, increased or decreased expression level of specific oncogenes and other transcription factors, activation or inactivation of a particular

energy pathway, or variable response rate to signaling modifications. Thus, a particular cancer cell signature, which in normal tissues is not modified or is almost absent [Zou et al., 2002], may provide a set of molecular and metabolic biomarkers.

Biomarkers, defined as any alterations in the molecular profile of non-healthy tissues and body fluids, provide a powerful tool to understand the behavior of multiple diseases from the earliest manifestations to the terminal stages. These changes or alterations may be used as signatures in observational and analytic epidemiology, randomized clinical trials, screening, diagnosis, and prognosis. The potential uses of biomarkers include (a) the identification of the illness stages of individuals, (b) the diminution of disease heterogeneity, and (c) the increment of reliable targets in clinical trials [reviewed in Mayeux, 2004].

In cancer, biomarkers are defined as the molecular characteristic (gene, gene product, and protein) that can be measured and evaluated as an indicator of the presence of neoplastic cells (Fig. 1) [Biomarkers Definitions Working Group, 2001; Lesko and Atkinson, 2001]. In turn, prognostic biomarkers or signatures are clinical baseline measurements that predict future outcomes, for untreated patients or those receiving standard treatment. Therefore, prognostic markers serve as therapeutic targets and they may be relatively easy to identify, although thorough validation is not usually done.

On the contrary, predictive biomarkers or signatures are baseline measurements that identify patients who are likely or unlikely to benefit from a specific treatment [Riley et al., 2009; Buyse et al., 2010], with the most significant and well-established example being HER2/neu amplification as a predictive signature for benefit from treatment with trastuzumab (see below) and perhaps with doxorubicin or paclitaxel [Hayes, 2005; Gennari et al., 2008]. A biomarker may be considered as predictive when changes in their baseline value over time forecasts the efficacy or toxicity of a treatment [Buyse et al., 2010]. Unfortunately, they require extensive data for validation, based on large randomized clinical trials, and meta-analyses that includes patients with both high and low levels of the biomarker. In consequence, few cancer biomarkers have been robustly validated, breast cancer included.

Validation of a prognostic biomarker involves the establishment of a strong correlation between the presence and absence of the marker at baseline, or changes in the biomarker over time, and a treatment [Buyse, 2007]. For instance, the estrogen receptor (ER) status has been validated both as a prognostic marker for outcome in breast cancer and, as a predictive marker, it has been established that endocrine therapies (such as tamoxifen and aromatase inhibitors) are only beneficial for patients with tumors expressing ERs (see below).

It should be noted that not-so-objective criteria are used to establish where the baseline is, how this can change from person-to-person and in the same person over time. This means that biomarker evaluation has to be carried out rigorously in parallel for normal, adjacent cells, and for suspicious cancer cells. Moreover, as pointed out by Riley et al. [2009], prognostic marker studies have been poorly designed, analyzed, reported, and subjected to biases such as selective reporting and convenient choice of cut-points, resulting in added confusion, rather than clarification, on the real value of an established or a new marker and on understanding of the clinical outcomes.
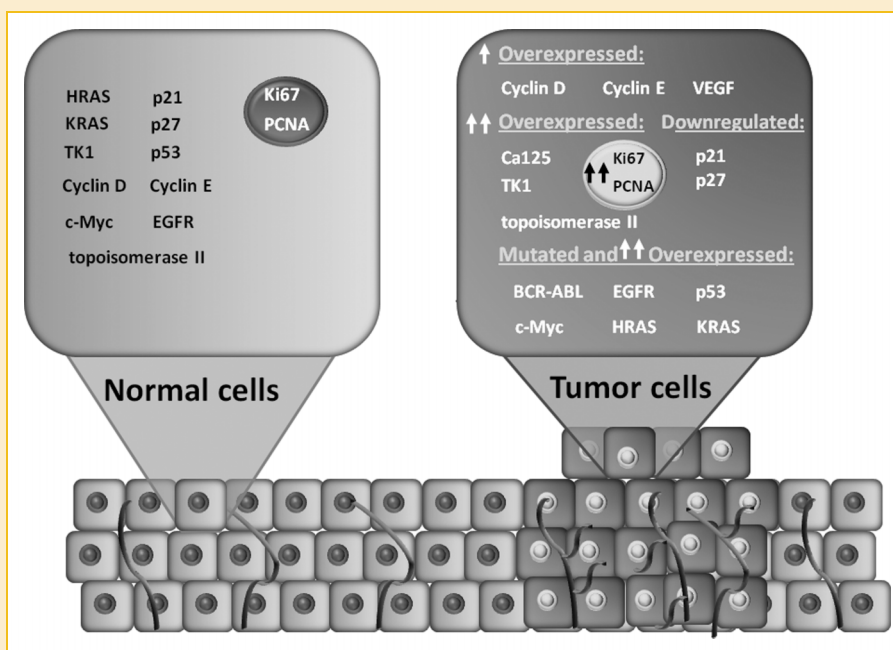

Fig. 1. Over-expressed proteins used as biomarkers in tumor cells compared with non-tumorigenic cells. Ribbons represent blood vessels. ↑, increment 1–5 fold; ↑↑, increment >10-times; Δ, mutated versus normal.

NEOPLASIC PROGNOSTIC AND PREDICTIVE BIOMARKERS USED IN CLINIC

For several years, the enhancement in (i) thymidine labeling index and S phase fraction/flow cytometry, (ii) the size of tumor, (iii) the lymph node status, or (iv) the amplification, over-expression, and/or mutation of proteins participating in (a) proliferation such as Ki 67, BIM1, proliferation cell nuclear antigen (PCNA), thymidine kinase, cyclins E, cyclin D, the cyclin inhibitors p27 and p21, epidermal growth factor receptor (EGFR), and topoisomerase II; (b) angiogenesis (vascular endothelial growth factor; VEGF); (c) oncogenes (CD1117, p53, and human ER-2 (Her-2)/neu); and (d) others (ER, Philadelphia chromosome) [Buyse et al., 2010] have been used in the clinical practice as prognostic or predictive cancer biomarkers (Table I; Fig. 1) in different types of human carcinomas. In addition, all or most of these genes are involved in driving the aggressive phenotype of the malignancy, and hence they are potential therapeutic targets [Buyse et al., 2010]. On the other hand, biomarkers not directly involved in the progression of the disease appear less likely to have a prognostic value, with *KRAS* mutations in colorectal cancer as an apparent exception (see below). Unfortunately, several limitations have been documented in their use (see below), suggesting that other biomarkers should be considered as an alternative for prognosis and prediction.

In the clinic, most of the biomolecular markers are detected by semi-quantitative estimations based on the staining intensity of positive cells (Histological Score System, HSS; Table I). The HSS value fluctuates depending on the biomarker characteristics, origin tissue, and tumor maturity. For example, in breast carcinoma the hormone receptors (estrogens and progesterone) score values are higher (50–100) [Allred et al., 2004; Papantoniou et al., 2004], whereas, for normal breast epithelium the ER HSS value is 30 [Allred et al., 2004]. In other tissues such as parotid gland, the presence of tumor does not alter the ER HSS value compared to normal tissues [Glas et al., 2002] suggesting that ER is a selective biomarker for breast carcinoma. For PCNA (invasive and metastatic marker) HSS value was around 300 in hepatocellular carcinoma, whereas, for health and cirrhotic liver score value are lower than 50 [Ng et al., 1994]. In progressive urinary bladder carcinoma, the score value for p53 was 160 but similar value was registered for non-tumor mucosa (around 130) [Soukup et al., 2007].

Unfortunately, several inconveniencies emerge from the H-score system analysis: (i) the criteria for selecting the range of score values (i.e., the dye intensity) are highly subjective and depend on the expertise and ocular appreciation of the pathologists; and (ii) high variability in H-score values is observed in the same tumor depending on the region where samples are taken for analysis. For example, for ER different H-score values have been determined in the periphery and in the center of the same tumor [Douglas-Jones et al., 2001]. In consequence, other analysis methods must be considered for tumor identification. On this regard, some biomarkers (E-cadherin, pyruvate kinase (PYK), apolipoprotein, and complement protein) have been analyzed by measuring the protein level by Western blot in human biopsies and compared with non-tumorigenic tissue (Table I) [Gasparini et al., 1992; Fan et al., 2010; Johann et al., 2010]. However, although significant increase in the level of these markers is attained, targeting these over-expressed biomarkers does not guarantee trial successful. For

TABLE I. Proliferation Biomarkers Employed in the Clinical Trial

Carcinoma biopsy	BM	Initial expression (%)	Treatment	Final expression (%)	Outcome	Reference
Positive cells detected by IHC						
Bladder	p53 Ki67	10–50	Surgery plus mitomycin C chemotherapy	10–50	Negative	Seo et al. [2010]
Breast	Ki67 S-phase fraction	0–43	Adjuvant radiotherapy; adjuvant chemotherapy (cyclophosphamide, adriamycin, 5-fluorouracil, and tamoxifen)	<8%	Positive	Pierga et al. [1996]
	Her2-neu	40–50	Lumpectomy, mastectomy, radiotherapy, and tamoxifen	40–50	Negative,	Stackievicz et al. [2010]
Cervical	Ki67 COX-2	40–87	Celecoxib	<20%	Positive	Ferrandina et al. [2003]
	Bax Bcl-2 p53	10–50	Radiotherapy	10–50	Negative.	Wootipoom et al. [2004]
Colorectal	Ki67 p53	40–100	5-Fluorouracil	40–100	Negative	Allegra et al. [2002]
	Ki67 VEGF p53	>10	Surgery	>10	Negative.	Padilla et al. [2007]
Glioma	PCNA Ki67 p53	32–44	Surgery	35–53	Negative	Berny et al. [2004]
Myeloid leukemia	Ki67	92	Cytosine arabinoside, idarubicin, and etoposides	40	Positive	Nowicki et al. [2006]
Lung	p53 p21 p27 Bcl-2	34–66	Neoadjuvant platinum-based chemotherapy	34–66	Negative	Morero et al. [2007]
WB detection						
Ovarian	Bcl-X	Over-expression	Platinum-based chemotherapy	Lower levels versus IST	Positive	Chaudhry et al. [2010]
	CA125	Over-expression	Surgery, chemotherapy: paclitaxel, cisplatino, and carboplatin	Lower levels versus IST	Positive	van Altena et al. [2010]
	Topoisomerase 1A, Her2/neu Ki67	Variable expression	Platinum-based chemotherapy	Variable levels of BM expression	Not clear	Surowiak et al. [2006]
Prostate	PSA	Variable	Androgen deprivation therapy	Variable levels of BM expression	Not clear	Takizawa et al. [2010]

BM, biomarker; IHC, immuno-histochemistry; PSA, prostatic-specific antigen; IST, initial stage before treatment.
Outcome indicates whether treatment was successful (positive) or unsuccessful (negative).

example, in breast cancer the increase in the number of de novo synthesized microwells (determined as factor VIII-related antigen content) correlates with a substantial increase in metastasis risk [Gasparini et al., 1992] but, regrettably, no significant response toward bevacizumab or anti-angiogenesis treatment is achieved [Miller et al., 2007]. This clearly indicates that alternative therapies and/or change in the approach concept are required for achieving improvement in cancer treatment [Moreno-Sánchez et al., 2010].

PROLIFERATION INDEXES

THYMIDINE AND BROMODEOXYURIDINE LABELING INDEXES

The nucleotide labeling-based method evaluates the proliferation capacity of solid tumors by following the radioactive analog incorporation to a new DNA synthesized chain with high precision [Levkoff et al., 2008]. However, fresh material, handling of radioisotopes, and time-consuming radioactive assay are required [Beresford et al., 2006] which limits its use.

S PHASE FRACTION/FLOW CYTOMETRY

This method identifies the relative cellular DNA content and its localization in phase S cellular cycle in both fresh- and frozen-samples [Pinto et al., 2008]. Although its use as prognostic indicator in breast cancer has been documented, results are inconsistent [Nunez, 2001]. In addition, debris and cellular aggregates must be discarded to avoid false negative signal. This method requires high amounts of sample to ensure adequate determination and data are not highly reproducible due to the high sample variability [Beresford et al., 2006].

SERUM THYMIDINE KINASE (TK)

TK catalyzes the phosphorylation of deoxythymidine to generate deoxythymidine-5′ phosphate in the pyrimidine synthesis pathway. There are two isoforms, cytosolic TK1 (fetal isoform) is exclusively cell cycle-dependent, whereas, the mitochondrial TK2 isoform is required for the initial phosphorylation of pyrimidine nucleosides for mitochondrial DNA synthesis [Sun et al., 2010]. Significant increase in serum levels of cancer-bearing tumor patients TK1 have been determined as a measurement of malignant proliferation and tumor aggressiveness [Li et al., 2005]. This method requires fresh samples and is time consuming.

NUCLEAR ANTIGENS ASSOCIATED WITH PROLIFERATION

KI67 AND MIB1 PROTEINS

The Ki67 is a nuclear protein expressed in G1, G2, S, and M phases but not in G0. A variety of malignant cancers (Table I) shows higher Ki67 index (measured as percentage of positive-staining cells with a specific monoclonal antibody) making this protein widely used as prognostic maker. Ki-67 antibody solely may be used on fresh or frozen tissue because tissue fixation abolishes immune-staining. Thus, to circumvent this difficulty, fixed or paraffin samples are incubated with MIB1, the new-generation anti-Ki67 antibody, which has shown a good correlation with tumor histological grade and mitotic index [Cattoretti et al., 1992; Spyratos et al., 2002].

Unfortunately, sample fixation alters staining which may lead to not so-reproducible results [reviewed by Colozza et al., 2005].

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA)

Like Ki67, PCNA is expressed in G1, G2, S, and M phases and it is also involved in DNA repair. However, poor correlation with other proliferation index such as Ki-67 expression and mitotic index has been observed [Sullivan et al., 1993].

CYCLIN D1, CYCLIN E, AND CYCLIN INHIBITORS

These cyclins maintain a changing expression pattern depending on the cell cycle phase. Cyclin E is expressed during G1 and early S phases, whereas, cyclin D1 is expressed exclusively in G1. In malignant tumors, both cyclins are over-expressed but only cyclin E increment has been associated with poor prognosis in breast cancer [Kuhling et al., 2003]. A disadvantage in the use of cyclins as biomarkers is their null positivity in some quiescent tumors [reviewed in Alsheyab et al., 2009].

p27 and p21 bind and inhibit the cyclin E-CDK2 and cyclinD-CDK4/6 complexes formation, which in turn modulates G1 phase progression. High proliferation rates, malignancy, and poor prognostic are associated with low p27 levels [Cianga et al., 2002], whereas, for p21 controversial results emerge from their role as prognostic marker (Table I) [Chow et al., 2000; Vassilopoulos et al., 2003].

HUMAN EPIDERMAL GROWTH FACTOR RECEPTOR–2/NEU (HER–2/NEU)

The oncogene Her-2/neu increases the proliferation rate and growth specifically of breast cancers in their early stages. The increment in this protein levels in tissues or sera indicates both poor prognosis and tumor mass enhancement [Kim et al., 2001]. In patients with Her2/neu positive tumors, the benefit of trastuzumab treatment has been established by several large randomized trials [Buyse et al., 2010]. Therefore, Her2/neu status is currently accepted in the clinical practice as a predictive biomarker for the efficacy of trastuzumab treatment, despite the lack of knowledge on the mechanistic interaction between the biomarker and the anti-cancer drug and that treatment might have similar effects in patients with Her2/neu negative tumors [Buyse et al., 2010]. Moreover, high variability is attained because the determination of the HER-2/neu levels depends on the reagents and technologies used to detect its amplification [Pegram et al., 1998].

MICROSATELLITE INSTABILITY AND 18Q LOSS OF HETEROZYGOSITY

Microsatellite DNA consists in repetitive short nucleotide sequences and is abundant in the human genome. Microsatellite instability (MSI; a change in the number of DNA repeat sequences) is a molecular signature of deficient mismatch repair in tumor DNA, and is present in 15–20% of sporadic colon cancers and >80% of hereditary non-polyposis colon cancers [Gangadhar and Schilsky,

2010]. In turn, MSI leads to increased rate of mutation in colon cells contributing to cancer progression [Gangadhar and Schilsky, 2010]. MSI has been found in other metastatic tumor types such as primary small cell lung carcinoma (76%) [Chen et al., 1996], and head and neck squamous cell carcinoma in stage III or IV (29%) [Nawroz et al., 1996], but not in breast carcinoma [Siah et al., 2000].

Early activation of oncogenes, such as *KRAS*, is followed by loss of tumor suppressor genes occurring late in the oncogenic process. Many of these tumor suppressor genes are located on chromosome 17p, which contains the *TP53* gene, and on chromosome 18q. Allelic loss of a maternal or paternal copy of chromosome 18q can be detected as a loss of heterozygosity; that is, one copy is missing upon amplification of PCR products corresponding to each chromosomal arm. Allelic loss from chromosome 18q is associated with a worse prognosis in patients with (a) stages II and III colon cancer [Gangadhar and Schilsky, 2010]; and (b) cohesive gastric cancer [Inoue et al., 1998].

KRAS

KRAS mutation is a well validated predictive marker for lack of treatment benefit from EGFR-targeted antibodies [Karapetis et al., 2008], although it does not seem to have prognostic value [Gangadhar and Schilsky, 2010], as a prospective study of stage III colon cancer patients showed that patients with tumors harboring *KRAS* mutations did not differ from patients with wild-type *KRAS* tumors with respect to disease free or overall survivals [Roberts et al., 2010].

CA125

As Ca125 is elevated in over 80% of women with epithelial ovarian cancer at the time of initial diagnosis, the Ca125 antigen is widely used to monitor patients after clinical treatment, thus its use has become a standard of care [Karam and Karlan, 2010]. The second generation Ca125 assay has allowed for improved sensitivity for low Ca125 levels and results in fewer day-to-day variations in Ca125 detection. It should be pointed out, however, that one single determination of Ca125 for the detection of early stage curable ovarian cancers still shows poor sensitivity and specificity [Karam and Karlan, 2010].

In general, most of the commonly used markers in the cancer clinical practice show some inconveniences and deficiencies which may lead to inaccurate identification, prognosis, prediction of malignancy, and treatment, thus indicating that there is not a significant number of reliable markers in the field (Table I). In addition, it now seems clear that the use of mono-therapy against one or two of these biomarkers does not bring about the expected result of arresting or at least slowing neoplasia development (Table I). In consequence, other molecular biomarkers should be considered to offer better-quality information for optimal clinical decisions. In this regard, cancer biomarkers research on other proteins or transcription factors whose over-expression is not linked with proliferation changes but with tumor microambient alterations [Smolková et al., 2010] should also be considered.

HYPOXIA AND CANCER

Hypoxia is defined as a physiological state in which oxygen availability diminishes compromising biologic functions such as oxidative phosphorylation (OxPhos) activity and cellular growth [Rodríguez-Enríquez et al., 2010]. The effect of hypoxia (at 1% O_2) has been widely analyzed in diverse tumor models. Unfortunately, the concentration of dissolved oxygen in mm Hg or μM corresponding to 1% atmospheric O_2 is not usually determined. Under hypoxia, the O_2 concentration is 0.5–10 mm Hg in tumor (neuroblatoma, Ehrlich ascites) and normal (Chinese hamster ovary) cells [Höckel and Vaupel, 2001], which corresponds to 0.6–13 μM O_2 [Horan and Koch, 2001]. On the other hand, we have calculated that 0.1–0.3% atmospheric O_2 corresponds to 20 μM dissolved O_2 [Rodríguez-Enríquez et al., 2010]; therefore, 1% atmospheric O_2 used in most of the studies where hypoxia has been assayed corresponds to an oxygen concentration which does not reflect the physiological hypoxia achieved in vivo.

In solid tumors, intermittent hypoxia (from 30 to 80 mmHg in well-oxygenated areas to 2.5–10 mmHg in the hypoxic regions [reviewed in Höckel and Vaupel, 2001; Toffoli and Michielis, 2008] is a recurrent feature associated with an inefficient, chaotic, and fragile neo-vasculature [Nagy et al., 2009]. Experimental evidence suggests that hypoxia promotes malignant transformation, metastasis, chemo-, immune-, and radio-therapy resistance, and a more aggressive phenotype, all which derivates into poor prognosis for the patient [Knowles and Harris, 2001]. It has been proposed that the hypoxic areas, detected with oxygen electrodes [Parker et al., 2004], may help to predict cancer incidence and its potential sensitivity toward radiotherapy and anti-angiogenic drug treatment, in a similar way to that applied when using prognostic markers [Sakata et al., 2006]. However, this in situ invasive method (i) very likely allows some degree of atmospheric O_2 infiltration into the inner cell layers leading to over-estimation of the tumor oxygen concentration [Parker et al., 2004]; (ii) it is unpleasant for patients; and (iii) it is not technically viable for some cancers (i.e., pancreatic cancer).

Therefore, other non-invasive alternatives for hypoxia determination are under investigation. For example, the use of radiolabeled 2-nitromidazoles (^{3}H, ^{14}C, ^{75}Br, ^{76}Br, ^{77}Br, ^{18}F, ^{123}I, ^{131}I, ^{99m}Tc) and its derivatives (*N*-(2-hydroxy-3,3,3-trifluoropropyl)-2-(2-nitro-1-imidazolyl) acetamide SR 4554, CRC 94/17) emerge as first-line candidates for hypoxic tumors detection. Under normoxia, 2-nitromidazole is rapidly reduced to nitro-radical anion which is efficiently oxidized back to 2-nitromidazol. However, under hypoxic conditions the nitro-radical anion continues complete reduction forming nitroso-, hydroxylamine-, and amine-derivatives [reviewed in Hodgkiss, 1998], whose rings and side-chains are rapidly fragmented and bounded to macromolecular components of hypoxic cells or tissues. These binding reactions allow that the hypoxic status be isotopically and immunologically recognizable and also detected by single photon emission tomography (SPECT) or positron emission tomography (PET) [Hodgkiss, 1998]. Interestingly, a clear correlation between the increase in 2-nitromidazole level and the proliferation marker BrdUrd in murine mammary carcinomas has been reported; although for other murine tumor types (SaF sarcoma) such correlation does not occur [Webster et al., 1998]. The

improvement in the assessment of this important parameter should help us to better understand the interaction cancer–hypoxia and their influence in the clinical response of tumors.

HIF–1α AND THEIR TARGET GENES AS INDICATORS OF CANCER ONSET AND DEVELOPMENT

The low oxygen concentration found in the core of solid tumors stabilizes the hypoxia inducible factor-1α (HIF-1α), which is a key transcriptional expression regulator of several proteins involved in oxygen-dependent processes (angiogenesis, erythropoiesis, cellular proliferation, and vascular remodeling) [Weidemann and Johnson, 2008]. In fact, it has been demonstrated a significant relationship of HIF-1α expression with tumor growth and angiogenesis. In consequence, HIF-1α and some of the HIF-1α-targets such as VEGF, angiopoietin-2 (Ang-2), insulin-like growth factor type 2 (IGF-2), and lysyl oxidase (LOX, metastasis) have been considered as prognostic markers of numerous neoplasias (Table II).

Unfortunately, controversial results emerge from the published literature making difficult the interpretation of clinical data which in turn, may interfere with adequate therapy selection. For example, in colorectal cancer, the loss of imprinting (an epigenetic alteration) of IGF-2 is a common characteristic widely accepted in the clinical practice [Cui et al., 2003; Vu et al., 2010]. However, for other neoplasias such as solitary fibrous tumors, ovary, and breast carcinomas IGF-2 levels may increase or decrease (Table II). The response to anti-IGF-2 treatment with dalotuzumab may yield encouraging results [Scartozzi et al., 2010], or may result innocuous [Reichert, 2011] for the same breast and ovary carcinomas. Similar results are observed for the rest of hypoxia markers including HIF-1α indicating that tumors may display heterogeneous response to hypoxic stress even within the same tumor type (Table II).

TABLE II. HIF-1α and Hypoxia-Induced Factors Used as Clinical Biomarkers in Cancer Human Biopsies

	Status	Carcinoma	Reference
VEGF	High level	Colorectal	Willett et al. [2005]
	High level	Liver	Kemik et al. [2010]
	No change	Liver	Goede et al. [2010]
	Low levels	Prostate	Tesan et al. [2008]
Ang-2	High level	Colorectal	Goede et al. [2010]
	No change	Astrocitoma	Ding et al. [2001]
	Low levels	Prostate	Tesan et al. [2008]
IGF-2		Colorectal	Cui et al. [2003]
		Breast	Van Roozendaal et al. [1998]
		Ovarian	Kim et al. [1998]
		Bladder	Byun et al. [2007]
	High Level	Ovarian	Huang et al. [2010]
		Solitary fibrous	Hajdu et al. [2010]
LOX	High level	Uveal melanoma	Abourbih et al. [2010]
	High level	Oral mucosa	Albinger-Hegyi et al. [2010]
	Low level	Melanoma	Timár et al. [1999]
HIF-1α	High Level	Breast, colon, lung, prostate, and breast	Zhong et al. [1999]; Generali et al. [2006]; Dales et al. [2010]
	Low Level	Brain, liver, breast, lymphoma, and prostate	Zhong et al. [1999]

Status versus normal tissues.

It has been determined that HIF-1α also regulates the expression of almost all glycolytic genes in monolayer cultured carcinomas, thus contributing to maintain the accelerated glycolysis in the majority of cancer types [reviewed in Marín-Hernández et al., 2009]. Therefore, the gene expression and protein content profiles of several glycolytic proteins have been determined in human biopsies as cancer biomarkers [Azuma et al., 2007; Koda et al., 2010]. Unfortunately, in some of these reports (1) the analysis of the expression pattern is performed by selecting one or two glycolytic genes assuming that the rest of the genes should be also over-expressed; and (2) analysis of protein content levels and HIF-1α is not always carried out, thus a strict relationship between HIF-1α and glycolytic-targets has not been rigorously evaluated.

For example, expression levels of glyceraldheyde phosphate dehydrogenase (GAPDH) and PYK (two glycolytic non-controlling enzymes in tumor cells [Marín-Hernández et al., 2006] were analyzed in different human carcinoma biopsies. In breast carcinoma, both enzymes significantly increase by 5–7 fold compared to non-tumor tissue, whereas, in squamous lung cancer solely GAPDH increase by 2.5-fold with no apparent change in the PYK level. In contrast, prostate, stomach, and esophagus cancer biopsies shown similar GAPDH and PYK expression patterns compared to normal tissue [Isidoro et al., 2004]. These results clearly indicate that GAPDH and PYK may be considered as reliable metabolic markers for breast cancer but not for stomach, lung, or esophagus carcinomas.

In other set of studies, the expression of diverse glucose transporters was analyzed in several carcinomas (Table III). In human early stage non-small cell lung carcinoma (NSCLC), GLUT-3 was the isoform with higher expression in 60% of studied patients, whereas, isoforms −1 and −2 were over-expressed in less than 25% of samples [Younes et al., 1997a]. Because in no more than 15% of biopsies no isoforms of GLUT were over-expressed, the authors concluded that glucose transporters expression may be a good prognostic cancer biomarker. Unfortunately, no expression of the rest of the glycolytic enzymes or HIF1-α was analyzed. On the other hand, enolase-α is severely down-regulated in the same NSCLCs. Survival analysis has shown that patients with low enolase levels had poor overall survival compared with those whose tumors had over-expressed enolase levels, suggesting that enolase may be considered as a marker in determining tumor aggressiveness in patients with NSCLC [Chang et al., 2003]. However, other enolase isoforms (β and γ), and most importantly, other glycolytic enzymes were not determined to definitively conclude that enolase-α is the most reliable marker for NSCLC. In conclusion, the analysis of solely one protein may be not indicative of malignancy in tumors until a pattern of markers be analyzed in parallel.

IS SCIENTIFICALLY AND CLINICALLY SOUND TO KEEP SEEKING THE "KEY OR WONDER BIOMARKER" FOR CANCER AND USING THIS CONCEPT FOR TREATMENT?

Cancer cells may contain several distinct cell signatures. However, the clinical research still continues to search for the specific "magic

TABLE III. HIF-1α-Induced Glycolytic Enzymes and Transporters in Human Cancer Biopsies

	Isoforms	Detection method	Carcinoma	Reference
GLUT + HK	−1, and I–III	IHC	55 breast cancers	Bos et al. [2002]
GAPDH + PK			Breast	Van Roozendaal et al. [1998]
GLUT	1	NB, IHC, ISH, PCR, IF	Liver, pancreas, breast, esophagus, brain, kidney, lung, skin, colon, endometrium, ovarian, stomach, and cervix	Cantuaria et al. [2001]; Altenberg and Greulich [2004]; Goldman et al. [2006]
	3	NB	Breast, lung, colon, ovarian, and larynx	Younes et al. [1997a]; Younes et al. [1997b]
HK	I	NB, IHC	Brain, testis, breast, and head and neck	Altenberg and Greulich [2004]
	II	NB, RTPCR	Liver, pancreas, breast, esophagus, brain, kidney, lung, skin, colon, endometrium, ovarian, and cervix	Altenberg and Greulich [2004]; Yasuda et al. [2004]
HPI		NB	Liver, pancreas, brain, kidney, lung, skin, ovarian, testis, head and neck, lymphatic nodules, prostate, stomach, uterus, and nervous system	Altenberg and Greulich [2004]
PFK-1	L			
ALDO	A			
TPI				
GAPDH				
PGK				
PYK	M2			
PGAM	B	WB,NB	Liver, breast, lung, and colon	Durany et al. [1997]; Altenberg and Greulich [2004]
ENO	α	WB, NB	Liver, pancreas, breast, brain, kidney, lung, skin, colon, ovarian, cervix, testis, prostate, stomach, uterus, nervous system, and eye	Durany et al. [1997]; Altenberg and Greulich [2004]
LDH	A	N, IHC	Liver, breast, kidney, colon, lung, skin, testis, head and neck, lymphatic nodules, prostate, stomach, uterus, endometrium, placenta, eye, and reticular lymphoma	Koukourakis et al. [2003]; Altenberg and Greulich [2004]; Giatromanolaki et al. [2006]; Koukourakis et al. [2006]
PFK-2	PFKFB3	IHC	Colon, prostate, breast, ovary, and thyroid	Atsumi et al. [2002]

ALDO, aldolase; ENO, enolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GLUT, glucose transporter; HK, hexokinase; HPI, hexosephosphate isomerase; IHC, immuno-histochemistry; IF, immunofluoesence; ISH, in situ hybridization; LDH, lactate dehydrogenase; NB, Northern Blot; PFK1, phosphofructokinase type 1; PFKFB3, phosphofructokinase type II; PGAM, phosphoglycerate mutase; PGK, phosphoglycerate kinase; PYK, pyruvate kinase; TPI, triosephosphate isomerase; WB, Western Blot.

cancer marker," that is, the single gene or protein involved in a particular cellular process or metabolic pathway (e.g., genes or proteins participating exclusively in the proliferation onset or in the transduction pathways) whose over-expression, or repression, may undoubtedly indicate malignancy and tumor progression (Tables I and II). However, although numerous targeted molecular drugs have been developed against each particular biomarker, clinical results have yielded poor percentages of success [Faratian et al., 2009]. This last observation clearly establishes that the assumption that a single protein or gene is the cause for tumor persistence is incorrect. Knowing that cancer is a multi-factor disease it seems counter-intuitive to design strategies for cancer detection and treatment based on a concept that assigns or localizes the cause of the perturbation to only one protein and/or gene.

It is noted that the key assumption beneath the use of either metabolic enzymes, kinases, transcription factors, receptors, or transporters as cancer markers is that their detection by determining mRNA by Northern blot or protein by Western blot, directly reflects *activity* and that these activities are essential for *function*. This last statement is erroneous considering that changes in the gene transcription may not correlate with changes in the biological function (i.e., protein content, enzyme activity, and pathway flux) [ter Kuile and Westerhoff, 2001; Moreno-Sánchez et al., 2008; Moreno-Sánchez et al., 2010] and proteins may also be modulated by post-transcriptional processes (enzyme's covalent modification and metabolic regulation) independently of the levels of RNA and protein content in the cell. Therefore, caution is required after interpreting the results based only on transcriptome, proteomic, and metabolomic analyses. In addition, to add specificity to the markers

proposed for a given cancer, it would be highly beneficial and mechanistically clarifying to have the biological function in which they are involved perfectly described and understood under both healthy and pathological states, emphasizing the relevant changes that have occurred.

According with the current scientific fashion, gene expression signatures are now being studied seeking to optimize treatment decisions for individual cancer patients. Recently, a quantitative multi-gene expression assay, or "gene-signature," based on RNA expression, has been developed with the aim of improving treatment decision-making in the setting of stage II colon cancer [Gangadhar and Schilsky, 2010] (Oncotype DX® colon cancer assay). Starting with a list of 761 candidate genes from published literature, real-time RT-PCR analyses were performed on tumor samples from 1,851 patients recruited in the gene-signature developmental phase. The use of real-time RT-PCR allowed for the quantification of RNA in tumor tissues, including formalin-fixed, paraffin- embedded tumor tissue as well as fresh frozen tissue, making the gene-signature assay more clinically feasible and accessible, as fresh frozen tissue is not always available. The 761 candidate genes were narrowed to seven potential recurrence genes (*FAP*, *INHBA*, *BGN*, *Ki-67*, *C-MYC*, *MYBL2*, and *GADD45B*), six potential treatment-benefit genes and five internal reference genes. This gene signature await for validation in clinical trials as a predictor of differential benefit from chemotherapy [Quasar Collaborative Group, 2007; Gangadhar and Schilsky, 2010].

Gene signature for breast cancer has also been recently developed, the so-called mammaPrint® signature. This 70-gene signature has shown high predictive power for an unfavorable

outcome (91% of patients who developed metastatic disease had the poor prognosis signature), although the specificity was modest (only 59% of patients who did not develop metastatic disease had the good prognosis signature) [van de Vijver et al., 2002]. On the other hand, the positive predictive signature value was 0.63, meaning that about two thirds of the patients with a poor prognosis signature were expected to develop metastases within 5 years; the negative predictive value was 0.9, meaning that one 1 of 10 patients with a good prognosis signature were expected to develop metastases within 5 years. These findings indicate that while the mammaPrintR signature may be useful to help avoid aggressive chemo therapy in patients with a good prognosis, it is not a sufficiently accurate predictor of which patients will, or will not, develop metastases to provide the sole basis for a treatment decision [Buyse et al., 2010].

Thus, in the absence of a theoretical framework that may support the application of these genetic approaches, which are mainly based on finding correlations between gene expression and disease incidence or response to treatment, leaving aside the mechanistic understanding of the molecular and biochemical processes involved, it appears that successful validated gene signatures will follow the same fate than that observed for many individual biomarkers, their lack of use or controversial use in the clinical practice [Subramanian and Simon, 2010].

A BIOCHEMICAL APPROACH TO IDENTIFYING THE OPTIMAL MARKERS PATTERN IN CANCER

The concept of the "rate-limiting step," "bottle-neck," or "key step" assumes that there is only one single enzyme or transporter, or even receptor, controlling the metabolic or signal-transducing pathway [Krebs, 1970; Rolleston, 1972; Newsholme and Start, 1973], whereas, all other members exert no control. The approach of identifying, and targeting, "the rate-limiting step" is the dominant theoretical support for an overwhelming number of cancer studies on drug-design, biomarkers identification, and treatment [see Moreno-Sánchez et al., 2010 for a review].

Metabolic control analysis (MCA) is a theoretical and experimental framework [Kacser and Burns, 1973; Groen and Westerhoff, 1990], which rationalizes the quantitative determination of the degree of control that a given step (or cellular process) exerts on pathway flux (or biological function). It helps to (i) identify and understand why a step (or cellular process) exerts significant or negligible control as well as to (ii) design experimental strategies for the molecular manipulation of a given physiological process in an organism [reviewed in Fell, 1997; Moreno-Sánchez et al., 2008; Westerhoff et al., 2009a; Moreno-Sánchez et al., 2010].

MCA of tumor energy metabolism has shown that the flux control of glycolysis in AS-30D hepatocarcinoma [Marín-Hernández et al., 2006] is distributed among all pathway components (71% by GLUT + HK; 29% by the ALD-LDH segment). Despite its elevated over-expression (100–500 fold in AS-30D hepatocarcinoma), tumor HK was stronger inhibited by its product G6P (which increased its concentration) thus keeping high flux limitation. On the other hand, PFK-1 was moderately over-expressed, but the tumor isoenzyme was highly activated by F2,6 BP and AMP, which surpassed the inhibition by citrate, ATP, and low pH. These observations provide a mechanistic explanation for the respective high and low flux control exerted by tumor HK and PFK-1 on glycolysis. Kinetic modeling of glycolysis in AS-30D and HeLa tumor cells has revealed that indeed GLUT and HK together with HPI are the main flux-controlling steps in both carcinomas [Marín-Hernández et al., 2010] and, therefore, these proteins are the most adequate targets for drug design as well as the most suitable biomarkers for early tumor detection. On this last regard, it has been demonstrated that HKII expression increases 70% in carcinomas grades I and II, and remains constant or slightly increases in intermediate (grade III) and severe carcinomas (70–80%) [Guo-Qing et al., 2010] compared to normal tissue. The results support the hypothesis that at least HKII may be a suitable marker for early stages of cancer which does not occur for most of the canonical and standard proliferation markers.

The control of the mitochondrial ATP synthesis (OxPhos) in cancer cells is also distributed among several steps. MCA of OxPhos in AS-30D hepatocarcinoma showed that the respiratory chain site 1 (30%) and the ATP-consuming enzyme block (protein and nucleic acid synthesis; ion ATPases; 34%) were the main controlling sites [Rodríguez-Enríquez et al., 2000]. The low respiratory site 1 content in AS-30D hepatoma [Rodríguez-Enríquez et al., 2000] seems the reason for its significant flux control. Flux control residing outside of pathway was originally proposed by Hofmeyr and Cornish-Bowden [2000].

MCA has been also applied to other very active cellular pathways in tumors [Boren et al., 2002]. For instance, glucose-6-phosphate dehydrogenase (G6PDH) and tranketolases (TKT) control (40% and 60%) the ribose synthesis flux in rat Ehrlich ascites cancer cells [Vizán et al., 2009]. In this last study non-specific inhibitors were used. However, it has been recently determined that the simultaneous inhibition of G6PDH and TK importantly diminishes human colon adenocarcinoma growth [Vizán et al., 2009]. In this sense, MCA provides a more rational and quantitative approach to the identification of drug-targets with higher therapeutic potential [reviewed in Moreno-Sánchez et al., 2010].

In summary, the application of MCA avoids the "trial and error" experiments for identifying the conceptually wrong and misleading "rate-limiting step" concept and may help to explain why there are multiple and variable cancer markers. On this last regard, MCA of cancer cells provides a mechanistic explanation for this phenomenon: several steps share the control of energy metabolism, that is, there is no single "rate-limiting step" and hence it is not expected to find a single key cancer biomarker. Targeting or selecting a particular enzyme or receptor or cellular process (i.e., apoptosis) that does not exert full control of the biological function is unlikely to be a successful paradigm for continued research into cancer biomarkers and treatment. In contrast, by applying MCA it should now be possible to identify the group of proteins (and genes) that are predominantly modified in the different cancer cell types to achieve successful tumor detection. Indeed, the oncologic clinical practice has shown that combination therapy, rather than mono-therapies, offers greater percentages of success [Shoshan and Linder, 2008; Savage et al., 2009], that is, there is not one single protein (or gene or cellular process) governing tumor growth and hence control of function is shared by multiple steps (or cellular processes).

BEYOND METABOLIC CONTROL ANALYSIS: SYSTEMS BIOLOGY APPROACH

The biological processes are rather complex multi-component systems and in consequence their analysis, understanding, and management result difficult for a researcher or team of researchers. Most macromolecules (i.e., genes, mRNAs, enzymes, and other proteins) that carry out the processes of life adjust their functioning to signals they receive from their microenvironment. Thus, most macromolecules engage strongly with their own networks, which in turn are all connected, with the relevant consequence that the behavior of the macromolecules is, therefore, determined by the cellular system as a whole [Lehar et al., 2008; Moreno-Sánchez et al., 2010].

Systems biology is a combined theoretical–experimental approach that allows for the integrative analysis of metabolic and cellular networks functioning with the ultimate goal of gaining full understanding of how complex biological systems work and how they can be perturbed. The mathematical procedure of integration multiplies the behavior of each particular component of the analyzed network for a short period of time, to then recalculate that behavior on the basis of all the new concentrations, interactions, rates, and fluxes reached, before integrating again. The procedure is then iterated, continuously updating the mathematical behavior in the light of the development in time of the environment that they sense. This is how the biology works and this is, therefore, how our understanding of biology should operate, that is, integratively. Genomic, proteomic, and metabolomic datasets with the information of the individual molecules and macromolecules is not sufficient to understand function [Moreno-Sánchez et al., 2010]; we rather need integrative systems biology [Alberghina and Westerhoff, 2005] in which all available databases are simultaneously used. Considerations of metabolism (pathway architecture, enzyme kinetics, reaction thermodynamics, metabolite–enzyme and enzyme–enzyme interactions, flux rates, and flux- and concentration control), gene expression, and signal transduction need to be integrated, because ultimately the behavior of systems depends on the responsiveness of all their components. An ultimate version of systems biology, therefore, is the silicon cell, that is, a precise replica of the networks in terms of kinetic equations of a component process [Snoep, 2005; Westerhoff et al., 2009b].

The current strategy of identifying individual biomarkers and validating their relevance to cancer is hardly reaching success, most probably because the approach and concepts behind are erroneous. Knowing that the oncogenic process involves multiple mutations in a variety of genes and hence that multiple nodes in the system have been modified to induce cancer is, therefore, comprehensible that a greater understanding of the disease network appears as essential for revealing the components that have been most profoundly altered to then identify the most adequate disease markers. Due to the strong robustness of the biological networks, single point mutations do not lead to disease onset by rather multiple perturbations are required [Lehar et al., 2008].

Having the courage, and the theoretical support, to leave behind the outdated and flawed concepts of the genomic era, to embark in an iterative process of experimentation and modeling, can help us to successfully identify the most appropriate cancer biomarkers with the highest therapeutic potential, although in the meantime, derived from the lack of popularity and understanding of these systems biology mathematical-based concepts and paradigms, we may not be able to publish our results in high impact journals and/or gain well-funded research grants.

CONCLUSIONS

Following with the precepts of MCA, the traditional approach of selecting individual proteins (Ki67, cyclins, HIF-1α, and HIF-1α targets) operating in a common or in different pathway (proliferation, angiogenesis, apoptosis, or glycolysis) results incorrect because *there is no single biomarker for any cancer type*. In fact, unsuccessful clinic outcomes emerge when treatment against individual biomarker is performed (Table I). Therefore, efforts must be oriented to the use of a cancer biomarker pattern, that is, a combination of several biomarkers from different altered pathways (proliferation + angiogenesis + hypoxia + glycolysis), to reach a better understanding of cancer progression and to achieve prognostic significance in solid cancers. Certainly this multi-site approach has been partially developed in clinical trials in which a few biomarkers (i.e., Ki67, p53, and HER-2/neu) are simultaneously analyzed in breast tumor bearing-patients [Arens et al., 2005]. Thus, a family or pattern of cancer biomarkers belonging to the most altered pathways in tumors now appears as a more rational and potentially successful approach. This global signature including HIF-1α, some glycolytic-targets in combination with a proliferation, apoptosis, and malignancy biomarker profile may offer a better alternative for prognostic identification of cancer development, resurgence, drug efficacy, etc. For example, some proliferation biomarkers such asKi67 are not over-express and maintain the same low level than normal tissues during the initial formation stages (grade I) of some tumors such as laryngeal squamous cell carcinoma; whereas, in other cancer types (intrahepatic cholangiocarcinoma) these same biomarkers are highly over-expressed in the same initial stage [Settarkorn et al., 2005]. Therefore, the strategy for selection of biomarkers, and hence for treatment, ought to be different depending on the tumor type, evolution, progression, and malignancy. It is clear that in those cancers where proliferation biomarkers maintain low level, analysis of other biomarkers such as HIF-1α or HK could help to attain better diagnosis in initial stages of cancer than other "classical" and "very much appreciated" Ki 67 or cyclins markers.

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