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Chapter III

Protein Isoforms and Their Special Role as Cancer Biomarkers

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Abstract

The discovery of biomarker molecules with utility in the detection of tumors, in the follow-up of diagnosed or intervened cancer patients, and in the decision over targeted therapies, has been traditionally occurring at a slow pace. Nevertheless, numerous efforts are being dedicated to the search for new and improved tumor markers. There are several reasons behind this low turnover of valid biomarkers with current clinical approval. One of the most important, but usually forgotten, is the existence of a plethora of mechanisms that convert a gene product into a variety of protein forms, the so-called protein isoforms. Although the term "isoform" is not contemplated by the IUPAC, the word is commonly used to refer to the various forms of a protein, whose charge or mass properties produce different electrophoretic mobility, irrespective of their genetic origin. Here we will address the origin (pre- or post-translational) of the protein isoforms. All those proteins, most frequently derived from the same gene, may be located at different subcellular compartments, function at different cellular conditions and situations, and be differentially regulated. The implication of these isoforms in the failure of the search for cancer biomarkers is seldom considered, but clearly understandable: variations affecting certain protein isoforms might be masked when detection methods measuring the whole set of protein forms are used. This is particularly easy to understand in the case of immunological techniques, such as the widely spread ELISA, which rely on the use of antibodies that generally cannot distinguish between isoforms, unless specifically raised and tested.

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In the field of cancer biomarkers, there are some examples of molecules with isoforms that experience opposing alterations due to the progression of the malignancy. The best known among the currently approved tumor biomarkers is the case of the prostate-specific antigen, employed in the detection of prostate cancer. However, the phenomenon is frequent among the experimental biomarkers, as haptoglobin, clusterin, prohibitin or vimentin (which our group has studied in detail). All these and other markers will be reviewed, and the role of their isoforms described, in the following chapter.

List of Abbreviations

2-DE	two-dimensional electrophoresis;
AFP	α-fetoprotein;
BPH	benign prostatic hyperplasia;
CLU	clusterin;
EMT	epithelial-mesenchymal transition;
ER	estrogen receptor;
fPSA	free prostate-specific antigen;
GPI	glycosylphosphatidylinositol;
hCG	human chorionic gonadotrophin;
HPRD	human protein reference database;
KLK	kallikrein;
MT	metallothionein;
PDE	phosphodiesterase;
PSA	prostate-specific antigen;
PTM	post-translational modification;
tPSA	total prostate-specific antigen;
VCAM-1	vascular adhesion molecule-1.

Introduction

The search for cancer biomarkers has been for years a hot topic of research. However, even attempts to use new and powerful technologies (as those derived from genomics and proteomics) in the discovery of better cancer biomarkers have failed. One of the reasons is that many of the promising molecules discovered did not prove useful when measured in the samples of choice for clinical diagnostics (as serum or other biological fluids). A frequent cause for this failure is that molecules that are significantly altered in tumors may be specific isoforms of a certain protein, which then merge with all other isoforms from different tissue sources, so that the alteration fades into the general pool.

Diversity of the Genome and Proteome

The term "isoforms", or more correctly "protein isoforms", refers to similar proteins which are in most cases derived from the same gene. Though this is a commonly used word in the field of biochemistry, still its definition is not reflected in the publications of the

International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry and Molecular Biology (IUBMB) Joint Commission on Biochemical Nomenclature (http://www.chem.qmul.ac.uk/iupac/bibliog/jcbn.html). However, multiple protein forms have been long considered in the case of protein enzymes [IUPAC-IUB Commission on Biochemical Nomenclature, 1977], defining "molecular forms" or "multiple forms of enzymes" as those proteins catalyzing the same reaction and occurring naturally in a single species. More specifically, they define "isoenzymes" or "isozymes" as those multiple enzymatic forms arising from genetically determined differences in primary structure, but not those originated by modification of a unique primary sequence. Noticeably, some authors prefer the term "form" when there is no definitive information about the structural differences between different protein products; the term "isoform" is used instead for different transcripts coding for the protein, when they have been identified and sequenced [Bettuzzi, 2009a]. On the other hand, some authors use the term "variant" when the changes are originated at the DNA level, to distinguish from the transcriptional and translational effects. Other current definitions of "isoforms", which can be found during online searches, are:

- Any of the proteins with the same function and similar amino acid sequence, encoded by different genes (or by RNA transcript) (www.biology-online.org/dictionary /Isoform).
- Different forms of a protein that may be produced from different genes, or from the same gene by alternative splicing (Genetics Home Reference: ghr.nlm.nih.gov /glossary=isoforms; from Unified Medical Language System -MeSH- at the National Library of Medicine, USA).
- The protein products of different versions of mRNA created from the same gene by employing different promoters, which causes transcription to skip certain exons. Since the promoters are tissue-specific, different tissues express different protein products of the same gene (Genetics Home Reference: ghr.nlm.nih.gov /glossary=isoforms; from: GeneTests from the University of Washington and Children's Health System, Seattle, USA).

Irrespective of the various definitions shown above, the fact is that the origins of diversity can be traced down to the genome level for some proteins, with their isoforms actually deriving from different codifying genes. However, results from the human genome sequencing projects revealed that we count with a lower number of codifying genes (25,000) than predicted, which do not account for the diversity of the proteome. Therefore it is dubitable that only one promoter can regulate one gene to yield one mRNA, as the traditional dogmas propose. At the transcriptional and post-transcriptional level, we know now a number of mechanisms adding complexity to that simple chain, such as the existence of alternative promoters, non-coding RNAs and proteins with regulatory functions, or epigenetic phenomena as methylation. Mechanisms like RNA editing and alternative splicing account for a high number of protein isoforms, as will be described in more detail in the next section. And once a protein is synthesized, the so-called post-translational modifications (PTMs) are responsible for generating further diversity.

The Human Protein Reference Database (HPRD) is an important resource for information about human proteins, including domain architecture, protein function, protein-protein

interactions, PTMs, enzyme-substrate relationships, subcellular localization, tissue expression and disease association. Interestingly, on the 2006 update this database included information on protein isoforms [Mishra *et al*., 2006a]. The criterion for inclusion of proteins as isoforms was that they should be entries from the RefSeq database with different coding sequence for the same gene. Thus, it only included those alternatively spliced forms where the splicing involved the coding region, and not the 5' or 3' untranslated regions. The 2009 update of the HPRD [Keshava Prasad *et al*., 2009] already included most of the human protein isoforms present in the RefSeq database, resulting in 25,661 protein sequences encoded by 19,433 genes. The phosphodiesterase (PDE) PDE9A, the cAMP response element modulator, collagen type XIII α1, and dystrophin, are examples of proteins with the highest number of isoforms (20, 20, 19 and 18, respectively). However, only data pertaining to the sequence, subcellular localization, mRNA/protein expression, biological motifs and domains are currently being annotated as isoform-specific, whereas protein-protein interactions and enzyme-substrate relationships are annotated as common to all isoforms, mainly due to lack of experimental data.

Cancer-related or cancer-specific isoforms have been assigned to different tumor characteristics, as the ability to evade apoptosis and immunological surveillance, unlimited replicative potential and immortality, independent growth and self-sufficiency, refractivity to inhibitory signals, angiogenic potential, invasion capacity, or metastasis. They can have new roles in these processes, or counteract the normal levels or action of the isoforms normally produced in the healthy state. For instance, there are many cases of isoforms with antagonistic function in apoptosis, as is the case of clusterin (CLU) in colon cancer (a nuclear isoform is proapoptotic, whereas a cytoplasmic one is antiapoptotic) [Leskov *et al*., 2003], or the Bcl proteins in prostate cancer (Bcl-xS is proapoptotic, whereas Bcl-xL is antiapoptotic) [Mercatante *et al*., 2002].

Isoforms Generated at the Genome Level

The generation of isoforms before transcription is not very frequent, yet there are several cases of protein forms arising from different genes. Besides, isoforms can be generated by alternative use of different promoters and terminators.

A common isoform case with origin at the genomic level, though far from the cancer field, is the metallothionein (MT) family. These are small metal-binding proteins induced by heavy metals as cadmium, copper and zinc. In several eukaryotes and prokaryotes it has been shown that different isoforms are induced by intoxication with different metals. For instance, the common mussel *Mytilus edulis* shows two distinct MT gene isoforms, called MT-10 (10 kDa) and MT-20 (20 kDa), and it seems that MT-20 is transcribed upon transient changes in metal concentrations in the cytosol due to environment fluctuations, whereas the synthesis of MT-10 is involved in the normal metal regulatory processes [Vasák and Hasler, 2000].

A well-studied example of cancer-related proteins with isoforms generated at the genome level is the kallikrein (KLK) family. These proteins originate from 15 different genes, which end up yielding more than 80 transcripts. In the next section we will describe some relevant examples from this family.

Isoforms Generated at the Transcriptome Level

RNA editing (chemical modification of specific nucleotide/s), alternative splicing, alternative promoter or start sites, variation in polyadenylation signals, etc., are common post-transcriptional events contributing to the proteome diversity. This regulation is typical for tissue- or time-specific (related to development) protein forms.

One of the most relevant phenomena in relation to the isoforms involved in cancer is the alternative splicing. This is defined as the production of multiple RNAs from a single gene. The average human gene contains 8 exons of variable length, interrupted by long non-coding regions called introns. During transcription, the RNA polymerases transcribe both types of segments to generate an RNA molecule. This is the substrate for the spliceosome (a complex formed by 5 small nuclear ribonucleoproteins and more than 100 other proteins), which removes introns and ligates exons, creating the mRNA molecule which would be finally translated into a protein. When alternative splicing occurs, exons and introns are arranged in different manners to produce alternative mRNA transcripts, eventually leading to alternative versions of the protein codified by the initial gene, thus creating protein isoforms. The alternative splicing for a given gene might be characteristic from certain tissues, cell types, of developmental stages, hence being an effective mechanism for regulating protein expression. This is the process pointed out as responsible for the complexity of mammalian species from a small number of starting genes. Alternative splicing was first predicted in 1978, and first reported after discovery of cDNA isoforms [Breitbart *et al*., 1987]. It has been estimated that approximately 60% of the human genes are alternatively spliced [Croft *et al*., 2000], but next-generation DNA sequencing data suggest that about 95% of all the human multiexon genes are actually alternatively spliced [Pan *et al*., 2008]. It has even been suggested that alternative splicing could generate more transcripts from a single gene than the total number of genes in an entire genome [Black, 2003].

However, aberrant and non-normal alternative splicing events play an important role in cancer processes, generating new mRNAs or varying the normal levels in the cell. Aberrant alternative splicing derives from misregulation of the splicing code, due to mutations in *cis*-elements of the mRNA transcripts codifying regulatory proteins, or changes on the expression, location or PTMs of *trans*-acting RNA binding proteins [Brinkman, 2004; Rajan *et al*., 2009]. Usually, aberrantly spliced mRNAs are targeted for degradation (nonsense-mediated mRNA decay), though they may end up being translated into proteins with oncogenic potential (involved in apoptosis, metastasis, etc.).

Alternative splicing has been reported for a large number of potential and existing cancer biomarkers. Nowadays there are several databases containing alternatively spliced sequences. One of the largest is the ECgene database (http://genome.ewha.ac.kr/ECgene/) [Kim *et al*., 2005], where entries are scored on the basis of the cumulative evidence supporting the existence of the variants annotated. In the review by Brinkman [2004], the readers can find a large collection of examples of alternatively spliced isoforms with a potential role as biomarkers. Relevant examples worth elaborating are *KLK3*, *CD44*, *WT1*, *BCRA1*, *MDM2* or *FGFR*.

From the KLK family, a well-known example is the *KLK3* that encodes the prostate-specific antigen (PSA), widely employed as a serum marker for prostate cancer. For this gene, 12 splice variants have been reported [Heuzé-Vourc'h*et al*., 2003], predicted to encode at

least 8 proteins, 5 of which could be theoretically secreted as the classical PSA. But the KLK gene family contains not only *KLK3*, but at least 15 genes leading to 80 transcripts [Yousef and Diamandis, 2001], many of which have been associated with tumorigenesis. From these, 41 mRNA variants are predicted to encode new proteins, and 93% of them would be truncated, 71% due to a shift on the open reading frame [Kurlender *et al*., 2005]. As regards other members of the KLK family, *KLK2* has been shown to predict positivity of lymph nodes in prostate cancer [Slawin *et al*., 2000]; *KLK13* has five splice variants that only appear in normal testis tissue but have never been found in testicular tumors [Chang *et al*., 2001]; *KLK5* and *KLK7* are overexpressed in ovarian carcinoma cell lines [Dong *et al*., 2003]; *KLK11* has two alternatively spliced forms, but only one of them is associated with more aggressiveness in prostate carcinomas [Nakamura *et al*., 2003].

The CD44 protein is a multifunctional cell receptor. The *CD44* gene is formed by 20 exons, the first 5 and the last 5 being conserved, whereas the intermediate 10 exons are subjected to alternative splicing, therefore constituting the variable region of the molecule. Alterations of the normal CD44 and specific variants have been associated with tumorigenesis and metastasis. For instance, the *CD44v10* expression was correlated with metastasis in pancreatic cells [Navaglia *et al*., 2003]; *CD44v6* is correlated with advanced-stage gastric cancer and lower survival rate [Xin *et al*., 2001]; in prostate cancer, an overexpression of *CD44v7-9* has been found [Iczkowski *et al*., 2003]; *CD44v5* is related to aggressiveness of thymic epithelial tumors [Lee *et al*., 2003]; and *CD44v3* decreased levels are associated with lung cancer [Shimbori *et al*., 2003].

The *WT1* gene (Wilms tumor 1) encodes a zinc finger transcription factor that seems to function in transcriptional activation of genes related with renal development and sex determination [Wagner *et al*., 2003]. Normal splicing of this gene involves the insertion of exon 5; in 63% of the Wilms tumors, it was found this splicing was disrupted [Baudry *et al*., 2000].

BRCA1 and *BRCA2* are two tumor suppressor genes involved in 90% of the familial breast cancers, but also in ovarian and prostate cancer. It is estimated that at least 4% of the carcinogenic alterations are splice variants, as for instance the elimination of a nuclear localization signal in some variants of the *BRCA1*, which then alters the cell location of the final protein product [Claes *et al*., 2003].

Regarding the *MDM2*, this gene encodes a nuclear phosphoprotein that binds p53, inhibiting thus its tumor suppressor activity. In tumors, about 40 splice variants of the *MDM2* have been found, and some of them have been related to tumor traits as higher aggressiveness in the case of breast cancer [Hori *et al*., 2000].

On the other hand, the global expression levels of mRNA variants have also been evaluated as tumoral markers. Most examples deal with prostate cancer, where cancer versus non-cancer cell lines, as well as normal versus tumoral tissue samples, have been studied by multiple comparison approaches and predictive models to capture splice features discriminating the tumor and normal cases [Li *et al*., 2006; Zhang *et al*., 2006; Murlidharan Nair, 2009].

Consequently, these new or altered molecules are valuable diagnostic and prognostic cancer biomarkers. There are several recent methods for detection of the alternative spliced forms typical of the tumor state, and for correlation of those isoforms with the clinical characteristics. One of these technologies is the splicing-specific microarrays, which can be further subdivided into exon arrays (to study all exons) and exon junction arrays (to study

specific splice junctions). A more recent method, relatively unbiased and based on next-generation sequencing, is the so-called mRNA-Seq [Rajan *et al*., 2009]. Detection in the clinical setting could be more simply tackled at the mRNA level by PCR amplification using variant-specific primers, though some of the previous techniques could be adapted to the requirements of the testing routine.

The tumor alternatively spliced transcripts can be targeted for degradation by use of small interfering RNAs (siRNAs) or synthetic modified oligonucleotides (SMOs). On the other hand, defective forms can be supplied through gene therapy strategies, delivering pre-mRNA to induce in *trans* the expression of a wild-type protein, or directly delivering antioncogenic recombinant proteins. Further down the synthesis lane, isoform-specific antibodies can be raised to block the function of the tumor-related splice variants, and they would be easier to introduce in the clinical setting, given the current equipments usually available in clinical laboratories.

Isoforms Generated at the Proteome Level

Polypeptide chains can experience covalent chemical modifications during or after their translation. More than 300 different types of these PTM events have been described (table 1), many of which perform crucial roles in normal cellular physiology, but also in disease [Zhao and Jensen, 2009]. This is due to their role altering both the physical and chemical properties of the polypeptides, which can in turn affect the folding, stability, activity and function of the final protein product.

On the other hand, PTMs are one of the sources of the so-called tumor autoantibodies. The reason is that the presence of PTMs in tumor-related proteins can induce an immune response, by generating a neo-epitope or by enhancing self-epitope presentation and affinity to the major histocompatibility complex or the T-cell receptor. The immune response against such immunogenic epitopes induces the production of autoantibodies which in some cases behave as serological biomarkers for the cancer [Tan *et al*., 2009].

The PTMs known until present have been divided into several classes depending on the nature of the modification:

a) Addition of a functional group. This is the class including a larger variety of modifications, which include acetylation (usually at the N-terminus), alkylation (as methylation, usually occurring at lysine or arginine residues), amidation at C-terminus, biotinylation, carboxylation, formylation, glutamylation, glycosylation, glycation, glycylation, addition of glycosylphosphatidylinositol (GPI), heme group attachment, hydroxylation, iodination, isoprenylation (prenylation), myristoylation, palmitoylation, attachment of nucleotides or derivatives (as ADP-ribosylation), nitration, S-nitrosylation, oxidation, addition of lipoyl and phosphopantetheinyl groups, phosphorylation, proline isomerization, transglutamination, tRNA-mediated addition of amino acids such as arginylation, sulfation (addition of sulfate groups to tyrosine), or selenoylation (co-translational incorporation of selenium).

b) Addition of a protein or a peptide. Relevant examples are ubiquitination, SUMOylation, neddylation or glutathionylation.

c) Chemical alteration of an amino acid, such as in the citrullination (deimination of arginine to citrulline), deamidation (conversion of glutamine to glutamic acid, or asparagine to aspartic acid), or pyroglutamate formation.

The PTM most studied, and also one of the most prevailing, is probably*phosphorylation*. The substrates of this modificationare mainly serine, threonine or tyrosine residues. More than 500 protein kinase genes have been identified in the human genome [Manning *et al*., 2002; Lu and Hunter, 2009] and it is considered that about one third of all the eukaryotic proteins bear modifier phosphates [Gevaert *et al*., 2007].

Phosphorylation is one of the best studied mechanisms of enzyme activity regulation and it has fundamental roles in the intermediary metabolism. Protein phosphorylation-mediated signaling networks regulate cellular responses to environmental cues including mechanical stress, growth factors and cytokines, cell-cell interactions, and cell-matrix interactions. Moreover, protein phosphorylation plays a key role in regulating proliferation, apoptosis, and migration [White, 2008]. Given the importance of this PTM, it is not surprising that deregulation of protein kinases and phosphatases has been linked to a vast number of pathologies including cancer. For example, recently Lee *et al*. [2009] described the establishment of a fully automatic online titanium dioxide (TiO2)-based 3-D LC (strong cationic exchange/TiO2/C18)-MS3-linear ion trap system, which resulted in high-resolution separationof phosphopeptides in a reproducible manner without noticeable sample loss. Using this system, they were able to identify and quantify phosphorylated biomarkers present in cultured cells (HepG2), tumor tissues and plasma of hepatocellular carcinoma patients. Two examples of potential hepatocellular carcinoma phospho-biomarkers, including plectin-1 (phospho-Ser-4253) and α-HS-glycoprotein (phospho-Ser-138 and -312), were identified by this analysis.

Glycosylation is defined as the introduction of monosaccharides, oligosaccharides, and polysaccharides as covalent components of proteins. Glycosylation is one of the most common PTMs, with implications in protein folding, stability, protease resistance, solubility and integrity. At least 50% of the human proteins are glycosylated, with some estimates being as high as 70% [An *et al*., 2009].Among the various modifications a protein can undergo in a living cell, the covalent attachment of carbohydrates is the most diverse since while the proteome is coded in the genome, no template exists for glycosylation.

The modification of proteins by glycans occurs primarily at asparagine residues (*N*-linked glycans), and at serine or threonine residues (*O*-linked glycans). Typically glycoproteins that have complex glycan structures are membrane-bound or secreted, and are involved in intercellular or systemic communication. Besides, *N*-acetylglucosamine linked to serine or threonine residues (*O*-GlcNAc) has been identified as an intracellular glycosylation event, which may have a regulatory role similar to phosphorylation, as it is quickly cycling in response to cellular signals [Krueger and Srivastava, 2006]. Moreover, this kind of glycosylation targets many proteins also modified by phosphorylation, and it is postulated that it may serve an antagonistic role [Ahmad *et al*., 2006].

Table 1. Most common PTMs, along with some examples of modified proteins

MODIFICATION	EXAMPLES
Acetylation	Histones, histone deacetylases, NF-κB, p53, Ras
Addition of GPI	Acetylcholinesterase, alkaline phosphatase, Thy-1
Addition of lipoyl group	Piruvate dehydrogenase complex
Addition of phosphopantetheinyl group	Fatty acid synthase
ADP-ribosylation	eEF-2
Amidation	Oxytocin, vasopresin
Biotinylation	Piruvate carboxylase
Carboxylation	Profactor IX, profactor X, prothrombin
Citrullination	Histones, myelin basic protein
Deamidation	MP26 (lens membranes)
Formylation	Histones
Glutamylation	α-tubulin
Glutathionylation	α-ketoglutarate dehydrogenase, caspase-3, GADPH, p53
Glycation	Hemoglobin
Glycosylation	AFP, CA-125, CA-19.9, CD44, MUC1, PSA
Glycylation	β-tubulin
Heme group attachment	Cytochromes, hemoglobin
Hydroxylation	Collagens, HIF-1α
Iodination	Thyroxine (T4 hormone), triiodotironine (T3 hormone)
Isoprenylation	GTPases of Rho and Rab families, k-Ras, rhodopsin kinase
Methylation (alkylation)	Calmodulin, cytochrome c, DNA-polymerase β, histones
Myristoylation	HIV Gag , PKA
Neddylation	Cullin-based E3 ligases, p53
Nitration	Manganese superoxide dismutase
Oxidation	Actin, enolase, myosin, peroxiredoxins, thioredoxin
Palmitoylation	CCR chemokine receptor, CD8α chain, tyrosine kinase
Phosphorylation	EGFR, glycogen synthase, MAP kinases, p53, PDGFR
Proline isomerization	Cyclin D1
Pyroglutamate formation	Bacteriorhodopsin
Selenoylation	Glutathione peroxidase
S-nitrosylation	GAPDH, β-hemoglobin
Sulfation	CCRC5 receptor, fibrinogen, gastrin
SUMOylation	Histone deacetylases, histones, NF-κB, p53
Transglutamination	Mitochondrial aconitase
tRNA-Arg addition (arginylation)	β-actin, calreticulin
Ubiquitination	Histones, p53, plasma membrane receptors

Glycans are produced by a set of competing glycosyltransferases. For this reason, the population of glycans occurring at a given site is often heterogeneous, as it may be occupied by a number of structurally distinct glycans. Therefore, if a protein contains 3 sites of glycosylation that can be occupied by 10 different glycans in each site, it can result in 1,000 different glycoforms of the protein [An *et al*., 2009]. The "glycoforms" are thus defined as the different versions of a glycoprotein, with diverse polysaccharides attached to them either co- or post-translationally. If the diversity resides in the glycoforms showing different site occupancy, the phenomenon is called "macroheterogeneity"; when the structure of the carbohydrates attached (or even their linkage position) to the same site is the one varying, then it is considered as "microheterogeneity". Both types of heterogeneity affect the size and charge of a glycoprotein. One commonly cited example of glycoforms is the chicken ovalbumin, which contains a single glycosylation site (the Asn-293 residue) where at least a dozen different oligosaccharides can bind [Carlsson, l993].

Research going back several years has yielded evidence that glycosylation is altered in cancer [Durand and Seta, 2000]. These cancer-related glycoproteins are frequently shed into the bloodstream, and there behave as easily detectable and non-invasive tumor markers for diagnosis, prognosis and follow-up. Hence glycosylation is one of the PTMs with higher clinical relevance. Furthermore, some cancer cells produce proteins with such differences in glycosylation from non-cancerous cells, that the proteins are categorized as tumor-associated antigens, and they may even elicit a humoral immune response, as reviewed several decades ago by Hakomori [Hakomori, 1984] and recently by others [see for example Dube and Bertozzi, 2005].Examples of glycoprotein diagnostic markersaccepted in the clinical oncological practice are the α-fetoprotein (AFP) for hepatocellular cancer [Poon *et al*., 2002], and the PSA for prostate cancer [Tabarés *et al*., 2006]. Altered and different glycosylation of serum AFP has been found in patients suffering from hepatocellular carcinoma and seminomatous germ-cell tumors. In those, AFP is re-expressed, and its serum concentration greatly increases. Moreover, in adults, glycoforms of the re-expressed AFP specific for hepatic regeneration,and glycoforms of yolk sac-derived tumors, are identified by the presence of a fucose on the first *N*-acetylglucosamine of the glycan core, or by an additional bisected *N*-acetylglucosamine on the first mannose, respectively [reviewed in Durand and Seta, 2000].

In general, alterations of the glycosylation patterns during carcinogenesis lead to a shift in the glycoform production, which nowadays is easily detectable, e.g. through proteomic techniques. Hence efforts to elucidate the spectrum of glycan alterations associated with particular tumor types have been accelerated thanks to the methodological improvements. For example, there have been substantial recent developments in sample preparation and MS methods. This has allowed a systematic analysis of the glycopeptides derived from biological samples, and quantification of their glycan-related isoforms, thus yielding identification of specific glycoforms associated with cancer. White and coworkers [2009] used a glycomic approach to characterize glycans cleaved from the PSA and prostatic acid phosphatase purified from seminal samples of normal controls, benign prostatic disease patients, and prostate cancer patients. The cleaved glycans were permethylated by chemically converting them to methylated derivatives, which improves MS analysis and stabilizes sialic acids and has, therefore, become a common method in glycomics. Analysis by HPLC/MALDI-TOF resulted in the discovery of 40 putative glycoforms of the PSA and 21 glycoforms of the prostatic acid phosphatase. Another example of a protein with glycoforms altered by

carcinogenesis is the haptoglobin. Regarding this protein, two studies by Miyoshi and co-workers [Miyoshi and Nakano, 2008; Nakano *et al*., 2008] characterized increased levels of fucosylated haptoglobin in pancreatic cancer. Subsequent LC/ESI-MS corroborated the increased level of the fucosylated glycan in the haptoglobin purified from pancreatic cancer patients' sera. Furthermore, they observed an increase of triantennary *N*-glycans containing LeX-type fucose at the haptoglobin Asn-211 site in pancreatic cancer, besides a new bifucosylated tetraantennary *N*-glycan related to the cancer process.

Various types of *lipids* can be found modifying proteins, including isoprenoids, fatty acids, GPI anchors, cholesterol and electrophilic lipids.One of the most common consequences of this PTM is an increased hydrophobicity of the modified protein, facilitating its interaction with hydrophobic environments like cellular membranes or certain protein domains [Melkonian *et al*., 1999]. In addition, the reversibility of the lipidic modification influences the dynamics of protein localization and function [Rocks *et al*., 2005]. The lipidic PTM has been also involved in protein-protein interactions, which again may have an effect in subcellular localization, but are also critical for cell signaling [Resh, 2006]. In consequence, the involvement of lipid modification of proteins in different human diseases, including cancer, has been described[Pereira-Leal *et al*., 2001; Winter-Vann and Casey, 2005].

Isoprenylation or prenylation is defined as the addition of an isoprenoid lipid (the 15-carbon farnesyl group or the 20-carbon geranylgeranyl group) to a cysteine residue located near the C- terminal end of a protein in a thioether linkage [reviewed in Pérez-Sala, 2007]. This modificationoccurs shortly after translation and it is irreversible. It seems to be key for the subcellular localization of the proteins it affects, besides altering their activity and interactions with other proteins. Several hundred isoprenylated proteins have been described. The list includes some names with known implication in carcinogenesis, as the H-Ras, N-Ras and K-Ras signaling proteins [Hancock *et al*., 1989], proteins from both the Rho and Rab families of GTPases [Kinsella and Maltese, 1992], or the γ subunits of heterotrimeric G proteins [Fukada *et al*., 1990]. Detection of isoprenylated proteins as potential cancer biomarkers has not been widely explored. Numerous cancers, however, are characterized by mutations in Ras proteins leading to persistent activation of their affected pathways. Because mutant Ras proteins are dependent on prenylation for their activation in promoting oncogenesis, considerable effort has been invested designing inhibitors of various stages of this process. Identification of tumors bearing mutations in Ras proteins can thus serve as a prognostic marker to predict which cancer may respond to chemotherapeutic agents interfering with prenylation [reviewed in Krueger and Srivastava, 2006].

Protein acylation is carried out by long-chain acyl~CoAs as donor substrates to nucleophilic side-chains in proteins. In this event, the long-chain myristoyl~CoA and palmitoyl~CoA act as donors for attaching the C14 and C16 acyl chains to the substrate proteins. These acyl chains will then behave as lipid anchors and direct the acylated proteins to the cellular membranes. The HIV Gag protein and the catalytic subunit of the cAMP-dependent protein kinase (PKA) are known examples of myristoylated proteins [Lakshmikuttyamma *et al*., 2008]. Palmitoylation is typically found in membrane receptors, though there are also cytoplasmic proteins modified with this acyl group. Typical examples of the latter are the mammalian H-Ras, N-Ras and K-Ras proteins, which display alterations in their trafficking, location and function [reviewed in Walsh *et al*., 2005]. Recently, estrogen receptor (ER) isoforms (ER α and ER β) belonging to the nuclear receptor superfamily have

been added to the palmitoylproteome by Marino and Ascenzi [2008]. These authors described that palmitoylation allows ER α and ER β localization at the plasma membrane, where they associate with caveolin-1. Upon 17β-estradiol stimulation, ER α dissociates from caveolin-1 allowing the activation of rapid signals relevant for cell proliferation. In contrast, 17β-estradiol stimulation increases ER β association with caveolin-1 and activates p38 kinase and the downstream proapoptotic cascade. These data highlight the physiological role of palmitoylation in modulating the ER α and ER β localization at the plasma membrane, and the regulation of different estradiol-induced non-genomic functions relevant for controlling cell proliferation. Although both ER isoforms undergo palmitoylation, their outcome effectsin cell physiology are opposite. Thus,the expression of each ER isoform and/or their co-expressionin the cells could account for the different 17β-estradiol-dependent modulation of cell proliferation.

GPI anchor refers to a glycolipid added to the C-terminal end of a protein. It consists of a hydrophobic phosphatidylinositol group bound through a carbohydrate-containing linker (glucosamine and mannose linked to a phosphorylethanolamine residue) to the C-terminal amino acid of the mature protein. Within the hydrophobic phosphatidylinositol group, the two fatty acids can anchor the protein to the cellular membrane. GPI-linked proteins are frequently located in the lipid rafts, which constitute organized microdomains in the plasma membrane [Taylor and Drickamer, 2006]. Examples of mammalian proteins attached to the membrane by GPI anchors are the T-cell marker Thy-1, the enzyme acetylcholinesterase, as well as both the intestinal and placental alkaline phosphatases. One GPI-anchored cell surface protein identified as a prognostic marker for prostate cancer is the prostate stem cell antigen (PSCA). This protein included in the Ly-6/Thy-1 family is expressed in normal human prostate and overexpressed in human prostate cancers, where the increased level has been correlated with advanced stage and bone metastasis [Jalkut and Reiter, 2002].

Protein acetylation is a PTM based on the addition of the short chain acetyl~CoA. The N-terminal acetylation is one of the most common PTMs, occurring on approximately 85% of the eukaryotic proteins [Polevoda and Sherman, 2000]. Nevertheless its biological significance is unclear. An example of its importance is given by the actin and tropomyosin, which require acetylation to form the actin filaments. Identification of diverse substrates for protein acetylation in both cytosolic and mitochondrial fractions opened new avenues for its functional studies in energy metabolism, signal transduction, and mitochondrial regulation [Kim *et al*., 2006]. On the other hand, it is known that acetylation of the lysine amino group of some proteins, especially transcription factors and histones, has an impact in transcription and in chromatin structure and rearrangement. These are all regulatory functions, which can be reversed by deacetylases [Walsh, 2006]. In this regard, histone hypoacetylation due to excess histone deacetylases has been described in patients with acute leukemia and the effect of phenylhexylisothiocyanate for correcting deficient acetylation has been analyzed [Xiao *et al*., 2010].

The *methylation* of proteins occurs when one carbon is transferred at one or more nucleophilic amino acid side-chains, usually on arginine or lysine residues. The best studied examples are the methylation of calmodulin [Barten and O'Dea, 1990] and the cytochrome c [Frost *et al*., 1990]. Arginine can be methylated once or twice, with either both methyl groups on one terminal nitrogen (asymmetric dimethylated arginine) or one on each nitrogen (symmetric dimethylated arginine). Lysine can be methylated once, twice or three times, and those that are di- or trimethylated are not available for other PTMs [Krueger and Srivastava,

2006]. The importance of methylation in protein function and tumorigenesis is still a field in its infancy. Protein methylation has been most well studied in histones. *N*-acetylation and *N*-methylation of side-chains in histone tails can have opposite effects on gene transcription. Increased levels of histone acetylation are generally associated with local activation of gene transcription [Zhang *et al*., 2002] whereas lysine methylation in histones has been shown to localize to the promoters of repressed genes [Berger, 2007]. Although much scientific effort has gone into studying these PTMs on histones, acetylation and methylation events occur on many other proteins.

Ubiquitination consists in the covalent addition of the small protein ubiquitin to the lysine side-chain. Usually this PTM functions in the way of polyubiquitination tagging the protein for degradation by the proteasome. When it consists of a monoubiquitination, proteins are generally not degraded, but involved in signaling pathways, as is the case of several histones and plasma membrane receptors [Schnell and Hicke, 2003]. It has been shown that monoubiquitination regulates cell division, differentiation, signal transduction, protein trafficking and quality control [Mukhopadhyay and Riezman, 2007]. The success of the drug bortezomib in clinical trials for treatment of multiple myeloma highlights the importance of ubiquitination in cancer biology [Einsele, 2010]. Bortezomib is a 26S proteasome inhibitor promoting cell cycle arrest and apoptosis, presumably by preventing degradation of critical transcription factors, tumor suppressors, and other regulatory proteins that may be subject to polyubiquitination during oncogenic progression. As better tools are developed to study ubiquitination, we should expect to find candidates for cancer biomarkers leading to additional drug targets based on this type of PTM. Another molecule that is similar to ubiquitin is the protein small ubiquitin-like modifier (SUMO). Although the two proteins are only 18% identical, they show similarity in their 3-D structures. The attachment of a SUMO peptide, the process of which is often referred as *SUMOylation*, is a transient PTM that targets nuclear as well as cytoplasmic and even plasma membrane proteins [Walsh, 2006]. More than 1,000 proteins have been identified as potential SUMO-conjugation targets, and this pathway has been implicated in controlling many aspects of cell physiology, including cell-cycle regulation, transcription, nucleocytoplasmatic transport, DNA replication and repair, chromosome dynamics, apoptosis, and ribosome biogenesis [Wang and Dasso, 2009]. Although its function is as diverse as its substrates, one generalization could be that modification of a protein substrate by SUMO alters its interactions with other proteins and DNA molecules. Furthermore, compromised SUMOylation has been linked to some cancers, through regulation of nuclear receptor-mediated gene expression (for example the androgen and estrogen receptors, with roles in prostate and breast cancer, respectively). A role for SUMO modification in cancer metastasis has been suggested through association of the SUMO pathway with the regulation of transcription of *KAI1*, a metastasis suppressor gene [reviewed in Andreou and Tavernakaris, 2009].

Nitration and nitrosylation of tyrosine, tryptophan, methionine and cysteine side chains comprise the bulk of nitrosative modifications. These additions are mediated by reactive nitrogen species produced during development, oxidative stress and aging. An increased amount of reactive nitrogen species result from the reaction of excess or deregulated nitric oxide with reactive oxygen species [Yeo *et al*., 2008]. Tyrosine nitrosative modifications are perhaps the best characterized by the adducts generated. Increased levels of tyrosine nitration have been implicated in age-related neurodegenerative diseases and can serve as a biomarker for oxidative stress [Yeo *et al*., 2008]. Accumulating evidence indicates important roles for

nitrosylation both in normal physiology and in a broad spectrum of human diseases, such as cardiovascular, pulmonary, musculoskeletal and neurological (dys)function, as well as in cancer. In many cases, pathophysiology correlates with hypo- or hyper-nytrosylation of specific protein targets. For example, Ras S-nitrosylation is implicated in the initiation of tumorigenesis and maintenance of established tumors [Foster *et al*., 2009]. Under normal physiological conditions, the formation of nitrosative tyrosine adducts is prevented by the presence of reducing agents such as glutathione [Chen *et al*., 2004].

The *proteolytic cleavage* of proteins occurs after translation. The most simple consists on the elimination of the initial methionine, but in many cases proteins are synthesized as inactive precursors that require cleavage of a peptide to be activated. This is for instance the case of pancreatic and blood clotting enzymes [reviewed in Walsh *et al*., 2005].

The PTMs described in this chapter are the most frequently found, but overall more than 300 types of covalent modifications have been reported to date. Noticeably, a single protein may be modified by more than one type of PTM. For instance, the transcription factor and tumor suppressor p53 has 18 serine/threonine sites that can be phosphorylated and 10 lysine residues that can accommodate acetylation, methylation and attachment of ubiquitin, SUMO and NEDD [Thomson and Gunawardena, 2009]. Another example is the two lipidations of Ras followed by region-specific proteolysis and C-terminal *O*-methylation [Walsh *et al*., 2005].

During the past several years, tremendous progress has been made in developing PTM-specific enrichment methods and MS-based proteomics technologies for PTM analysis [Zhao and Jensen, 2009]. These enrichment techniques can alleviate the problems created by rare modifications. Among the variety of affinity enrichment strategies available, there are two major categories. First are approaches that use antibodies to recognize a specific PTM or uniquely modified peptide. For example, antiphosphotyrosine antibodies are used to enrich for peptides with phosphotyrosine residues [Rush *et al*., 2005]. Second, there are existing and emerging technologies to enrich for PTMs based on the chemical affinity of a modification for an immobilized resin. Such techniques include immobilized metal affinity chromatography (IMAC) for phosphorylations and lectin chromatography for glycosylations [Ito *et al*., 2009]. Another widely used proteomic technique useful in the identification of PTMs is two-dimensional electrophoresis (2-DE). PTMs can alter the isoelectric point and electrophoretic mobility of a protein in a 2-DE experiment. When such a change is detected between different cell types or growth conditions, the protein can be isolated and sequenced to identify its PTMs. For example, a differentially phosphorylated protein may appear as a train of 2-DE spots with different isoelectric points but with similar molecular weights. This pattern is sometimes referred to as "pearls on a string".

Specific protein staining methods for revealing protein PTMs have been devised over the years. These include fluorescent methods for the direct detection of phosphoproteins and glycoproteins in gels. The proteins can then be in-gel digested, and the recovered peptides can be analyzed by MS to identify, validate, and map the expected PTMs. However, the 2-DE approaches to identify PTMs are not trivial, since the recovery and analysis of the modified peptides are often problematic. Moreover, there are other limitations that must be considered when using MS-based proteomics to identify PTMs. Some PTMs, such as phosphorylation of serine, tyrosine or threonine, and *O*-linked or *N*-linked glycosylation, are labile, and maintaining the modification during sample preparation can be difficult [Farley and Link, 2009].

Relevant Examples of Isoforms as Cancer Biomarkers

1. Prostate-Specific Antigen (PSA)

Detection in serum of the PSA is currently used in prostate cancer screening and diagnosis. PSA is a serine protease, member of the human KLK family. It is produced in the epithelial prostate cells and secreted into the seminal fluid as pro-PSA, and subsequently activated. In prostate disease, disruption of the prostatic basement membrane allows PSA to access the peripheral circulation and be detected in blood [Nogueira *et al*., 2009]. PSA circulates in free and complexed forms. The predominant molecular form in blood is the 80-90 kDa complex of PSA with α-1-antichymotrypsin (70-90%). Complexes of PSA with other protease inhibitors (such as α-2-macroglobulin or α-1-antitrypsin) are present in minor quantities, whereas free forms (fPSA) represent 10%-30% of the total PSA content (tPSA) [Balk *et al*., 2003]. The current immunoassays can detect both free and complexed PSA.

In 1990, the upper threshold for normal PSA level was established at 4.0 ng/mL [Cooner *et al*., 1990]. Nevertheless, the use of this cut-off point has been under continuous re-evaluation, since a number of men with prostate cancer actually have PSA values lower than 4.0 ng/mL. Similarly, a PSA level above 4.0 ng/mL does not always indicate the presence of prostate cancer, but can be associated with other non-malignant prostatic diseases such as benign prostatic hyperplasia (BPH), inflammation or prostatitis [Lilja *et al*., 2008]. Thus, several parameters have been proposed to improve the use of PSA as a tumor marker: age-adjusted PSA; PSA density (serum PSA regarding prostate volume); PSA kinetics (doubling time and velocity); and the proportion of different PSA isoforms as the most promising tool [Nogueira *et al*., 2009].

As the content of sialic acid on the PSA *N*-glycan chain can modify the isoelectric point of PSA [Tabarés *et al*., 2006], 2-DE has been used by several authors to study PSA forms from different sources. Charrier *et al*. [1999] determined that the pattern of fPSA isoforms in serum of prostate cancer and BPH was different, observing a lower proportion of cleaved isoforms in cancer than in BPH, probably due to the incremented addition of *N*-glycan chains in PSA during oncogenesis. More recently, isoforms of fPSA and PSA released from the complex with α-1-antichymotrypsin were evaluated by 2-DE in serum of prostate cancer and BPH patients, and it was reported that the percentage of one of the five detected isoforms, named as F3 by the authors, showed a higher sensitivity and specificity for prostate cancer diagnosis than the currently used tPSA and fPSA-percentage tests [Sarrats *et al*., 2010]. In other current study, the use of p2PSA, a pro-PSA isoform containing two amino acids in the propeptide leader, in combination with tPSA and fPSA, showed a better diagnostic accuracy in prostate cancer [Jansen *et al*., 2010]. Therefore, there is a growing belief that the differential isoform behavior of the tumor marker PSA could improve prostate cancer diagnosis.

2. Haptoglobin

It has been claimed that haptoglobin may not be specific enough for cancer diagnosis as elevated serum concentrations of this well-known acute phase glycoprotein also occur in other diseases involving inflammation or tissue necrosis. For this reason, it has been traditionally discarded as a valid disease marker, as other high-abundance serum proteins. However, it is now known that many of these proteins suffer PTMs related to disease development, and thus certain isoforms could be more indicative of the disease condition than the whole pool of protein.

Haptoglobin is a heterotetramer composed of two α and two β subunits connected by disulfide bonds, normally secreted by liver cells, as well as by tumor cells in cancer patients. It prevents renal damage by binding to hemoglobin and recycling the iron, protects DNA and tissues against oxidative stress, suppresses T-helper cell type-2 cytokine release, and regulates both epidermal cell transformation and immune responses [Oh *et al*., 1990]. In humans, there are three major phenotypes of haptoglobin due to two different types of α subunits: 1-1, 2-1 and 2-2, where numbers 1 and 2 stand respectively for α-1 and α-2. The β chain (40 kDa) is heavier than the α chain, and is identical in all haptoglobin types.

The appearance and different behavior of haptoglobin isoforms has been reported in many proteomic studies of disease, particularly in relation to cancer. For instance, using the SELDI-TOF technology, dissociated fragments of haptoglobin were found elevated in serum of patients with ovarian cancer, suggesting that these fragments inhibit the native haptoglobin-hemoglobin complex, and interfere with immune cellular responses through their potent immunosuppressant activities [Ye *et al*., 2003]. In sera from head and neck cancer patients, the expression levels of two isoforms of haptoglobin α-2 chain were also found to be upregulated [Chen *et al*., 2008]. Using a glycoproteomic approach, Ang *et al*. [2006] clearly demonstrated that specific haptoglobin glycoforms, with altered sialylation and fucosylation, were not only increased in the sera of patients with hepatocellular carcinoma, but also positively associated with the degree of tumor progression. Moreover, the study of Hamrita *et al*. [2009] presented for the first time evidence of the increased expression of different isoforms of the haptoglobin precursor in sera of breast cancer patients. These isoforms may have different biological function and their occurrence may be related to a disease-specific defective intracellular processing.

3. Clusterin (CLU)

The highly heterodimeric CLU is a conserved and ubiquitously expressed glycoprotein. The *CLU* gene is located in chromosome 8, and has 9 exons that encode three protein forms with distinct subcellular locations. The secreted form or sCLU (70-80 kDa) presents two subunits, α (34-36 kDa) and β (36-39 kDa), held by five disulfide bonds; it exhibits pro-survival activity, and is found in the extracellular milieu. Its intracellular precursor (60 kDa) is located in the cytoplasm and matures to the sCLU through glycosylation and proteolytic cleavage [De Silva *et al*., 1990]. Lastly, there is a CLU form that accumulates in the nucleus of apoptotic cells, called nCLU (50-55 kDa) [Leskov *et al*., 2003]. Though it is still under discussion, it seems sCLU is translated from the full-length mRNA, and nCLU from an mRNA lacking exon 2 [Leskov *et al*., 2003]. On the other hand, as our group described

[Rodríguez-Piñeiro *et al*., 2006], sCLU presents a large number of isoforms with slight differences in mass and isoelectric point (at least 18 isoforms in serum). CLU is implicated in many different biological activities, as apoptosis, cell cycle regulation, DNA repair, tissue remodeling, lipid transport, membrane recycling, cell matrix formation and immune system regulation, and these functions seem to be accomplished by its different forms. For instance, now it seems clear that while sCLU acts as an antiapoptotic molecule, nCLU is proapoptotic.

Elevated CLU levels are found in tissues under stress or during disease, including many cancer types. In colorectal cancer, we and others have found an altered profile of CLU isoforms, and thus we described 6 isoforms upregulated and 15 downregulated or absent in the serum of patients [Rodríguez-Piñeiro *et al*., 2006]. Hence we have proposed a diagnostic role for CLU, and other authors have found is a useful prognostic biomarker for stage-II patients [Kevans *et al*., 2009]. Moreover, we recently found there is an increased synthesis and secretion of CLU in undifferentiated Caco-2 cells, and in SW-480 cells, which resemble non-metastatic colonic tumor cells. Noticeably SW-620 cells, which are metastatic, seemed to have lost the expression of sCLU and its intracellular precursor, and thus the lack of the protective and prosurvival activity might be related to the invasion [Rodríguez-Piñeiro *et al*., manuscript in revision].

In other cancer processes, the behavior of the CLU forms is as complicated, though it can be generalized saying that, in the majority of neoplasms, sCLU is downregulated in early steps whereas its levels increase in advanced stages. This is the case with prostate and breast cancer, for example. However, there are some pathologies as colon and lung cancer, where an initial increased expression seems to be related to prosurvival mechanisms [Bettuzzi, 2009b]. Changes in the nCLU forms have not been so far reliably characterized.

4. Vimentin

Vimentin is a 54-kDa type-III protein of the intermediate filament network that exists between the cell periphery and the nucleus. Hence it serves as a dynamic link between the nuclear matrix, the actin filaments, and the extracellular matrix. Its function was long thought to be purely structural, but now it is well known that vimentin protects the cell against mechanical stress and is also involved in signal transduction, as well as in carcinogenesis and apoptosis [Helfand *et al*., 2005]. Although it is expressed mainly in cells of mesenchymal origin, such as fibroblasts and endothelial cells, it can also be found in a variety of cells of non-mesenchymal origin [Osborn, 1983]. It seems that phosphorylation has a major role in regulating the structure and assembly of vimentin and other intermediate filaments [Eriksson *et al*., 2004].

A large number of reports on the isoforms of vimentin have been published during the last years. By electrophoresis and Western blotting, Molero *et al*. [2005] found a downregulation of vimentin in bovine vascular smooth muscle cells (BVSMCs) incubated with 17β-estradiol, regarding control BVSMCs. However, using 2-DE they were able to detect that the reduction in vimentin expression mainly occurred in the isoforms called 2 and 3. This report reflects that isoform-specific antibodies are necessary to allow detection and distinction of protein isoforms in order to further clarify their specific cellular roles.

Regarding cancer disease, vimentin has been particularly reported to be altered in colon cancer. For instance, Alfonso *et al*. [2005] detected 4 isoforms of this protein (with different

mass and/or isoelectric point) using DIGE (a 2-DE method) followed by immunoblot, suggesting they reflected the different levels of its phosphorylation. In another study in our laboratory, Western blot analysis showed 3 isoforms of vimentin with different mass, which were named as 1, 2 and 3 from larger to smaller [Álvarez-Chaver *et al*., 2007]. Discrepant results have been published about the expression of this protein in colorectal cancer, since both increases as well as decreases have been described. In the work of Alfonso *et al*. [2005] mentioned above, authors reported an upregulation of vimentin in tumors, but they could not confirm the result obtained from 2-DE gels by immunohistochemistry. On the contrary, other studies showed a lower expression of vimentin [as in Friedman *et al*., 2004; Polley *et al*., 2006]. In our study, electrophoresis followed by immunoblot showed a 38% and 26% reduction in the abundance of two isoforms in colorectal tumors, whereas the other isoform could not be observed in the tumor tissue [Álvarez-Chaver *et al*., 2007]. Moreover, subsequent 2-DE and immunoblotting demonstrated many isoforms of vimentin with different mass and isoelectric point in the normal tissue, whereas none of them were visualized in the tumor [Rodríguez-Piñeiro *et al*., 2007].

Overexpression and downregulation of vimentin was also reported in other cancers. For instance in human neuroblastoma cell lines, Urbani *et al*. [2005] detected by 2-DE three overexpressed vimentin isoforms, probably involved in the chemoresistance of these cells to topoisomerase inhibitors. In another previous work, vimentin was identified as an underexpressed spot in 2-DE maps of human gastric carcinoma cells under heat stress, suggesting that this protein may lead to the development of thermoresistance [Sinha *et al*., 2001]. Using different proteomic techniques, vimentin was found increased in micrometastatic breast cancer cells compared with normal breast cells [Willipinski-Stapelfeldt *et al*., 2005]. Troiani *et al*. [2005] identified one phosphorylated isoform of vimentin as a substrate of a mitotic serine/threonine kinase overexpresed in different human tumor types, contributing to the oncogenic transformation. Hong *et al*. [2006] detected a specific vimentin isoform that induces an antibody response in pancreatic cancer. Interestingly, none of several other isoforms of vimentin detectable by these authors in 2-DE gels exhibited reactivity with patient sera. More recently, a glycoproteomic analysis of human lung adenocarcinoma tissues suggested that vimentin from normal and lung tumor tissue differs in their content of sialic acid and terminal *N*-acetylglucosamine [Rho *et al*., 2009].

5. Prohibitin

Prohibitin is a highly conserved protein of about 30 kDa, present in a variety of forms and implicated in cell growth, ageing and cell transformation. It is mainly located in mitochondria, but also in cytoplasm, nucleus and plasma membrane, and it has been identified as a cell surface receptor [Mishra *et al*., 2005]. Prohibitin has been shown to be required for Ras-induced Raf-MEK-ERK activation in the epidermal growth factor receptor signaling pathway [Rajalingam *et al*., 2005].

In the last years, several studies reported different prohibitin isoforms produced by different PTMs. The central and C-terminal parts of prohibitin contain tyrosine residues (Tyr-114 and Tyr-259) that can be phosphorylated and are highly conserved across species, suggesting a functional role for these residues [Mishra *et al*., 2006b]. Han *et al*. [2008] suggested that the serine/threonine protein kinase Akt might regulate the cellular function of

prohibitin via its phosphorylation. Studying the membrane fraction of Ras-transformed fibroblasts, Alfonso *et al*. [2006] identified prohibitin from two spots with different isoelectric points in 2-DE gels, which might correspond to two different phosphorylation states. Other authors reported that distinct changes in the expression of the prohibitin isoforms were observed in the involuting thymus of pregnant woman, with higher expression of the basic forms [Dixit *et al*., 2003]. However, the functional significance of prohibitin phosphorylation remains unclear. In addition to tyrosine phosphorylation, prohibitin can also undergo *O*-linked β-*N*-acetylglucosamine modification, which has been shown to play a regulatory role in tyrosine kinase signaling pathways [Ande *et al*., 2009]. Moreover, Thompson *et al*. [2003] reported that ubiquitination of prohibitin in sperm mitochondria during spermatogenesis is implicated in the targeted degradation of paternal mitochondria after fertilization. This mechanism is consistent with the prevailing view that the inheritance of mitochondrial DNA in mammals is predominantly maternal. Both the expected 30-kDa prohibitin isoform, and a 64-kDa isoform suggestive of tetraubiquitination (each molecule of ubiquitin increases the molecular mass by 8.5 kDa), were largely expressed in the sperm tail fraction and were absent from the sperm head fraction, suggesting an important role of prohibitin in the regulation of mitochondrial inheritance and sperm quality control. More recently, Donoghue *et al*. [2010]observed isoforms of prohibitin with differential changes in rat skeletal muscle of young adult versus aged gastrocnemius tissue, indicating altered PTMs in this protein during muscle aging.

In relation to cancer, prohibitin was found by 2-DE to be overexpressed in membrane fractions from superinvasive human breast cancer cells [Dowling *et al*., 2007], suggesting a potential role of this protein in aggressive metastatic behavior. Prohibitin upregulation was also found in different cell lines of hepatocellular carcinoma [Seow *et al*., 2000], in esophageal squamous cell carcinoma [Ren *et al*., 2010], in colon cancer [Roblick *et al*., 2004], and in prostate cancer [Dart *et al*., 2009], among others. In this last mentioned disease, prohibitin was proposed as a potential biomarker to distinguish BPH and prostate cancer [Ummanni *et al*., 2008]. Moreover, prohibitin can be shed into circulation from colonic malignant cells [Mengwasser *et al*., 2004], although the nature and function of the circulating prohibitin remains unclear. Interestingly, in the study reported by Ren *et al*. [2006] using SELDI-TOF-MS, increased levels of prohibitin were detected in the preoperative serum of patients with gastric cancer, descending to normal levels after surgery. However Kang *et al*. [2008] proposed prohibitin as a potential biomarker for tissue-based detection of gastric cancer, but not for serum-based detection. These contradictory results suggest that an isoform-specific regulation of prohibitin might exist, but to our knowledge there are no studies regarding prohibitin isoforms in cancer. In a previous work performed in our laboratory, prohibitin was identified from a spot increased in the colorectal tumor tissue [Álvarez-Chaver *et al*., 2007]. Afterwards, in a first validation step of this biomarker candidate, at least 7 isoforms with different molecular mass (30-32 kDa) and isoelectric point (5.5-5.9) were detected by Western blotting (figure 1). Overall, the amount of prohibitin was found increased in the tumor tissue, corroborating the 2-DE result. Most of the isoforms were observed in the membrane-associated protein fraction of tumors, showing that prohibitin is located predominantly in cellular membranes during colorectal cancer development. Nevertheless, some isoforms were detected in the soluble fraction, showing a different pattern in tumors compared to healthy tissues.

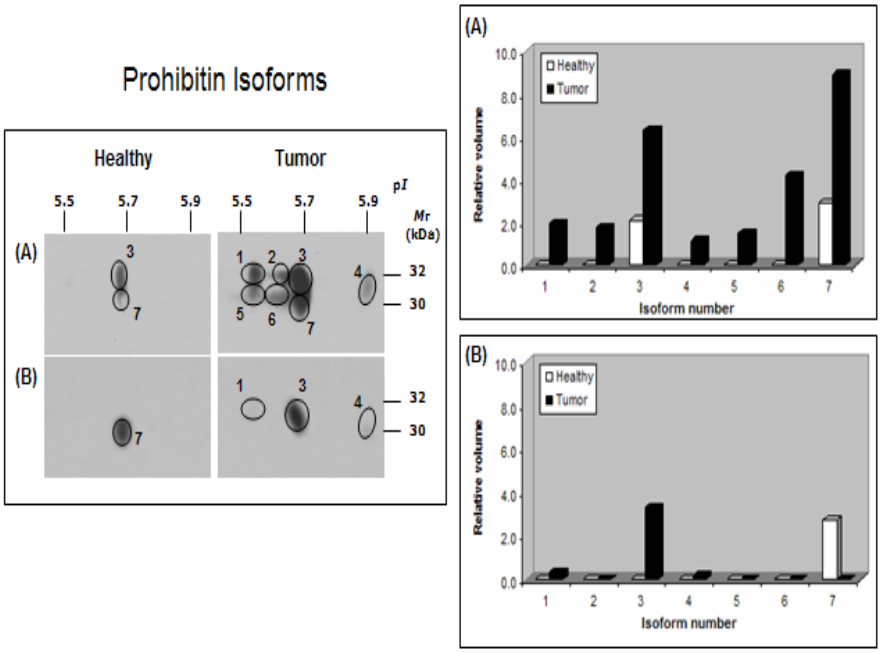

Figure 1. Prohibitin isoform pattern on bidimensional blots from healthy mucosa and tumor tissue. (A) Membrane-associated protein fraction. (B) Soluble protein fraction. Bar diagrams represent the relative volume of the 7 prohibitin isoforms detected. Mr: relative molecular weight; pI: isoelectric point.

6. p53

p53, also known as protein 53 or tumor protein 53, is a tumor suppressor protein involved in preventing carcinogenesis, encoded in humans by the *TP53* gene [Matlashewski *et al*., 1984]. Up to ten p53 isoforms have been identified, originated by transcriptional and post-transcriptional regulation, and by translational control. Moreover, as it has been described early, p53 suffers many PTMs, including phosphorylation, acetylation, ubiquitination, SUMOylation, neddylation, methylation and glycosylation. In addition, there is a post-translational control of protein degradation in response to stress [Hollstein and Hainaut, 2010]. All these factors influence the quantity in the cell and generate changes in its structure, intracellular localization, DNA-binding activity, and interactions with other proteins, and thus regulate its activity.

Many studies have been focused on the biological role of the p53 isoforms [see Marcel and Hainaut, 2009]. However, as Hollstein and Hainaut [2010] indicate, the full range of their biological functions is only beginning to emerge. For instance, the isoform named as ΔNp53 (lacking parts of the N-terminus) acts as antagonist of the canonical p53 (called TAp53α, because it retains the entire N-terminal transactivation domain) by binding to p53 response elements without activating transcription. On the other hand, another isoform named Δ133p53 (initiated at an internal AUG triplet located at position 133) may also exert antagonistic effects, although it does not appear to bind DNA. Recently, it has been proposed that this isoform, together with the p53β isoform (that differs in the C-terminal region respect

to the canonical protein) may be involved in the regulation of replicative senescence [Fujita *et al*., 2009].

Changes after translation of the protein may also confer specific roles to p53. For instance, the induction of apoptosis in non-small cell lung carcinoma cells is dependent on the upregulation of specific phosphorylated p53 isoforms [Mukhopadhyay *et al*., 1998]. It has also been observed that phosphorylation produces differential effects on the ability of p53 to transactivate promoters [Lohrum and Scheidtmann, 1996].

7.Phosphodiesterases (PDEs)

Cyclic nucleotide PDEs comprise a family of related proteins that include at least 18 different isoforms, which can be distinguished on the basis of their amino acid sequences, sensitivity to different activators and inhibitors, and their ability to preferentially hydrolyze different substrates. Considering that epithelial-mesenchymal transition (EMT) is a critical event in the pathogenesis of organ fibrosis and cancer, Kolosionek *et al*. [2009] evaluated the potential role of PDEs in TGF-β1-induced EMT in the human alveolar epithelial type II cell line A549. After induction with TGF-β1, they detected changes in the relative expression of PDE isoforms by means of real-time RT-PCR, immunoblotting, and immunofluorescence, with an increased expression of PDE4A and PDE4D, and a decreased level of PDE1 and PDE3 relative to control A549 cells. The authors claimed there is a relevant biochemical and physiological role for PDE isoforms in the EMT in alveolar epithelial cells; based on its high expression level and activity, PDE4 seems to be a novel target to attenuate EMT-associated lung diseases such as pulmonary fibrosis and lung cancer. On the other hand, the decreased expression of the isoforms PDE1 and PDE3 could be a marker of phenotypic switch.

8. Vascular Adhesion Molecule-1 (VCAM-1)

The VCAM-1 is a cell surface glycoprotein expressed on the apical membrane of endothelial cells activated by cytokines. Two isoforms of VCAM-1 have been reported, a full length protein and a smaller version lacking exon 5. Their expression occurs in response to inflammatory mediators and is dependent on the translocation of the transcription factor NF-κB into the nucleus [Holmes-McNary, 2002]. Recently, Montes-Sánchez *et al*. [2009] analyzed *N*-glycosylation variants of VCAM-1 in primary human endothelial cells stimulated with either TNF or tumoral soluble factors derived from the human breast cancer cell line ZR75.30. Western bidimensional blots revealed three new VCAM-1 isoforms differing in mass and isoelectric point, probably due to the result of differential glycosylation states, expressed in primary human endothelial cells in response to the tumoral soluble factors stimulation. These isoforms could serve as biomarkers to discriminate between inflammation and metastasis.

9. Human Chorionic Gonadotropin (hCG)

The hCG is a hormone produced by placental trophoblastic cells during pregnancy, which maintains the production of progesterone in the corpus luteum. In oncology, it is used as a marker for detection of choriocarcinoma, testicular and ovarian germ cell tumors, and other non-gonadal cancers [Stenman *et al*., 2006]. The hCG protein is highly glycosylated, with about 38 kDa, and composed by two subunits covalently attached and stabilized by three disulfide bonds forming a cysteine knot. The α subunit (14 kDa) is identical to that of other hormones (as the luteinizing hormone), whereas the β subunit (24 kDa) is specific for the hCG and confers its biological specificity [Lapthorn *et al*., 1994]. The form of the protein expressed depends on the biological process, and for instance during pregnancy, the protein is fully expressed, raising the form named as intact hCG heterodimer (intact hCG) that can be found in serum and urine, and the degradation product free β-subunit core fragment (hCGβcf) that can be found in urine. Both forms alter their relative amounts in urine during pregnancy. From the clinical point of view, there are other interesting isoforms, as the hyperglycosylated hCG (hCGh), the nicked hCG (hCGn) and the free β-subunit (hCGβ) [Stenman *et al*., 2006; Cole, 2009]. In cancer conditions, the pattern of isoform expression becomes more complex. Recently Lund *et al*. [2009], using an immuno-MS method, established specific peptide signatures for the hCG that distinguish serum normal pregnancy and cancer serum. This methodology could be further extended to differentiate hCG-related cancer types, as the specific isoforms detected would prove valuable cancer biomarkers.

10. Metallothionein-3 (MT-3)

As described earlier in this chapter, the MTs are a family of small, cysteine-rich, intracellular proteins that bind transition metals. In humans, there are four classes of MTs (named MT-1 through MT-4) with small differences in their sequence, and therefore in their charge. The most studied are the ubiquitously expressed MT-1 and MT-2, which intervene in the homeostasis of zinc and copper during growth and development, and in detoxification of heavy metals as cadmium and mercury [Hamer, 1986].

The MT-3 isoform was isolated in 1992; it shows 63-69% of homology with the other MTs, though its sequence presents a unique stretch of 8 amino acids [Palmiter *et al*., 1992]. Unlike the other MTs, it is capable of inhibiting neuronal cell growth, and it appears not to be related to metal binding in this tissue [Uchida *et al*., 1991]. Similarly, it seems to be related to cell growth in the urothelial tissue, and thus MT-3 is upregulated in human bladder cancer, and its level increases with tumor grade [Zhou *et al*., 2006]. Noticeably, the MT-3 mRNA level does not show a correlation with tumor grade, hence indicating a complex regulation of the protein expression or the PTMs it suffers (as acetylation). More importantly, MT-3 is undetectable in normal bladder biopsies and in biopsies from non-malignant lesions, whereas its expression is highest in carcinomas *in situ*, which unlike other cancers, in the bladder is highly malignant and aggressive [Sens *et al*., 2000]. These features, alongside the possibility of raising an isoform-specific antibody against its unique 8-amino acid sequence, make the MT-3 isoform a firm marker candidate for bladder cancer diagnosis and prognosis.

Conclusion

Current research for new cancer biomarkers yields every year a large number of candidates for different malignancies. However, not many of them eventually prove their utility in the clinical setting. This failure is clearly understandable considering the existence of protein isoforms, since alterationsaffecting certain variants might be masked when the detection methods employed measure the whole set of isoforms. As described in this chapter, the number of modifications that lead to the existence of isoforms and their level of penetrance is such, that virtually all marker candidates would present at least two isoforms. Thus the cancer biomarker research field should learn from successful examples as the PSA, where the evaluation of only certain isoforms improves the diagnostic parameters for prostate cancer. A critical step towards the implementation of isoform-specific detection techniques would be the generation of antibodies against characteristic isoform epitopes. This strategy will hopefully open a new and more successful era in the cancer biomarker field.

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