ISSN 1669-9106

SPECIAL ARTICLE - REVIEW

MEDICINA (Buenos Aires) 2019; 79 - N° 6/1 -
NÚMERO ESPECIAL 80 ANIVERSARIO: 570-575

MODIFICATIONS IN THE CELLULAR PROTEOME AND THEIR CLINICAL APPLICATION

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Abstract Post-translational modifications (PTMs) are covalent modifications in proteins during or after their synthesis. Among them, the best known are phosphorylation, methylation, acetylation, and also cleavage or binding of small peptides (ubiquitination, SUMOylation and NEDDylation). Often the protein is modified in multiple sites and these modifications are coordinated generating a PTMs crosstalk. Altered patterns of PTMs have been related to several pathologies. Currently, advances in mass spectrometry have made it possible to study multiple PTMs simultaneously. Oncology is one of the disciplines that incorporated these technologies for the need to better characterize tumors. In cancer, several alterations related to the ubiquitin-like PTMs have been described, such as SUMOylation. In particular, the interaction between different PTMs with SUMOylation has been studied in the context of the von Hippel Lindau (VHL) multitumoral syndrome, generating new putative biomarkers for the evolution of these tumors. RSUME or RWDD3, an enhancer of SUMOylation that acts on VHL and HIF proteins, shows a correlation with malignant parameters in this type of tumors, such as angiogenesis. Regulators of PTMs are becoming relevant as biomarkers in cancer.

Key words: post-translational modifications, cancer, biomarker, SUMOylation, RSUME/RWDD3

Resumen ***Modificaciones en el proteoma celular y su aplicación en la clínica.*** Las modificaciones postraduccionales (PTMs por sus siglas en inglés) son modificaciones covalentes en las proteínas durante o posteriormente a su síntesis. Las más conocidas son fosforilación, metilación y acetilación, también clivajes o unión de pequeños péptidos (ubiquitinación, SUMOilación y NEDDilación). Frecuentemente la proteína es modificada en múltiples sitios y estas modificaciones se coordinan generando una interacción de PTMs. Patrones alterados de PTMs han sido relacionados con varias enfermedades. En la actualidad los avances en la espectrometría de masas han hecho posible estudiar en simultáneo múltiples PTMs. La oncología es una de las disciplinas que ha incorporado estas tecnologías por su necesidad de caracterizar a los tumores. En cáncer se han descripto varias alteraciones relacionadas a las PTMs del tipo ubiquitina como la SUMOilación. En particular la interacción entre distintas PTMs con la SUMOilación ha sido estudiada en el contexto de la enfermedad multitumoral de von Hippel Lindau, generando posibles nuevos biomarcadores para la evolución de estos tumores. RSUME o RWDD3, un *enhancer* de SUMOilación que actúa sobre las proteínas VHL y HIF, ha mostrado una correlación con parámetros malignos en este tipo de tumores, como la angiogénesis. Los reguladores de las PTMs están cobrando relevancia como biomarcadores en el cáncer.

Palabras clave: modificaciones postraduccionales, cáncer, biomarcador, SUMOilación, RSUME/RWDD3

Post-translational modifications (PTMs) are covalent modifications that occur throughout the life of a protein, in the amino acids side chains or at the ends of the protein. These changes can take different forms: small chemical modifications such as phosphorylation, methylation, acetylation and also cleavages or binding of small peptides (ubiquitination, SUMOylation and NEDDylation), among others[1]. They can be considered as the last stage of gene expression and, more importantly, they allow diversify the repertoire of proteins that can be generated and also understand the great discrepancy between the genotype and phenotypes observed in both physiological and pathological conditions.

The PTMs can be classified as irreversible (proteolytic cleavages, cysteine oxidation, etc.) or reversible (phosphorylation, methylation, ubiquitination and SUMOylation, among others). Reversible modifications are particularly interesting since they are dynamically regulated for both specific conditions and the cell environment allowing to modify the function, the interactions with DNA or with other proteins and macromolecules, as well as the activity, the subcellular localization and the

Received: 14-VI-2019 Accepted: 10-IX-2019

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assembly in complexes of higher order of the proteins on which they occur[1, 2]. Historically, reversible PTMs were considered those catalyzed by enzymes and irreversible those that occur in response to spontaneous chemical reactions, such as the oxidation of cysteine to sulfonic acid. However, it is now known that some of these non-enzymatic reactions previously considered as irreversible are regulated, for example, by the cellular redox state, and thus are in some cases reversible, reflecting a particular state of the cell and activating a response to this.

To date, more than 200 PTMs have been found in humans[3]. Although the study of them occurs in a first stage at the level of individual modification in a protein residue, often the protein is modified in multiple sites, generating a code in which, through positive and negative interactions, these modifications are coordinated to determine the response, process called PTM crosstalk, as is clearly depicted in the histones code[2].

Post-translational modifications in medicine: relevance of post-translational modifications in pathologies

As a consequence of the individual variability in response to therapies, the medical clinic approach has changed to a personalized medicine. This one and the translational research are focusing on finding the connection and relationship between genotype and phenotype. The study of the proteome, a protein set present in a cell at the time of analysis, arises from this perspective, which is ultimately, what defines the phenotype. For a given protein the combination of PTMs generates a variant different from the original and defines the function, the proteome includes as different variants all those proteins that have different PTMs. Altered patterns of PTMs have been related to several pathologies, whether they are cause or result, which makes them relevant targets for biomarkers studies and also pathogenesis studies[1, 4]. Currently, advances in mass spectrometry (MS), a technique that separate and identify proteins or fragments, has made it possible to simultaneously study multiple PTMs from small amounts of sample. This new approach, the quantitative PTMomic, has a great potential to discover biomarkers from body fluids and tissues, as well as elucidate the mechanisms of the disease through the subsequent investigation of signaling pathways[1]. This new field also includes bioinformatics tools both for the identification of new PTMs and for the interpretation of results. Recently, numerous databases have been developed (reviewed in Pacovici, 2018) and can be classified into categories such as those used for the location and prediction of possible sites for modifications, for prediction of modified protein function and interaction and for visualization of the protein structure resulting from a PTM[5].

Among the post-translational modifications related to pathologies and studied in clinical samples have been described phosphorylation associated with Alzheimer's, diabetes and cancer, glycosylation that plays an important role in cancer, in neurodegenerative diseases and inflammation, proteolytic cleavage that has been linked to cardiovascular disease, neurodegenerative inflammation and cancer[4] and also ubiquitylation associated with-autoimmune, neurodegenerative and cancer diseases[5] .

Post-translational modifications and cancer

The relevance of studying PTMs in cancer relies in the searching for biomarkers for early detection[5] and in the ability to select the appropriate therapeutic strategy to target molecular alterations. These tumor molecular alterations are continuously changing, increasing the heterogeneity of the cellular populations within it. These changes reflect the physiology of the patient and the different treatments he was exposed, among other factors. In some cases, these modifications are determined by mutations, a situation in which the genomic approach will provide important information but, in other cases, the spectrum of complex and heterogeneous mutations makes it difficult to predict whether the presence of genetic anomalies will result in different levels of gene expression and in the cell phenotype. In addition, the tumor responds dynamically to a constantly changing environment, as the tumor microenvironment is, through the regulation of gene expression specified, ultimately, by PTMs. Thus, research in oncology has incorporated recent advances in technologies associated with proteomics[5], such as the of PTMs study for biomarkers in plasma of patients with glioblastoma recently approved by the FDA (Food and Drug Administration, USA)[6].

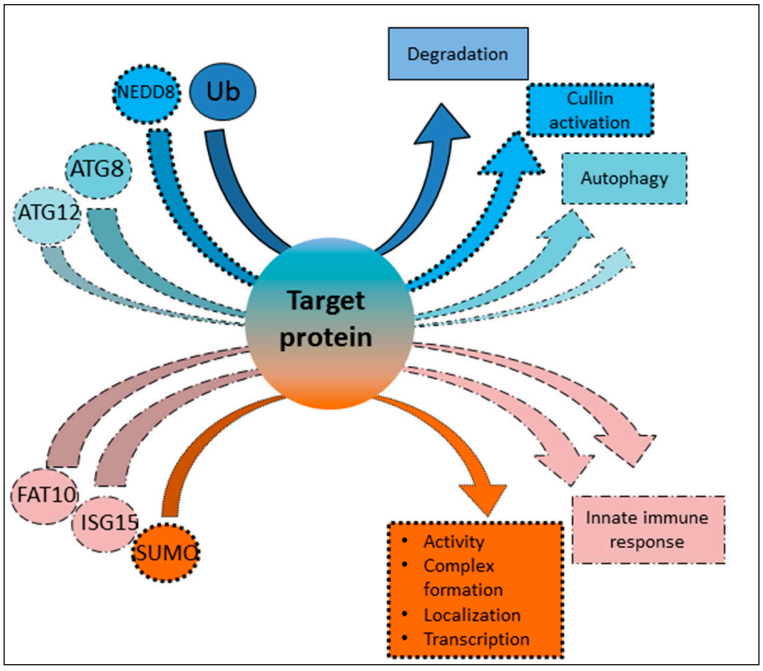
Ubiquitin and Ubls (ubiquitin-like proteins) in cancer

Ubiquitylation and conjugation of ubiquitin-like peptides (Ubl) involve a class of evolutionarily conserved peptides that are covalently conjugate to proteins and affect almost all aspects of cell regulation[3]. These include ubiquitin (Ub), Small ubiquitin-like modifier (SUMO), Neural precursor cell expressed, developmentally down-regulated 8 (NEDD8), Autophagy-related protein 8 (ATG8), Autophagy related 12 (ATG12), HLA-F adjacent transcript 10 (FAT10), and Interferon-stimulated gene 15 (ISG15), among others[7]. The conjugation pathways of these small tags typically require a cascade of reactions carried out by: Ub or Ubls activating enzymes (E1), conjugating enzymes (E2) and ligases (E3). These three types of enzymes act sequentially. The tag is first activated by an E1 and then transferred to an E2 conjugate

enzyme. Under certain conditions, E2 can conjugate directly to the target protein, but frequently it requires the cooperative activity of E3 ligases that interact at the same time with an E2 and substrate protein[3]. The different modifications with Ubls have differential roles. In the ubiquitylation process, depending on the Ub bond configuration, the polyUb chains can label the proteins for degradation through the 26S proteasome or they can act as intermediates for the formation of protein complexes. On the contrary, conjugation with a single Ub (monoubiquitylation) can regulate the activity or localization of proteins. Moreover, conjugation with the peptide SUMO is associated with the regulation of protein transcription and activity, complex formation and nuclear or cytoplasmic localization. While the NEDD8 peptide is mainly conjugated with Cullin proteins to activate them for Ub conjugation, the Ubls ISG15 and FAT10 play a role in the innate immune response. Other notable Ubls are ATG8 and ATG12, which function in autophagy[7] (Fig. 1).

As a consequence of the diverse type of functions of this type of PTMs, alterations in the pathways associated with Ub and Ubl have been implicated in the pathogenesis of numerous human diseases such as cancer. Overexpression of several Ub E3 ligases has been reported in acute myeloid leukemia (WWP1), renal cell carcinoma (UBE3C), lymphoma and breast, pancreas, colon, and prostate cancer (Ubc13)[5]. Cancer has also been linked to the expression of enzymes involved in NEDDylation (e.g. NEDD8 E1, NAE1/UBA3 and NEDD8 E2, BE2M/UBE2F) in several cancers such as lung, liver cancer and colorectal cancer, intra-hepatic cholangiocarcinoma, glioblastoma, and nasopharyngeal and esophageal squamous cell carcinomas[8]. Currently there are numerous chemotherapeutic agents that are being developed or that have already been approved and are involved in clinical trials focused on these PTMs. Although proteasome inhibitors (bortezomib/velcade/PS-341 and carfizomib) are being used in the clinic, they are nonspecific and may cause side effects. In contrast, the substrate specificity of Ubls conjugating enzymes makes them an attractive target for treatment[3]. Since the E1 of Ub also show no specificity, similar to the proteasome inhibitors, the only inhibitor that is in phase II of trials is the MLN4924 (E1-inhibitor of NEDD8)[9]. In opposition, multiple drugs based on E3 ligases have been developed given the high specificity of these enzymes. GDC-199 (venetoclax) is a compound that inhibits

Fig. 1.– Ubiquitin-like post-translational modifications (PTMs)



There are different small peptide tags such as NEDD8, SUMO, ubiquitin (Ub), ATG8, ATG12, FAT10 and ISG15, which generate PTMs in a target protein and generally each family of tags is associated with a role on the protein.

SCFSKP2 activity, an E3 ligase of cell cycle regulators such as p27Kip1 and p21/Cip1, and has been shown good results in animal tests[9]. Nutlins is a cis-imidazoline analogue that inhibits MDM2 (E3 ligase of p53), whereas MI-219 inhibits the interaction between MDM2 and p53. Both inhibitors are currently being tested in clinical phase[9]. Related to the Ub system, the chemotherapy drugs development is currently focused on compounds known as PROTACs (PROteolysis-TArgeting Chimeras) based on artificial molecules that recruit an Ub ligase to selectively degrade target proteins, not targeting the Ub system but rather using it as a tool.

Role of SUMOylation in cancer

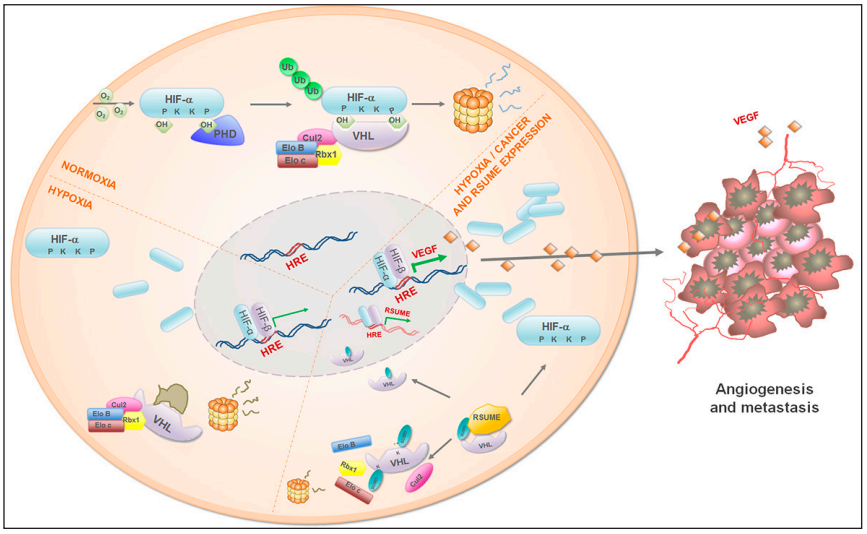
SUMOylation modulates a variety of biological processes whose deregulation is closely related to the pathogenesis of cancer such as DNA damage repair, cell cycle progression and apoptosis, as well as processes that have effects on tumor progression such as immune response, mitochondrial division regulation, voltage channels regulation and biological rhythms. The components of the SUMOylation pathway include the four paralogs of the SUMO peptide (SUMO1, SUMO2, SUMO3 and SUMO4), which require a cleavage by SENPs (setrin-specific proteases) proteins to be active. It also includes activating enzymes (SAE1 and SAE2) that transfer the peptide to the next enzyme called Ubc9 conjugating enzyme, which binds the peptide to the substrate (to date, only one conjugating enzyme has been described: Ubc9). In turn, there are enzymes E3 ligases (PIAS, RanBP2 and Pc2, among others) that favor conjugation. The expression of these ligases has been observed elevated in several malignant neoplasms and it is associated with a poor prognosis of the patient. By make use of SAE or UBC9 shRNAs, several groups have provided evidence that inhibition of the SUMO pathway inhibits tumor growth in mouse models[10]. In addition, the reduction of SAE confers synthetic lethality in tumors with high MYC activity or KRAS mutations, although several inhibitors of UBC9 (GSK145A, 2-D08 and spectomycin B1) have been reported, their specificity is unclear and they seem to have low activity[10]. However, given the relevance of this PTM in cancer, the search for new inhibitors of these pathways remains a very active field. Considering that in comparison to ubiquitylation, SUMOylation confers greater stability and its main feature is modify the surface of its target proteins to regulate protein-protein interactions and thus, mediate the location and function, an alternative to the study of components involved in the SUMOylation is the study of interactors with the different enzymes of this pathway.

RSUME role in cancer

RSUME (RWD-containing SUMOylation enhancer) or RWDD3 is a protein identified from a tumor pituitary cell line with increased angiogenic capacity and acts as a enhancer of SUMOylation cascade through a direct interaction with the conjugating enzyme of SUMO, Ubc9[11]. This protein has a RWD domain (RING finger and WD repeat containing proteins and DEAD-like helicases) which is essential for its activity and although exerts its effect mainly during the formation of the Ubc9-SUMO-1 thioester bond, also acts in the transfer of SUMO-1 of the thioester bond to a specific substrate. It has been found expressed in different types of tumors such as gliomas, pituitary and pancreas neoplasias[12, 13]. In addition, RSUME has been associated with high risks of recurrence and metastasis in some tumors[14]. RSUME is intimately connected with the hereditary multitumoral syndrome of von Hippel Lindau (VHL), caused by mutations in the VHL gene, since it is expressed in organs predisposed to the formation of VHL tumors and in hemangioblastomas and pheochromocytomas, characteristic of this disease[15]. A bioinformatic analysis conducted by The Human Protein Atlas with a recent data set from The Cancer Genome Atlas Research Network (TCGA) shows that 20.1% of the 528 tumors samples of clear cell renal cancer studied, the main causes of death of VHL patients, expresses high levels of RSUME, and correlates with a 23% decrease in patient survival[16]. The VHL protein is a tumor suppressor. Interestingly, it is part of an Ub E3 ligase complex, which most studied target in cancer is the Hypoxia Inducible Factor, HIF-α, a protein that plays an important role in promoting tumor angiogenesis. Recently, RSUME has been reported as a regulator of this process, which makes it a promising target for future therapies as well as a biomarker in these types of cancer[16] (Figure 2). Under normal oxygen conditions, enzymes called Prolyl Hydroxylases (PDH) catalyze the hydroxylation of prolines on HIF and allow its interaction with VHL, which acts as the recognition element of an Ub3 E3 ligase complex and thus leads to HIF final degradation by the proteasome. Multiple components of this system are SUMOylated, in the case of HIF-α this modification enhances its stability. RSUME is induced in hypoxia, increases the SUMOylation of HIF-α and VHL. VHL SUMOylation regulates its spatial distribution and reduces its interaction with HIF-α[16].

This regulation system involving VHL, HIF-α and RSUME is an example of multiple regulation nodes that occur in cellular processes and clearly illustrates the interrelation between a variety of PTMs in the cellular response induced by internal and external stimuli. The greater knowledge of these interconnections is an important tool for defining new cancer biomarkers based on

Fig. 2.– Crosstalk of SUMOylation with other posttranslational modifications (PTMs) in cancer



The stability of the hypoxia inducible factor alpha (HIF-α), that regulate the response to hypoxia in cells, is strictly regulated. In normoxia, HIF-α is hydroxylated in proline residues by the prolyl hydroxylases (PDH) enzymes that require oxygen. This hydroxylation allows VHL, which together with Elo B, Elo C, Cul2 and Rbx1 form E3 Ubiquitin ligase, interact with HIF-α and poly-ubiquitinate it allowing to be degraded by the proteasome. In this way, the binding of HIF-α to the HIF-response elements (HRE) in the DNA that promoted the expression of hypoxia- responsive genes, is prevented. Under low oxygen conditions, HIF-α is not hydroxylated and cannot be recognized by VHL and ubiquitinated, allowing its migration to the nucleus and activate gene transcription. In cancer, different situations occur simultaneously, an intermittent hypoxia, due to the tumor vessels leakiness, and expression of several proteins that affect the PTMs, as RSUME. This protein acts in both HIF-α and VHL, which is the main target of its action, by promoting SUMOylation carried out by the E2 conjugate enzyme of SUMO, Ubc9. Therefore, HIF-α and the other components of the E3 Ubiquitin ligase and VHL interaction decreases. The result is an accumulation of HIF-α that promotes angiogenesis through its regulated targets.

proteomic and PTMomic, as well as for the development of new therapeutic strategies.

Acknowledgments: This work was funded by *Universidad de Buenos Aires* (UBA), *Consejo Nacional de Investigaciones Científicas y Técnicas* (CONICET) and *Agencia Nacional de Promoción Científica y Tecnológica* (ANPCyT) Argentina, and *Fondo para la Convergencia Estructural del Mercosur* (FOCEM) (COF 03/11).

Conflict of interests: None to declare

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