New Perspectives of "omics" Applications in Melanoma Research

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Abstract: *Background*: Oncoproteomics is the study of proteins and their interactions in a cancer cell by proteomic technologies and has the potential to revolutionize clinical practice, including cancer diagnosis. Recent technological advances in the analysis of the human genome have opened the door to improving our primitive understanding of the gene expression patterns in cancer. The examination of the phenotypic and (epi) genetic changes in cutaneous melanoma has identified several genes deemed central to the development and progression of melanoma.

Methods: A review of the literature was performed to determine the role of epigenetic modifications in human melanoma. The role of array-based high-throughput gene expression analysis in understanding the specific genes involved as well as the pathways and the comparative gene expression patterns of primary and metastatic melanoma. The development and clinical application of selective pharmacologic agents are also discussed.

Results: We identified several articles that have extensively studied the role of epigenetics in melanoma, further elucidating the complex processes involved in gene regulation and expression. Other studies utilizing gene microarray analysis and other whole genome approaches reveal a wide array of genes and expression patterns in human melanoma. Several genes have been identified as potential prognostic markers of tumor progression and overall clinical outcome.

Conclusions: High-throughput gene expression analysis has had a major impact in melanoma research. Several gene expression platforms have provided insight into the gene expression patterns in melanoma. Such data will provide foundations for the future development of prognostic markers and improved targeted therapies for patients with melanoma.

Keywords: Melanoma, genomics, epigenetics, miRNA, proteomics.

INTRODUCTION

Melanoma is an aggressive and often fatal type of cancer that arises from transformed melanocytes. These long-lived pigment-producing cells typically colonize the basal epidermis during embryonal development. Melanoma, one of the most invasive and metastatic human cancers, is the major cause of death from skin cancer and is more likely to be reported and accurately diagnosed than non-melanoma skin cancers. According to the World Health Organization (WHO) statistics, the incidence of malignant melanoma in white populations generally increases with decreasing latitude, with the highest recorded incidence occurring in Australia where the annual rates are 10 and over 20 times the rates in Europe for women and men, respectively. Since the early 1970s, malignant melanoma incidence has increased significantly, for example an average of 4% every year in the United States [1]. In addition, melanoma is the fifth most common cancer for men and the sixth most common for women. The incidence of many common cancers is falling, but the incidence of melanoma continues to rise significantly at a rate faster than that of any of the 7 most common cancers.

The tumor-node-metastasis (TNM) staging system for node negative melanoma is based on survival data from more than 27,000 stage I and II melanoma patients. These data identified primary tumor thickness (also known as Breslow depth), ulceration, and a mitotic rate higher than 1/mm^2 as factors associated with worse survival. For example, primary tumor thickness was a strong predictor of survival, and 92% of patients with node negative, T1 (of <1 mm thickness) melanoma primary tumors survived 10 years compared with only 50% of patients with node negative T4 (of >4 mm thickness) tumors. The new staging guidelines from the American Joint Committee on Cancer (AJCC) also identified primary tumor ulceration as an independent prognosticator of survival, and in a pooled meta-analysis of 3 large adjuvant interferon trials E1684, E1690, and E1694, primary tumor ulceration was associated with worse relapse-free survival (RFS, HR = 1.54) and overall survival (OS, HR = 1.73). Interestingly, post-hoc analysis of 2 large European trials, EORTC 18952 and EORTC 18991, also suggested that patients with ulcerated primary lesions, particularly those with no more than minimal nodal metastases, may benefit more from treatment with adjuvant interferon than patients with non-ulcerated primary lesions. In these patients, interferon substantially decreased the risk of relapse by 25%, the risk of developing distant metastasis by 31%, and the risk of death by 31% [2].

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Although the pathogenetic mechanisms underlying melanoma development are still largely unknown, several genes and metabolic pathways have been shown to carry molecular alterations in melanoma

The mitogen-activated protein kinases/extracellular signal–regulated kinase (MAPK-ERK) pathway (including the cascade of neuroblastoma RAS viral (v-ras) oncogene homolog (NRAS) , v-raf murine sarcoma viral oncogene homolog B1 (BRAF), mitogen-activated protein kinase 1/2 (MEK1/2, also known as MAP2K1/2), and mitogen-activated protein kinase 3/1 (ERK1/2, also known as MAPK3/1), which is involved in the control of cell growth, proliferation and migration, has been reported to play a major role in both the development and progression of melanoma and seems to be implicated in rapid melanoma cell growth, enhanced cell survival, and resistance to apoptosis [3]. Therefore, existing evidence suggests that activating BRAF mutations induce cell proliferation and cell survival, which represent 2 biological events occurring in both melanocytic expansion of nevi and malignant progression from superficial to invasive disease [4].

In recent years, a common mechanism for the induction of cell senescence has been described: a progressive-reduction in the length of telomeres (often in conjunction with overactivity of specific oncogenes such as v-myc myelocytomatosis viral oncogene homolog (avian) (*MYC)* and ataxia telangiectasia mutated *(ATM*) seems to exert DNA damage signaling with activation of the p16 cyclin-dependent kinase inhibitor 2A (CDKN2A) pathway [5]. Nevertheless, cancers including melanomas cannot grow indefinitely without a mechanism to extend telomeres. The expression and activity of telomerase is indeed upregulated in melanoma progression. This evidence strongly suggests that both telomere length and $p16^{CDKN2A}$ act in a common pathway leading to growth-arrest of nevi. In particular, the $p16^{CDKN2A}$ protein acts as an inhibitor of melanocytic proliferation by binding the cyclin-dependent kinase 4/6 (CDK4/6) and blocking phosphorylation of the RB protein, which leads to cell cycle arrest [6]. The dysfunction of the proteins involved in the $p16^{CDKN2A}$ pathway has been demonstrated to promote uncontrolled cell growth, which may increase the aggressiveness of transformed melanocytic cells [7].

The $p14^{CDKN2A}$ protein exerts a tumor suppressor effect by inhibiting the oncogenic actions of the downstream Mdm2 p53- binding protein homolog (mouse) (MDM2), whose direct interaction with p53 blocks any p53-mediated activity and targets the p53 protein for rapid degradation. Impairment of the $p14^{CDKN2A}$-MDM2-p53 cascade, whose final effectors are the B-cell CLL/lymphoma 2 (Bcl-2)- associated X proteins (Bax), has been implicated in defective apoptotic responses to genotoxic damage and thus to anticancer agents (in most cases, melanoma cells present concurrent high expression levels of Bax/Bcl-2 proteins, which may contribute to further increasing their aggressiveness and refractoriness to therapy) [8].

Currently, thanks to the use of "omics" technologies (genomics, epigenetics, proteomics, etc.), new potential biomarkers are raising and broadening the possibilities of application for a future improvement of specificity and selectivity in prognosis and diagnosis at different stages of melanoma disease. This review tends to show latest knowledge uncovered in this field.

GENOMICS

The invasive potential in skin melanoma strictly depends on the thickness of the primary tumor, since there is a progressive increase in invasive and metastatic potential with the thickness of the primary tumor. In addition, the growth pattern also reflects the invasive potential: vertical growth pattern (VGP) represents invasiveness, while radial growth pattern (RGP) is the reflection of non-invasive phenotype. Gene signatures associated with thin and thick primary tumors or VGP versus RGP tumors characterize predominantly invasive potentials (corresponding to metastasis initiators) but may also contain signatures for lymphatic and vascular metastatic ability as well as signatures for lymphatic and vascular metastasis maintenance. A quick overview of the microarray studies on human skin melanoma revealed that they were performed on a very heterogenous patient cohort and pathological sample collections containing primary tumors, cutaneous metastases, and lymphatic and in some instances visceral metastases. Only 1 study defined the cutaneous metastasis signature [9], while none were able to identify the lymphatic metastasis signature [10]. It is remarkable that these metastasis-signatures of human skin melanoma are predominated by downregulated genes and only by a much more limited list of upregulated genes [9].

A meta-analysis of genome-wide association studies was found. Studies investigating cell lines or publishing association studies focusing on a set of pre-selected genes were not included in the study. All together 2,475 transcripts were associated with melanoma metastasis (cutaneous or lymphatic). Of these, 350 genes were identified in more than 1 study. Only 19 probe sets representing 17 genes were identified in 3 studies [10] and only cell division control protein 28 (CDC28) was identified in 4 studies. Another potential marker, desmocollin 3 (*DSC3)* gene, is a cell adhesion molecule involved in melanocyte-keratinocyte communication, suggesting the importance of this gene in the development of invasiveness and RGP/VGP transition in melanoma [11]. Early events in melanoma invasion and metastasis may involve alterations in cell proliferation [12] where genes involved in cell cycle regulation are important: cell division cycle 6 homolog (*CDC6)* and protein kinase regulatory subunit 2 (*CDC28/CKS2)* are 2 representative of those genes. It is remarkable that 2 probe sets of epidermal growth factor receptor (EGFR) can be found in this consensus signature. EGFR was considered to be an important regulator of the biology of melanocytes and melanoma; however, a few experimental or pathological studies were able to define its precise role in progression [13]. S100 calcium binding protein B (S100B) is a marker of melanoma, while S100 calcium binding protein A (S100A) family members are more related to progression, mostly the downregulation of their expression [14]. Important factors in early phase of melanoma progression may also involve various nuclear proteins such as

H2A histone family, member V (H2AFV), the neuroblast differentiation-associated protein (AHNAK), or β-catenin-binding protein. The stromal involvement in early phase of

melanoma progression is indicated by the presence of chemokine (C-X-C motif) ligand 14 (CXCL14) in the signature. The interconverted phenotype of melanoma is characterized by cytokin expression involved in melanoma progression. The role of wingless-type MMTV integration site family, member 5 A (WNT5A) in melanoma progression is well documented in the literature [15] similar to the anti-apoptotic protein B-cell CLL/lymphoma 2 (BCL2) [16]. Moreover, the integrin ligand opsin (OPN) was repeatedly shown to be involved in the motility signaling of melanoma cells [17], indicating its role in progression.

Mutations and aberrations can be identified through the use of a variety of biotechniques such as array comparative genomic hybridization (CGH) and fluorescent in situ hybridization (FISH). Using FISH data, investigators evaluated melanocytic lesions, including 3 cohorts of unequivocal melanocytic nevi and melanomas of varying levels of atypia, and a fourth cohort of ambiguous melanocytic tumors. A discriminatory algorithm was established and validated and was found to correctly classify melanoma with 86.7% sensitivity and 95.4% specificity [18]. Array CGH allows for the quantification of DNA copy number variations, including the detection of single copy deletions and duplications. This can be performed on paraffin-embedded specimens. In melanomas evaluated using CGH, 96% had multiple genetic aberrations, while nevi had none with the exception of Spitz nevi where a single specific aberration characterized by a single 11p gain was found. This correlates with v-Ha-ras Harvey rat sarcoma viral oncogene homolog *(HRAS)* activation and MAPK activation. Typical congenital nevi evaluated with array CGH have no aberrations, while melanomas arising in congenital nevi have genetic patterns similar to de novo cutaneous melanoma. Proliferative nodules have frequent numerical chromosomal aberrations that differ from those seen in melanoma [19]. In another study, *BRAF* or *NRAS* mutations were observed in 86% of Spitzoid melanomas and Spitzoid melanoma metastases, but in none of the Spitz nevi and atypical Spitz nevi evaluated [20]. Using array CGH and FISH, histologically and clinically normal appearing skin adjacent to acral melanoma was found to have genetic amplifications in 84% at distances up to an average of 6.1 mm from visible melanoma in situ and 4.5 mm from invasive malignant melanoma [21].

In a large expression study of human primary melanomas, it was found that 23 of 24 genes involved in DNA repair have increased expression correlated with thickness, as did all the examined genes associated with cell cycle (8 genes), protein folding (10 genes), chromatin remodeling (10 genes), and heat shock protein activity (11 genes). In contrast, decreased expression with increasing thickness of primary melanoma was observed for all examined genes involved in serine-type endopeptidase inhibitor activity (15 genes), cell adhesion (15 genes), cell-cell signaling (8 genes), and transcription factor activity (33 of the 36 genes examined) [22].

Among the intrinsic properties of melanoma that may favor both ulceration and dissemination are proliferative activity of the tumor and overexpression of c-myc [23]. In a work of dendritic cell (DC) maturation in the sentinel lymph nodes (SLNs) draining melanoma, it was found that the maximum mature DC density in the SLNs correlated significantly and inversely with ulceration of the primary melanoma ($P = 0.0005$) [24]. It is of interest to put this finding parallel to a more pronounced impact of pegylated interferon-a2b (PEG-IFN) on recurrence-free survival in patients with ulcerated melanoma, as compared with patients with non-ulcerated melanoma [25].

Genes identified in another validated and reproducible signature prognosticating metastases or death are mainly associated with DNA replication or DNA repair. In DNA replication, genes of 2 pathways are over-represented: replication origins firing (ROF) genes and the separation of sister-chromatids by securin [22, 26]. Melanomas with poor prognoses are characterized by a global overexpression of ROF-related genes. The minichromosome maintenance complex component 4/6 (MCM4/6) expression is strongly correlated with metastasis-free survival and overall survival [22]. Securin has 3 known activities: it blocks the sister-chromatids separation in stabilising separase, it stimulates angiogenesis and, it decreases p53 transcription. Securin acts as an oncogene, and its expression is observed by immunohistochemical staining in the vertical growth phase but not in the radial growth phase of melanoma [27]. Table **1** shows a selection of candidate biomarkers from tissue microarrays that need confirmation from independent data sets and/or prospectively collected data sets preferentially from clinical trials. Table **2** summarizes a set of prognostic serum biomarkers [28] .

Table 1. Prognostic Cutaneous Melanoma Biomarkers Detected by Immunohistochemical Analysis of Tissue Microarrays

Prognostic Tissue Biomarkers
Cellular heat shock protein chaperone (HSP90)
Regulator of G protein signaling 1 (RGS1)
Osteopontin
Epidermal growth factor receptor family member 3 (HER3)
Inhibitor of growth family member 4 (ING4)
Inhibitor of growth family member 3 (ING3)
Nuclear receptor coactivator-3 (NCOA3)
Minichromosome maintenance protein family member 4 (MCM4)
Minichromosome maintenance protein family member 6 (MCM6)

Table 2. Prognostic Serum Biomarkers of Cutaneous Melanoma

Prognostic Serum Biomarkers
Lactate dehydrogenase (LDH)*
Calcium-binding protein S100B
Melanoma-inhibiting activity (MIA)
Tumor-associated antigen 90 immune complex (TA90IC)
Mammalian chitinase-like proteins member YKL-40

*Included in the AJCC staging system, and patients with distant metastases and elevated LDH are considered as stage IV M1c.

EPIGENETIC AND MICRO-RNA STUDIES

Recently, the epigenetic mechanisms involved most often in gene silencing in melanoma have come to the forefront of research, highlighting unique perspectives on the gene regulation of melanoma, and how this relates to tumor suppression and metastatic potential. The hallmarks of epigenetic gene regulation are DNA methylation and histone modifications [29].

DNA hypermethylation contributes to gene silencing by preventing the binding of activating transcription factors and by attracting repressor complexes that induce the formation of inactive chromatin structures. Currently, more than 50 genes have been identified to be aberrantly hypermethylated during some phase of melanoma progression and metastasis [29]. A cytosine nucleotide next to a guanine nucleotide (CpG) island DNA hypermethylation seems to play a central role in melanoma progression and metastasis. A recent work reports that the methylation events involved in advanced-stage melanoma was examined by extensively screening the methylation status of the promoter regions of 30 "cancer-related" genes utilizing a sensitive and quantitative methylation-specific polymerase chain reaction (PCR)-based assay (Q-MSP), performed on 20 melanoma cell lines and 40 human melanoma samples [29]. The analysis of this large panel gave raise to identify 4 new genes: decoy receptor 1 (*DcR1)*, decoy receptor 2 (*DcR2)*, lysyl oxidase *(LOX)*, and tropomyosin 1 (alpha) (*TPM1*) implicated as hypermethylated in human melanoma, with an overall methylation frequency of 60%, 80%, 50%, and 10%, respectively [28]. Another gene stratifin (also known as 14-3-3σ) was first identified as an epithelial cell antigen (HME-1) exclusively expressed in human epithelia. Experimental data demonstrate that the 14-3-3σ gene is highly expressed in normal skin but undetectable in normal melanocytes and in most melanoma cells. We have found that the promoter CpG islands in the 14-3-3σ gene are heavily methylated in both normal melanocytes and most melanoma cells in a cell-lineage specific manner [29].

Little is known about the role of hypomethylation in the initiation and development of melanoma. In melanoma, a group of cancer-testis antigens (CTAs) and several other genes have been identified to be aberrantly hypomethylated [30-32]. Expression of these genes is repressed in normal human skin melanocytes, primarily due to heavily methylated promoter regions in a cell lineage-specific manner. Conversely, these same genes can exist in a demethylated state and are aberrantly re-expressed in subsets of melanoma cells. Current evidence would suggest that reactivation of these genes may contribute to overall tumorigenesis. It is clear that the expression of these tumor antigens can result in their recognition and possible destruction by the host immune system, with the products influencing a range of cellular processes including cell signaling, transcription, translation, and chromosomal recombination. Testis-specific protein Y-encoded (*TSPY*) is a repeated gene mapped to the critical region harboring the gonadoblastoma locus on the Y chromosome (GBY); the only oncogenic locus on this male-specific chromosome and TSPY is considered a putative oncogene [29].

DNA-binding histones are directly modified to affect the strength with which they bind and subsequently segregate DNA from transcriptional machinery. The acetylation of lysine residues on histones is associated with transcriptionally active DNA where deacetylation is associated with tightly bound inactive DNA. Additionally, promoter methylation can affect histone activity through the stepwise recruitment of other transcriptional modulators to the methylation site, as histone acetylation similarly affects promoter methylation levels. Each of these processes provides a means of controlling gene expression for the maintenance of cell homeostasis and has been shown deregulated in many cancer types [29]. The enzymes responsible for acetylating and deacetylating lysine are histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. In this manner, chromatin can switch between being open (euchromatin) or closed (heterochromatin) to transcriptional activity. A group of HATs named MYST for family members of K (lysine) acetyltransferase 8 (MOZ, Ybf2-Sas3, Sas2) and K(lysine) acetyltransferase 5 (Tip60) also includes K(lysine) acetyltransferase 7 (HBO1), MOF (also known as MYST 1), and monocytic leukemia zinc finger (also known as MYST histone acetyltransferase (monocytic leukemia) 3 (MORF) or (MOZ-related factor) have a characteristic highly conserved 370 bp MYST domain with acetyl-CoA binding site and function in numerous nuclear processes including transcriptional activation and DNA repair [29]. Interestingly, MYST members Tip60 and HBO1 are present in a complex with the putative melanoma tumor suppressors inhibitor of growth family, member 3/4 (ING3/ 4), respectively [33, 34]. Additionally, both Tip60 and HBO1 functionally link the nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB), while acetylation by Tip60, K(lysine) acetyltransferase 2A (GCN5), and P300/CBP-associated factor (also known as K (lysine) acetyltransferase 2B (PCAF) can stabilize the transcription factor c-Myc, each of which are putative melanoma oncoproteins [35]. The other groups of HATs, E1A binding protein p300 /CREB-binding protein (p300/CBP) (that groups adenoviral E1A-associated protein, 300 kDa and CREB-binding protein and are involved in cellular proliferation, differentiation, and apoptosis), have been shown to associate with microphthalmia-associated transcription factor (MITF), a melanocyte lineage survival oncogene [36] that transcriptionally regulates melanoma invasiveness, proliferation, and apoptosis [37], is mostly upregulated in metastatic melanomas [38] and is associated with decreased survival in metastatic melanoma patients [39]. In the complex malignant transformation process of melanoma, gene expression profiles reveal a loss of expression of genes that act to counter tumor cell formation including cell cycle regulators and proapoptotic genes [40]. Many of these tumor supressor genes (TSGs) are shown to be downregulated jointly through promoter hypermethylation and the reversible deacetylation of lysine residues by HDACs of local histones [41]. Furthermore, HDACs are known to act on proteins that regulate cellular differentiation, proliferation, gene expression, and death; that is, the reason why HDAC inhibitors are currently being studied as treatment against the development of malignant melanoma [30].

A new area of recent progress is the study of micro-RNA (miR) as factors that mediate in gene expression regulation of potential biomarkers in melanoma. Micro-RNA-200 (miR-200) and miR-205 are both highly expressed in normal

skin and have been shown to specifically target the mRNA of the transcriptional repressor of E-Cadherin, zinc finger E-box binding homeobox 1/2 (ZEB1/2). Thus, they promote the expression of E-Cadherin. Consequently, downregulation of the miR-200 family and miR-205 leads to the inhibition of E-Cadherin, promoting an epithelial-mesenchymal transition [42]. Consistent with these studies, miR-205 is often significantly downregulated in human epithelial tumors when compared with normal tissues [43]. However, in other profiling studies in human epithelial cancer, the miR-200 family and/or miR-205 were found upregulated [44]. These apparently contrasting results suggest that miRNAs may have different functions in different cells in which the local mRNA content may be an important determinant for their functions. Future studies are required to dissect the molecular circuit that underlies these observations.

Recent studies have begun to show miRNA expression and functions in the development and progression of melanoma [45]. Interestingly, the members of the Let-7 family were implicated in the suppression of melanoma development [46, 47], reminiscent of their functions in other human cancers. Given their critical functions in cancers and other human diseases, it is conceivable that miRNAs possess great potential as therapeutical targets as well in melanoma. Owing to their small size (~19–24 nucleotides), short oligonucleotides complementary to miRNA sequences, for example, antagomir, can effectively block their functions in animals, whereas miRNA mimics can enhance miRNA functions [42].

PROTEOMICS

An innovative approach to identify new and better serological biomarkers in melanoma is the serum proteomic profiling. This methodology offers the possibility to screen the whole serum proteome for markers that match different criteria such as prognostic significance and prediction of therapy response.

The first promising results showed that patients with stage I and stage IV disease can be differentiated by their serum proteomic profiles. In this study of serum samples from patients with stage I or IV melanoma analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-ToF) utilizing protein chip technology and artificial neural networks (ANNs) correctly identified the disease stage in 84 of 96 (88%) samples. Forty-four of 55 (80%) stage III serum samples were correctly assigned as progressors or non-progressors using random sample cross-validation statistical methodologies. Twenty-three of 28 (82%) stage III progressors were correctly identified by MALDI-ToF combined with ANN, whereas only 6 of 28 could be detected by using the S100B marker [48].

Another recent study using proteomic profiling in larger sets of sera succeeded in the identification of serum amyloid A (SAA) as a new prognostic serum biomarker in melanoma. Using an immunonephelometric assay, low serum levels of SAA showed a favorable survival compared with patients with high serum levels of this protein. By associating a second proinflammatory protein, C-reactive protein, to the dosage of SAA, it was showed that both proteins have higher sensitivity and specificity to predict progression-free as well as overall survival than the current standard serum marker, S100B [49].

One of the key elements for biomarker discovery will be the assays for the identification of low-abundance serum proteins. Pre-processing steps have been devised including depletion columns for removal of albumin and other major proteins, molecular weight cut-off cartridges, acetone precipitation, gel filtration, ion-exchange chromatography, isoelectric focusing, and electrophoresis. More recently, multidimensional separation techniques were developed for low-abundance protein identification [50]. Coupled with tandem mass spectrometry (MS), these approaches allow high-throughput protein identification. Owing to its good compatibility with online MS detection, nano-liquid chromatography is typically used to separate peptides in shotgun proteomics. Even if analytical challenges remain in trying to resolve the problems of complexity and dynamic range, serum proteomic analysis using multidimensional liquid chromatography and MS for shotgun proteomic analysis will have an important role in serum clinical biomarker discovery [28, 51].

CONCLUSIONS

With the implementation of new "omics" technologies (genomics, epigenetics, and proteomics) in the biomarkers discovery area, a wide range of possibilities have opened for the uncovering of specific targets of melanoma disease and the development of new potential drugs that could improve the survival rate and quality of life in patients with malignant melanoma. To date, many efforts are being employed in multidisciplinary groups for establishing better diagnosis and prognosis of the disease. Furthermore, most recent clinical trials are testing the laboratory outcomes emerging from the application of "omics" platforms. It is a challenging field of rapid acquisition of evidence-based knowledge that promises an optimistic future for solving the main problems of the clinical management of cutaneous melanoma.

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