Editorial Commentary

The control of tumor progression by circular RNAs: novel prognostic and therapeutic insights resulting from the analysis of the circAGO2/human antigen R complex

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Circular RNAs (circRNAs) represent a relatively novel class of non-coding RNA (ncRNA) characterized by a covalently bound loop (1). Due to their circular structure, circRNAs are very stable and resistant to the action of exonucleases and this characteristic distinguishes them from the other linear RNAs (2). They belong to the largest class of long ncRNA (lncRNA), and consist of several hundred nucleotides; they are endogenous, abundantly expressed, conserved and able to perform own peculiar functions (3,4). Depending on where they are located on the genome, members of this class are distinguished in exonic, intronic, and eso-intronic (3). As far as their biogenesis is concerned, it has now been found that they are co-transcriptional products, being generated through back-splicing, unlike messenger RNA of genes coding for proteins (5). Interestingly, they are able to regulate gene expression both at transcriptional and post-transcriptional levels, essentially through three main mechanisms: (I) they can function as endogenous sponge for microRNAs (miRNAs), (II) they can bind to RNA-binding proteins (RBP) and, finally, (III) they can interact with other RNAs through base-pairing (3).

CircRNAs take part in cellular physiology, as it has been observed that they regulate either physiological (proliferation and migration) and pathological (invasion and modulation of therapeutic response to antitumoral drugs) mechanisms (6-8). In fact, recent studies have shown that circRNAs play an important role in the development of certain human pathologies including neurological disorders, diabetes, Parkinson's and Alzheimer's disease, multiple sclerosis and even cancer (7-9). In the latter case, it has been observed that, like other ncRNAs, circRNAs can also behave both by oncogenes and tumor suppressors (10).

The work by Chen and colleagues (6) recently published on *Cell Death & Differentiation* sheds new light on elucidating functions of the circRNA *circAGO2* in human cancer. Interestingly, *circAGO2* appears able to physically interact with the RBP called "human antigen R" (HuR), then recruiting it into particular cellular districts and modulating its function (6).

C*ircAGO2* is over-expressed in tumor cell lines in culture and in tumoral tissues (stomach, colon, prostate) compared to adjacent non-tumoral tissues (6) and essentially acts as an oncogene. As it is evident from its name, *circAGO2* is generated from an intron of the *Argonaute 2* (*AGO2*) (6,11) and, in particular, its circular structure (consisting of 391 nucleotides) encompasses a piece of *AGO2* first intron (6). C*ircAGO2* is not able to modulate the expression of its cognate gene *AGO2* in this system, but interestingly, the proteomic approach used in this work elucidates how it exerts its oncogenic properties by interacting with RBPs. Among the several putative interacting partners identified, the HuR protein is finally confirmed to be an interactor of *circAGO2* (6).

HuR belongs to the RBP family of the Elav type (12). At the resting state HuR is located in the nucleus, but when activated, it plays its role mostly in the cytoplasm.

Following different types of stimuli, HuR binds to particular mRNAs, whose sequence is rich in adenine and uridine (AU), through protein domains called "RNA recognition motifs" (RRMs). As a result of this interaction, the protein/RNA complex is moved to the cytoplasm where HuR is able to stabilize mRNAs and regulate their translation into proteins (13). Several studies have interestingly shown that HuR is abundantly expressed in a variety of human cancers, and that its expression is associated with several characteristics of tumors, such as development and progression, migration and invasion, prognosis and resistance to therapy (12). This could be likely explained by considering that HuR tends to stabilize a large series of mRNAs associated with human tumors (12). Additionally, it is worth to note that a series of post-transcriptional modifications control the abundance, the localization and the binding of HuR to the different target mRNAs (12). In this context, the interaction with *circAGO2* represents a further and interesting control mechanism exerted by the HuR protein. In fact, the enforced expression of *circAGO2* in prostate cell lines induces its translocation from the nucleus to the cytoplasm, without modulating its relative expression (6). This observation is of paramount importance for understanding the mechanism since *circAGO2*, by inducing this translocation, is able to activate the HuR protein on the 3'-UTRs of genes targeted by miRNAs. Recently, it has been reported that HuR can modulate the function of AGO2/miRNA complexes (14), therefore, it is intriguing to envisage an involvement of *circAGO2* in this regulatory circuitry. Interestingly, AGO2 and HuR also interact in MKN-45 cells and this interaction is restrained by ectopic over-expression of *circAGO2* (6). Noteworthy, the over-expression of *circAGO2* is able to interfere with the expression of *circAGO2*/HuR downstream genes, since as many as 6 cancer progression-related genes (*EIF4EBP3*, *HNF4A*, *MAP4K1*, *NOTCH4*, *SLC2A4* and *SLC44A4*) are modulated. Additionally, the binding sites for up to 5 miRNAs (miR-224-5p, miR-143-3p, miR-181a-5p, miR-503-5p and miR-125a-3p) are located in a site close to the AU-rich elements (ARE) in the 3'-UTR of these 6 target genes (6). The emerging mechanism of regulation is quite remarkable, in fact, while the over-expression of miRNAs induces an enrichment of both HuR and AGO2 on these 3'-UTRs, the over-expression of *circAGO2* is able to impair this occurrence, therefore preventing the decrease of the 6 target genes (6). Thus, it is worth noting that *circAGO2* promotes the repressive function of HuR on the miRNAs/AGO2 complex. The interaction between *circAGO2* and HuR on the 3'-UTRs of target genes most likely generates a steric hindrance which, in turn, prevents the positioning of the miRNA/AGO2 complex on adjacent sites located in the 3'-UTR and, consequently, blocks the translational repression driven by miRNAs (6).

The interaction with AGO2 represents an additional mechanism of circRNAs function. AGO2, plays a crucial role in the gene silencing process mediated by miRNAs, by interacting with them and driving translational repression or cleavage of target mRNAs. Several authors have already shown that a lot of circRNAs are able to interact with AGO2 and miRNAs, thus acting as miRNA sponge, and then inhibiting the block of the expression due to miRNAs (15). AGO2, therefore, represents a typical example of RBP that not only interacts with miRNAs, but also mediates the function of specific circRNAs. Nevertheless, the mechanism proposed by Chen *et al.* is somewhat different from miRNA sponge since *circAGO2* has no putative base-pairing sites shared with the miRNAs identified in the present work. This observation, however, further strengthens the importance of the circRNAs/AGO2 interaction, which as a matter of fact, can exert its regulatory function in many different ways. The miRNA sponge mechanism exerted by circRNAs has however been questioned, since the putative binding sites of miRNAs on circRNAs are not sufficient, as required by such a model (6).

The mechanism involving *circAGO2* and HuR shows very interesting similarities with the lncRNA *LINC00707*. It has just been reported that *LINC00707* is able to bind HuR forming a complex able to increase the stability of several mRNAs associated with gastric carcinoma (16). Considering that HuR and *LINC00707* are also over-expressed in gastric carcinoma, it is likely to envisage that this interaction can lead to the development and the progression of gastric carcinoma (16). Another similar mechanism has been observed in epithelial ovarian carcinoma, where *LNCARSR* is able to simultaneously interact with HuR and with the β-catenin mRNA in the cytoplasm of carcinoma cells. This interaction is reflected into an increase of the β-catenin levels, with a substantial progression of the carcinoma (17). Indeed, it seems that the HuR protein represents a true nodal center in diseases like human cancer and, currently, researchers are developing new therapeutic approaches based on the HuR blocking.

Oncogenic properties of *circAGO2* are specifically mediated by the activation of HuR protein, since HuR silencing leads to a lower stability of the 6 target genes mRNAs, with a reduced expression of them. Conversely, it

is noteworthy that the over-expression of *circAGO2* is able to sustain their expression (6). On the other hand, *in vivo circAGO2* silencing is able to significantly reduce growth, tumor mass, expression of Ki-67 and CD-31 positive microvessels in subcutaneous xenografts and the ability to form metastases to the lungs (6).

More in general, the characterization of the circRNAs/RBPs interplay sheds new light on current therapeutic strategies and future developments in the field of oncological diseases. The possibility to target RBPs is certainly desirable in the case of cancer, as these proteins simultaneously modulate multiple characteristics of the disease (18). However, it is proving very difficult to block specifically this type of proteins. In this perspective, a combinatorial therapy, which also takes into account RNA interactors, could provide further validity. In this framework, the circRNAs play a leading role since they show remarkable features which make them exploitable not only as molecular targets, but also as therapeutic vectors (19). In the latter case it is noteworthy that molecular vectors expressing circRNAs can be engineered in laboratory to carry binding sites both for miRNAs and for RBPs (19). This allows, at least in principle, to obtain a therapeutic advantage due to a multiple block of a same pathway. Novel therapeutic strategies must move along a path in which ncRNAs and the respective RBPs must be considered as central nodes, whose targeting is reflected in a better therapeutic response of patients.

A therapeutic use of *circAGO2* silencing is also envisaged, in fact the treatment of AGS cells with a peptide mimicking the binding site of HuR to *circAGO2* (HIP-13) is able to block the endogenous interaction between HuR and *circAGO2*, with a consequent effect on the viability, proliferation and invasive ability of cells. Additionally, *in vivo* treatment with HIP-13 has the same effect of *circAGO2* silencing, namely a significant reduction of growth, tumor mass, expression of Ki-67 and CD-31 positive microvessels in subcutaneous xenografts, and also a reduced ability to form lung metastases (6). Thus, this investigation undoubtedly shows that the block of *circAGO2* could be exploited as a novel therapeutic strategy in cancer management. The characterization of *circAGO2*/HuR interaction certainly offers new therapeutic opportunities for the treatment of gastric carcinoma and, more generally, in the different human carcinomas. In fact, the idea of using protein fragments able to penetrate the cell, as potential drugs, is very interesting. The small size of peptides would also allow a very rapid clearance, with a reduction of side effects. More importantly, such an approach would be even more specific and personalized, since peptides would only block *circAGO2* and, consequently, the pathological functions of HuR, but not the whole physiological functionality of HuR. This eventuality would be very advantageous in terms of drug efficacy and decrease of side effects.

It is clearly obvious that the block of *circAGO2* could also be obtained by other approaches, such as the administration of other ncRNA fragments able to bind it. A further aspect to consider is the possibility to take advantage of the novel delivery opportunity offered by nanoparticles and, more generally, by nanotechnologies.

The characterization of the oncogenic role of *circAGO2* in gastric carcinoma further emphasizes the importance of circRNAs in the development and progression of human diseases such as cancer. It is well known that a series of circRNAs are aberrantly expressed in human carcinomas (7,8,10). Their improper expression is mostly reflected in the interaction with miRNAs with subsequent sponge effect and this aberrant interaction seems to be a fairly general phenomenon in human carcinogenesis (15). Additionally, several works tend to extend the concept of sponge also to RBPs, as it has been observed that some circRNAs can bind and relocalize other proteins, preventing their functioning (20). This is quite similar to what is observed for *circAGO2* and its interaction with HuR.

The expression analysis of *circAGO2* and HuR in human gastric carcinomas shows that their levels are significantly associated with the presence of metastases and with a poor overall survival and, in particular, the over-expression of HuR in carcinoma samples is associated with a worse clinical condition of patients (6). If we consider only gastric carcinoma, we can certainly say that a large list of circRNAs are deregulated and show both an oncogenic and a tumor suppressor role. For instance, it has just been reported in the literature that *circPSMC3* can suppress proliferative and metastatic abilities of gastric carcinoma cells (21). Conversely, *circNRIP1*, *HSA_circ_0067997* and *circNF1*, are all able to sustain the progression of gastric carcinoma (22-24). Likewise, RBPs also play a general and very important role in cancer (25). It has been hypothesized that single RBPs are virtually involved in all the various pathological processes leading to cancer development (25). For example, the IMG type RBPs are mainly associated with an increase of cell proliferation (25). Sam68, on the other hand, due to its splicing regulatory ability, is also involved in the enforcement of breast and prostate

cancer cells proliferation (25). The RBPs of the LARP family, again, are mainly associated with the acquisition of resistance phenomena to apoptosis. Finally, ESRP1/2 and KHSRP proteins are involved in the onset of several human carcinomas, as they are associated with the acquisition of mesenchymal phenotypic characteristics, with consequent increase in the migratory and invasive capacity of tumor cells (25).

In conclusion, we can recapitulate the main implications of *circAGO2*/HuR interaction as follows: the over-expression of *circAGO2* in a series of human tumors, including gastric carcinoma, promotes the translocation of HuR from the nucleus to the cytoplasm and induces its enrichment on the 3'-UTR of target genes related to the proliferation and the cell cycle. The presence of *circAGO2*/HuR complex on the 3'-UTR increases the stability of target mRNAs by competing with the AGO2/miRNAs complex. Then, the final effect is to promote tumorigenesis and aggressiveness (6). For the future, a new window opens in the design of personalized anti-cancer therapies based on the inhibition of *circAGO2* and HuR in specific types of human cancers.

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Footnote

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