Editorial

circRNA meets gene amplification

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Lung cancer remains the most common cause of cancer related death among both males and females globally accounting for 1.69 million deaths in 2015 (1). Of the different types of lung cancer, lung adenocarcinoma (LAC) is the most common (2). Given the prevalence and lethality of lung cancer the need to elucidate the mechanisms of this disease and to develop novel therapeutic targets remains paramount. The understanding of the biology and genetics of LAC is essential to learning more about this disease.

The recognition of copy number variation (CNV) across the genome, leading to gain (amplification) and loss (deletion) of function of DNA segments has been well studied. These CNVs have been found to play a role in the tumorigenesis of a number of cancers including lung cancer (3). Most previous research has investigated the gain of oncogenic proteins, such as MYC (4), or the loss of tumor suppressors, such as PTEN (5), however, oncogenic proteins represent only a small portion of the genomic regions exhibiting CNV. While protein coding genes occupy less than 5% of the genome, more than 50% of the genome is transcribed (6). The non-coding portion of the transcriptome has been gaining increasing attention as numerous studies have implicated non-coding RNA in the development and regulation of cancer (5), and, as the majority of the transcriptome, non-coding RNAs represent a tremendous area of unexplored opportunities.

In addition to the classical small noncoding RNA species that are essential for RNA metabolisms (e.g., small nuclear and nucleolar RNA), recent advances in genomic analysis have to led to further discoveries of a variety of non-coding RNA: long non-coding RNA (lncRNA), microRNA (miRNA), enhancer RNA (eRNA) and circular RNA (circRNA) (7,8). Specifically, circRNAs are naturally occurring non-coding RNAs generated possibly through exon circularization from precursor RNAs and are mainly found in the cytoplasm. They have been shown to modulate a number of biological processes such as cell proliferation and cell cycle regulation (9). circRNAs have also been identified in cancer cells, such as circPVT1 in gastric cancer (10) and circMTO1 in hepatocellular cancer (11). The most common mechanism by which these circRNAs regulate cell function is through microRNA (miRNA) sponging. miRNA plays a significant role in the regulation of gene expression through binding to mRNA and causing either degradation or inhibition of that particular mRNA. A single miRNA can have multiple targets across the transcriptome and a single mRNA can be the target of numerous miRNAs and vice versa (12). As such, the abundance of miRNA is an important part of post-transcriptional regulation. One mechanism to disturb the regulation is through miRNA sponging where different forms of RNAs can bind to the complementary sequence of a miRNA to "absorb" miRNA and prevent the binding of the miRNA to the target mRNA. Such miRNA sponging function has been demonstrated first for the circRNAs from *CDR1* and *SRY* loci (13). Since then, the miRNA sponge by circRNA has been shown to regulate pluripotency, function, proliferation and apoptosis in cells (13-16). miRNA sponging has also been shown to function in several cancers such as: gastric (10), hepatocellular (11) and colon cancer (17). However, the presence and/or function of circRNAs in LAC is largely unknown. Furthermore, circRNAs arising from CNV regions have not been identified before. The recent study by Qiu *et al.* is the first

to report the significance of circPRKCI in LAC (18). The authors eloquently identified circPRKCI as a prognosticator of LAC and elucidated the mechanism of circPRKCI, providing a potential treatment target for this lethal disease.

They initially cross referenced tumor-specific circRNAs from 5 LAC patients with common CNVs in LAC from TCGA data and found the circRNA associated with the gene *protein kinase C iota (PRKCI)* (18). *PRKCI* is located within the 3 Mb region of 3q26.2 that is frequently amplified in lung cancer (3,19). *PRKCI* and *SOX2* are leading candidates of the target genes within the 3q26.2 amplicons and have been shown to promote the stem-like phenotype in squamous cell carcinoma of the lung (20). However, it is possible that non-coding RNA within the amplicon can also be a target in LAC. Based on the integrated screening of circRNA expression and CNV, the authors hypothesized that circPRKCI arising from the *PRKCI* gene may play a role in lung cancer and further characterized circPRKCI.

In addition to identifying the presence of circPRKCI, the authors correlated circPRKCI levels and outcomes in LAC. circPRKCI was found to be overexpressed in tumor tissue compared to normal tissue with an average fold change of 6.89 (18). They further found circPRKCI expression was higher in larger tumors, and those with stage II-III tumors had higher circPRKCI expression than those that were stage I. Furthermore, they verified this finding using chromogenic in-situ hybridization (CISH) which showed that higher CISH expression of circPRKCI correlated with size and TNM stage (18). While this was an important finding, most interesting was that patients with higher CISH expression in their LAC tumors had a shorter overall survival and on multivariate analysis circPRKCI expression level is an independent poor prognostic factor (hazard ratio 2.66) (18). Prognostic factors are important in determining treatment algorithms and surveillance of patients, however, a prognostic characteristic holds much more importance when the characteristic can be acted on to alter the prognosis of the patient. Previous studies have identified circRNAs as prognostic biomarkers in cancer (10), including lung cancer (21). The importance of circRNAs as prognostic biomarkers in cancer cannot be understated as they represent not just a prognostic characteristic but also a potential therapeutic target.

In order to identify whether circPRKC has more than just prognostic value, Qiu *et al.* further investigated the mechanism by which circPRKCI may actually influence tumor characteristics, and how targeting this circular RNA may be used to improve outcomes. LAC often presents with advanced disease that requires treatment beyond just surgical resection, as such, improved treatments in order to curtail the effects of this terrible disease continues to be important.

Qiu and colleagues found that circPRKCI contributes to cell growth and migration in vitro. They clearly showed that knockdown of circPRKCI significantly impaired cell proliferation while overexpression promoted proliferation (18). Further, knockdown of circPRKCI arrested cells in G1 but did not affect cell apoptosis. When examining the invasion of these LAC cell lines they found that si-circPRKCI treatment significantly impaired the invasion capacity of the cells (18). Overall, these findings suggest that circPRKCI promotes cell proliferation and migration of LAC cells. The implication of these results further suggests the importance of circPRKCI in maintaining the malignant environment of LAC, and again, suggests a possible target for modifying the biology of LAC tumors.

The authors determined that circPRKCI was predominantly present in the cytoplasm of the cell (18). Based on this finding they hypothesized that circPRKCI may be functioning as a miRNA sponge. miRNA bound to circRNA can form a complex with Argonaut 2 (Ago2). Qiu *et al.* was able to show that circPRKCI was pulled down with Ago2, and among putative target microRNA, miR-589 and miR-545 bound to circPRKCI in LAC cells (18). The direct interaction of circPRKCI and miR-545 and miR-589 was further established by mutating the binding sites to miR-545 and miR-589 (18). Additionally, silencing of circPRKCI did not actually effect the expression of miR-545 or miR-589, and transfection with miR-545 and miR-589 mimics did not affect expression of circPRKCI (18). Thus, circPRKCI likely acts as a sponge but does not downregulate the expression of these miRNAs. As the mechanism of these two miRNAs are relatively unknown, the authors further investigated the biologic function of miR-545 and miR-589 finding that they inhibited cell proliferation and induced G1 arrest *in vitro* (18). These findings further support the hypothesis that absorbing miR-589 and miR-545 by circPRKCI's sponging function could promote tumor cell proliferation by inhibiting these two microRNA in LAC cells.

Next, the authors were able to determine the mRNA target of miR-545 and miR-589 that can be rescued from degradation by circPRKCI. By either silencing of circPRKCI or transfection with miR-545 or miR-589

mimics they identified the E2F7 gene was significantly downregulated (18). E2F7 is a transcription factor that plays an essential role in the regulation of the cell cycle. This transcription factor negatively regulates cyclin-dependent kinase inhibitor p21 leading to upregulation of Cyclin D1 (22) and promote cancer cell proliferation (23). miR-545 and miR-589 mimics indeed led to the upregulation of p21 and downregulation of cyclin D1 (18). Silencing of E2F7 inhibited LAC cell proliferation and led to G1 arrest. Modulation of circPRKCI recapitulated these phenotypes (18). While silencing of circPRKCI led to upregulation of p21 and downregulation of cyclin D1, overexpression of circPRKCI increased E2F7 protein levels and promoted cell proliferation (18).

The above findings delicately show that *in vitro* circPRKCI functions by acting as a miRNA sponge to miR-545 and miR-589 thereby increasing expression of E2F7. With this strong evidence in vitro, Qiu *et al.* further investigated this mechanism *in vivo*. First using a xenograft tumor model and patient-derived tumor primary cultures, they found that downregulation of circPRKCI led to smaller tumors (18). This is consistent with their earlier finding that patients with lower circPRKCI expression in their tumors had lower T stage tumors. Further, the cells collected from si-circPRKCI injected mice showed lower E2F7, lower Cyclin D1 and higher p21 expression, again consistent with findings *in vitro* (18). Lastly, circPRKCI inhibition on the effectiveness of already developed EGFR tyrosine kinase inhibitors was assessed using proliferation assays *in vitro* of EGFR mutant LAC cells. As expected, both the EGFR tyrosine kinase inhibitor and si-circPRKCI treatments inhibited cell growth, but combined treatment with both an EGFR directed drug and si-circPRKCI had a stronger inhibition, indicating that circPRKCI promotes tumor growth independently from EGFR signaling (18). These findings validate the previous *in vitro* results using an *in vivo* model, and identify a potential therapeutic target for patients with LAC both as a standalone treatment or in conjunction with already known targeted therapies.

circRNAs are unique non-coding RNA structures that predominantly occupy the cytoplasm and function as miRNA sponges, thereby regulating transcription and affecting cell function. Qiu *et al.* have identified a novel circRNA, circPRKCI, that is derived from a recurrent CNV locus in lung adenocarcinoma. The 3q26.2 amplicon harbors two candidate targets PRKCI and SOX2 (20) and the authors have shown that circPRKCI may not function through SOX2 signaling (18). In this regard, it would be interesting to see whether the poor prognosis of patients with high level of circPRKCI expression is independent of the amplification of 3q26.2 in the future study. Another interesting question is how circRNAs are generated from amplified genomic regions. A large number of circRNAs have been identified in the human genome, and back-splicing of the transcript from the native gene locus is considered to be a dominant mechanism for producing circRNA. However, cancer genomes, in particular genomic amplification consist of complex rearrangements (24). Considering the efficient generation of circPRKCI under the strong promoter in the transgene system (*Figure 3A*) (18), it is plausible to envision a similar situation can be created somatically by rearrangements in the cancer genome and continuously produce circRNAs that promotes tumor growth.

Through elucidating the presence of and mechanism by which circPRKCI functions in lung adenocarcinoma, the authors have not only identified a biomarker for lung adenocarcinoma but they have also awoken the opportunity for the discovery of other circRNAs across the transcriptome that may be derived from CNVs with similar prognostic and therapeutic potential. Additionally, in a focused setting this has tremendous implications for the future treatment of LAC. The ability to combine targeted therapies with independent targets may improve anti-tumor efficacy and potentially limit medication toxicity, often experienced with various chemotherapy regimens. Further, from a biologic standpoint, the ongoing advances in understanding how circRNAs are involved in cancer development, proliferation, and aggressiveness through their post-transcriptional regulation of gene expression provides great opportunity and promise for future treatment advances and therapeutic development across many cancer types.

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