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RESEARCH HIGHLIGHT

miRNAs as tools for tailoring personalized therapeutic strategies in ovarian carcinoma

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Ovarian carcinomas are associated with an extremely poor prognosis, resulting in a 5-year survival of 30% for advanced-stage disease. Unlike other malignancies, no significant improvement in disease management and overall survival has been achieved in the past three decades, underlining the urgent need of new therapeutic opportunities. The cooperation of Bcl-xL and Mcl-1 is required to protect ovarian cancer cells against apoptosis. Given that miRNAs target the expression of several genes, in a coordinated manner, often at the nodes of important regulatory pathways, we hypothesized that among miRNAs able to kill chemoresistant ovarian cancer cell lines some may target both Bcl-xL and Mcl-1. Recently, we demonstrated that miR-491-5p induces apoptosis in several ovarian cancer cells by inhibiting directly Bcl-xL and indirectly Mcl-1 through the targeting of EGFR leading to BIM activation. Interestingly, the apoptotic effect of miR-491-5p could be mimicked by a combining an EGFR inhibitor with a BH3-mimetic molecule (two drugs already used in clinical practice). In addition, the knowledge of the precise molecular effect of miR-491-5p provided ways to identify downstream genetic alterations impeding the pro-apoptotic effect of miR-491-5p in another cellular context. The resistance to this combination treatment was finally counteracted by associating pharmacological molecules affecting the involved pathways at pertinent levels. Altogether, our work highlights the potential of phenotype-based miRNA screening approaches to decipher, in the absence of a preconceived idea, new relevant therapeutic targets harboring synthetic lethal interactions to finally propose new rationale drug combinations.

Keywords: microRNAs; ovarian cancer; Bcl-2 family; EGFR inhibitors; ABT-737; MAPK; Akt

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Introduction

Ovarian cancer is the most aggressive gynaecological malignancy^[1]. The yet unpredictable recurrence observed in more than 75% of the ovarian carcinoma patients, associated to the fact that the relapsed disease generally becomes unresponsive to conventional chemotherapy

(carboplatin/paclitaxel), is responsible for the poor overall survival rate. More than 230,000 new cases are diagnosed each year worldwide, at a late dissemination stage in the majority of cases, leading to the death of about 140,000 women^[2]. Despite advances in surgery and chemotherapy practices, the ultimate rate of cure has not been strongly impacted during the past three decades, the

mortality/incidence ratio thus remaining dramatically elevated. Improved ovarian cancer therapeutic care needs the development of both improved early detection methods and innovative therapeutics susceptible to overcome chemoresistance.

More and more efforts are made to develop targeted therapies, specifically dedicated to the treatment of patients with identified genetic alterations, but ovarian cancer therapeutic care lags behind other cancer types in incorporating such therapies into standard treatment. The angiogenesis inhibitor bevacizumab (Avastin[®]) was the first "targeted therapy" which has been introduced in clinical practice and was shown to only modestly increase progression-free survival (PFS)^[3]. In addition, guidelines for the therapeutic use of bevacizumab in ovarian cancer differ worldwide. For example, the European Medicines Agency (EMA) has approved bevacizumab in combination with carboplatin and paclitaxel as first-line therapy^[4, 5] whereas the Food and Drug Administration (FDA) has approved bevacizumab only for use in combination with other treatments for women with recurrent and platinum-resistant ovarian cancer^[6]. Very recently, the PARP inhibitor, Olaparib (Lynparza[™]) has been approved for monotherapy in the treatment of advanced ovarian cancer. However, its recommendation is limited to patients harboring *BRCA1* or *BRCA2* gene mutations (10-15% of cases) who have previously received three or more lines of therapy^[7]. Other molecular targeted therapies are under further evaluation in ongoing clinical trials. The most suitable strategies for these innovative approaches will thus be defined in various settings [first-line, relapse (platinum-sensitive and resistant)] in a near future^[8]. Moreover, the definition of interaction between these agents and various pathways as well as the associated predictive markers is a major objective for safe and efficient treatment.

However, while the initial results of targeted therapies in laboratory and clinic have proved promising in ovarian cancer, and globally in the field of oncology, they also provided researchers and clinicians with a cautionary tale. Indeed, in most of the case, the redundancy of survival pathways, the cross-talk and feedback loops which sustain tumor cells survival are often at the origin of tumor escape from current targeted therapies. In addition, the emergence of alterations and mutations in the targeted pathways can result in the appearance of resistance phenotypes. Perhaps, using combination of targeted therapies will limit the probability of the development of resistance. The detailed characterization of the pathways involved in ovarian cancer cells survival, chemoresistance and response to treatments is then of utmost importance to design new innovative treatment options.

In this context, microRNAs (miRNAs) represent an interesting area to explore. Indeed, miRNAs are a class of endogenous small non-coding RNAs and they act through imperfect sequence complementarity by binding in the 3' untranslated region (UTR) of target messenger RNAs (mRNAs), and negatively regulate mRNA translation and stability^[9]. Now there are over 2,500 potential human miRNAs recorded in miRBase (version 21, June 2014), and it is predicted that more than 60% of protein coding genes could be targeted by miRNAs. Consequently, it is not surprising that miRNAs have been found to play a crucial role in all basic biological processes and that their dysregulation is implicated in the development and progression of cancer, including ovarian carcinoma^[10]. Importantly, one miRNA could target several hundreds of target mRNAs, and one transcript can be targeted by multiple miRNAs, leading to the formation of complex regulatory networks. Moreover, the most remarkable feature of miRNAs is that they often target proteins at the nodes of important regulatory pathways, opening new avenues in the perspective of drug target identification.

miRNA as a tool to identify synthetic lethality

Hallmarks of cancer have underlined the ability of tumor cells to escape from apoptosis as a major contributor of drug resistance and tumor progression [11]. Cancer cells often harbor alterations in the expression and activity of BCL-2 family members leading to an increase in the apoptotic threshold. As a consequence, inhibition of the expression and/or activity of anti-apoptotic members of BCL-2 family represent an attractive way to selectively kill cancer cells. In ovarian carcinoma, Bcl-xL and Mcl-1 cooperating to protect ovarian cancer cells from cell death against apoptosis, their simultaneous inhibition is required to trigger apoptosis in chemoresistant ovarian cancer cell lines (IGROV1-R10 and SKOV3)^[12, 13]. Postulating that miRNAs may coherently modulate genes involved in networks regulating apoptosis, we hypothesize that among miRNAs able to kill the chemoresistant ovarian cancer cell lines some may target both Bcl-xL and Mcl-1^[14].

Using *in silico* miRNA targets prediction algorithms together with functional studies, we tried to identify miRNA(s) that could induce apoptosis in ovarian cancer cells by targeting directly Bcl-xL and Mcl-1. Among the 11 putative miRNAs identified by our approach, some down-regulated either Bcl-xL or Mcl-1, but none was shown to decrease both in our cellular models. However, the transfection of one miRNA, miR-491-5p, was found to induce apoptosis in IGROV1-R10 cells. We next demonstrated that Bcl-xL is a direct target of miR-491-5p as previously demonstrated by others in different cellular

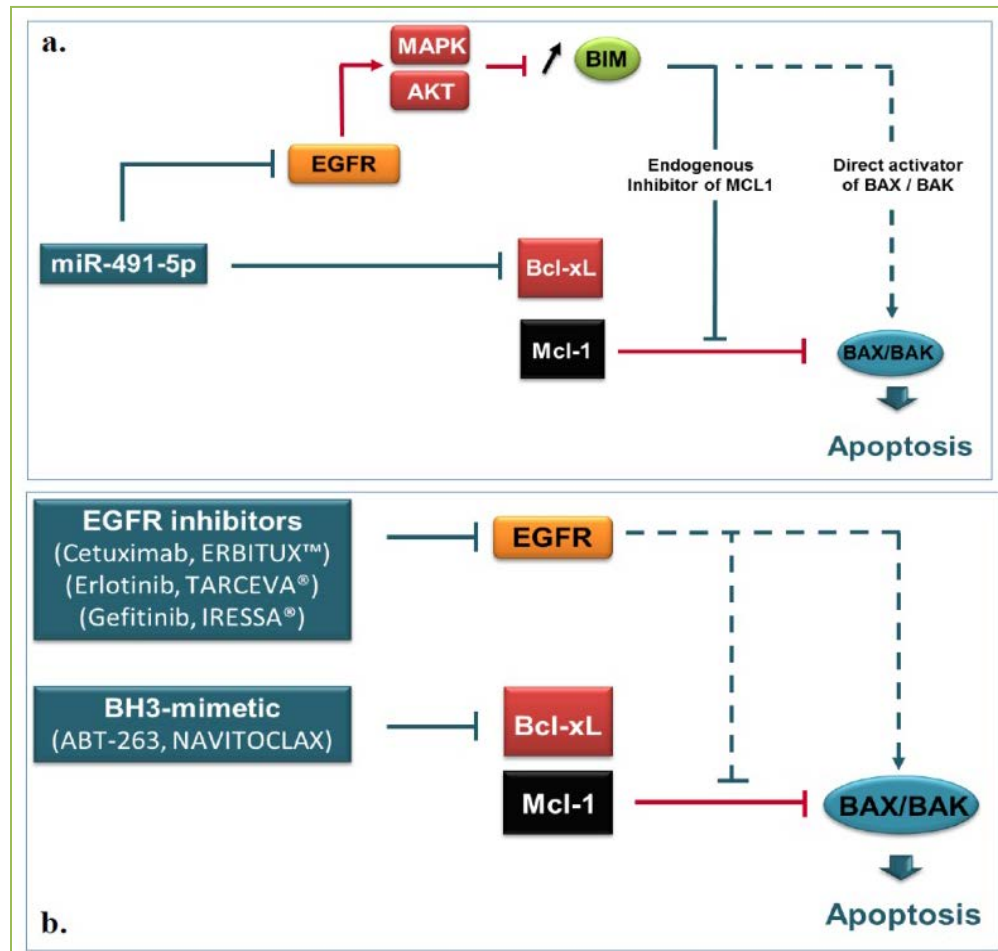


Figure 1. Potential of miRNA functional studies to decipher cell signalling pathways involved in cell survival to propose the rationale design of new strategies based on pharmacological combinations. (a) miR-491-5p induces apoptosis in ovarian cancer cells by inhibiting Bcl-xL and by targeting EGFR leading to BIM activation and subsequent Mcl-1 inhibition and possibly BAX/BAK activation. (b) The effect of miR-491-5p can be mimicked by a combination therapy of an EGFR inhibitor with a BH3-mimetic molecule.

contexts ^[15-17]. Whereas previous observation showed that a siRNA targeting Bcl-xL induces only a mild apoptotic effect, miR-491-5p induces a drastic cell death in IGROV1-R10 cells, suggesting that other targets, either with direct/indirect or without relations with Mcl-1, may be involved in miR-491-5p mediated cell death.

miR-491-5p exerts its cytotoxic effect by inhibiting directly Bcl-xL and indirectly Mcl-1 through the targeting of EGFR leading to BIM activation

We demonstrated that the apoptotic effect of miR-491-5p is related on one hand to the down-regulation of Bcl-xL (direct effect) and on the other hand to the indirect inhibition of Mcl-1 activity. Indeed, miR-491-5p was shown to decrease directly EGFR expression which diminishes subsequently activation of both AKT and MAPK pathways leading to the BH3-only protein BIM induction and

dephosphorylation, allowing finally the inhibition of Mcl-1 activity and possibly direct activation of BAX/BAK (Figure 1a). Of note, this study demonstrated, for the first time, that EGFR is a direct target of miR-491-5p in ovarian cancer cells. Although our target-driven approach did not lead to the identification of miRNA with direct effect on both Bcl-xL and Mcl-1, an interesting learning is the identification of the pathways involved in the death phenotype in response to miR-491-5p. Another study used a miRNA screen to identify miRNAs able to down-regulate Mcl-1, a determinant of response to a BH3-mimetic molecule (ABT-263). In HCT-116 cells, the authors identified several miRNAs able to trigger cell death but Mcl-1 down-regulation was not a direct effect for several of the miRNAs identified ^[18], paralleling our observations that a phenotype instead of a target-directed approach could be more efficient in characterizing the determinants of cancer cells survival.

Toward a phenotype-based consideration of miRNA effects

One can hypothesize that screening of individual miRNAs against the induction of a specific phenotype (including cell death) might allow the identification of molecular determinants sustaining this phenotype, and thus of potential pharmacological targets. This requires miRNA to be implicated in coordinated regulation of gene networks as supported by several reports. For example, miRNAs may regulate proteins in the same complex, in the same signalling pathway or in the same biological function. A first experimental demonstration was provided by Linsley *et al.* [19]. Using miRNA transfection in DICER-deficient cell lines followed by comparative gene expression profiling, they showed that miR-16 affects many targets that function in a coordinated manner to regulate the G0/G1 to S cell cycle transition. Accordingly, simultaneous siRNA-mediated down-regulation of several miR-16 targets did block cell cycle progression in more efficient way than the silencing of individual miR-16 targets. In another study, the consequences on global gene expression pattern of the transfection of two individual miRNA, miR-7 and miR-128, in an ovarian cancer cell line, were monitored by microarrays and qPCR [20]. Modifications of gene expression were not random, and functional pathway analysis showed that genes modulated by miR-7 and miR-128 transfections were predominantly involved in distinct regulatory pathways: cell adhesion, EMT and development for miR-7, cell cycle regulation for miR-128. Experiments on cell adhesion and cell cycle gave results consistent with those predictions. Interestingly, functional and binding interaction network analysis suggested that transfection of individual miRNA, by impacting the regulation of hub genes (often directly), may indirectly affect the expression of a large number of downstream genes, and thus trigger cascades of coherent regulatory changes. Further evidence that miRNAs regulate functionally related genes within distinct networks has been reviewed recently [21]. A study integrating publicly available mRNA expression data from numerous cellular systems and tissues was able to establish co-expression clusters among predicted targets of individual human miRNA, reinforcing the hypothesis that the targets of a given miRNA belong to the same gene regulatory network [22]. A statistical analysis of enrichment of miRNA-targeting signatures in functionally annotated gene sets compared to random gene sets provided evidences that many miRNAs coordinately regulate several components of pathways. Some miRNAs can also function in multiple canonical pathways and mediate crosstalk between these pathways, to coordinately regulate cellular functions [23]. Otherwise, screening the effects of miRNA mimics on the viability of isogenic KRAS-wild type and KRAS-mutant colorectal cancer cell lines led to the identification of

miR-126, which specifically reduced the growth of multiple KRAS-mutant cell lines, and inhibited clonogenicity and tumorigenicity. This selective effect, and the reduced expression of miR-126 in colorectal tumor with mutant KRAS as compared to wild type, led to the hypothesis that miR-126 regulates a coherent gene network that is required for the viability of KRAS-mutant cells. This postulated that synthetic lethal interaction between miR-126 target genes and mutant KRAS was compatible with the fact that transcriptome profiling failed to identify genes down-regulated by miR-126 only in KRAS-WT or KRAS-mutant cells. By analyzing the overlap between miR-126 targets with previously characterized synthetic lethal genes in KRAS-mutant cells, the authors identified that a subset of miR-126 regulated genes was required for the survival of KRAS mutant cells. SiRNA-down-regulation of individual miR-126 targets was less efficient than miR-126 to decrease the clonogenicity of KRAS-mutant cells [24]. This work, in addition to the study by Lam *et al.* [18] and our recent work suggests that a phenotype- instead of a target-orientated strategy could prove itself successful to identify contextual synthetic lethal interactions. The intrinsic capacity of miRNAs to modulate the expression of a network of target genes in a comprehensive way to trigger a specific phenotype makes them very good candidates for a screening-based approach. This way, it would be possible to bring out relevant target associations harboring a synthetic lethal phenotype, in the absence of a preconceived idea about the targets.

Using a “death” phenotype triggered by a miRNA to characterize synthetic lethality

The characterization of the precise pathways regulated by a miRNA of interest can be achieved through the identification of its direct targets. To this end, different biochemical strategies based on co-precipitation of RNA with Ago proteins and/or a specific miRNA have been described [25]. For example, the transfection of a biotinylated miRNA and subsequent streptavidin based purification of direct targets have been successfully used to identify new direct targets of miR-34a [26]. Moreover, integration of information relative both to direct targets of a miRNA and to perturbation induced by a miRNA at transcriptomic and proteomic levels would allow the precise characterization of the pathways involved in a death phenotype, and thus provide opportunities for combinatory treatments. According to this strategy, our laboratory has performed a miRNA screen on several chemoresistant ovarian cancer cell lines, which identified several miRNAs able to trigger cell death (unpublished observations); the precise characterization of the pathways involved is currently under investigation.

Two possibilities exist with the identification of several miRNAs presenting a death phenotype in a given context (e.g. a specific cell line): (i) these miRNAs share an effect on identical pathways – although possibly at different levels, or (ii) they target different pathways. We hypothesize that these two options will appear to be true to some extent. Moreover, the combinatory aspect of the approach we present might identify targets within the network of a single miRNA whose individual regulation is harmless to cancer cells, and thus not yet identified as critical for their survival.

Context dependence of the synthetic lethality revealed by the miR-491-5p: a problem or a source for tailoring new combinatorial therapeutics?

It is worth noting that a given combinatory approach will not be effective in every cellular context. Screening for miRNA mediated-toxicity towards ovarian cancer cells cannot be tested on an individual basis in patient's biopsies. However, the choice of several cell lines representative of ovarian cancer subtypes diversity will provide more combination options; it will reveal as well the extent of sensibility to a given combination among a range of ovarian cancer subtypes. A miRNA may have the same targets in different cellular contexts without inducing the same phenotype because of specific alterations in the involved-pathways. Intervention at different levels in these pathways can provide means to recover the phenotypic effect. As an example, we could evidence that miR-491-5p has a differential effect in SKOV3 cells without inducing apoptosis. Intriguingly, miR-491-5p decreases both Bcl-xL and EGFR expression similarly to the observation made in IGROV1-R10 cells but fails to inhibit both AKT and MAPK signaling and do not induce BIM in SKOV3 cells. These observations allowed us to hypothesize that BIM degradation was induced by a continuous activation of AKT and MAPK pathways downstream of EGFR. Indeed, we noticed that in SKOV3 cells ERK is more strongly activated than in IGROV1-R10 cell line. In addition, SKOV3 cells are characterized by an activating mutation (H1047R) in the *PIK3CA* gene, leading to continuous phosphorylation of AKT. Importantly, the use of specific inhibitors of AKT and MAPK pathways led to BIM dephosphorylation and induced apoptosis in SKOV3 cells with a siRNA targeting Bcl-xL. This observation demonstrated that the precise characterization of pathways involved in miRNA-induced phenotype can provide the opportunity to counteract context dependent resistance.

Opportunities for therapeutic interventions based on miRNA biology

It is now well established that there is substantial interest

in exploiting miRNAs for therapeutic applications. However, most of studies were focused on the fact that miRNAs, themselves, could be considered as therapeutic agent (replenishing tumor suppressor miRNAs) or therapeutic target (silencing an oncomiR). For instance, restoration of miRNA levels can be achieved with 'miRNA mimics' or miRNA-expressing plasmids whereas miRNA knockdown is reached using chemically modified antisense-oligonucleotides [27]. Notably, one miRNA-based cancer therapy (MRX34, a liposome-based miR-34 mimic) has recently entered a Phase I clinical trial in patients with advanced liver cancers to evaluate its safety (NCT01829971). However, although considerable progresses have been made in this field, the clinical development of such strategies is still facing major hurdles of inefficient delivery, biodistribution and biostability [28].

Based on our study, we proposed that miRNA functional approach may facilitate the identification of new relevant therapeutic targets, effective drug combinations, and eventually enable the proposition of new clinical trials. Indeed, we demonstrated that a BH3-mimetic molecule (ABT-737) binding and neutralizing Bcl-xL in combination with an anti-EGFR monoclonal antibody (cetuximab) or EGFR TKIs (erlotinib and gefitinib) mimic the apoptotic effect of miR-491-5p in IGROV1-R10 cells (Figure 1b), whereas each drug alone had no obvious effect. This observation is of importance in the context of ovarian carcinoma because to date, EGFR-directed therapies were used as single agents and have yielded disappointing results. EGFR inhibitors are widely used in clinical practice for the treatment of colorectal and non-small cell lung cancer [29] and Navitoclax (ABT-263, the orally bioavailable analogue of ABT-737 used in clinical trial) presents some activity in haematological and solid cancers [30], both molecules are therefore already available for a clinical use. Consequently, EGFR inhibitors in combination with Navitoclax would be a feasible and interesting way to explore in ovarian carcinoma. Accordingly, combination of Navitoclax with targeted therapies could be efficient for the treatment of various solid tumors as suggested by recent preclinical studies. In addition, although the development of direct Mcl-1 inhibitors is currently an active research field, these molecules are still dramatically lacking for clinical application. Therefore, BIM-inducing strategies, as observed with EGFR inhibitors, may represent an alternative interesting way to inhibit Mcl-1.

Our study also demonstrated that some downstream alterations triggering constitutive activation of AKT and MAPK signaling pathways abolish the effect of EGFR inhibitors as observed in SKOV3 cells. In this context, the use of specific inhibitors of AKT and MAPK will lead to BIM accumulation and its sequestration by a BH3-mimetic

molecule will lead to cell death. Accordingly, it was shown in our team that the combination of BEZ-235 (a dual PI3K/mTOR inhibitor) and CI-1040 (a MAPK pathway inhibitor) with ABT-737 led to apoptosis of SKOV3 chemoresistant cancer cell line [31]. Importantly, in both cancer cell lines, BH3-mimetic molecules are useful to sensitize ovarian carcinoma cells to BIM induction through the inhibition of EGFR signaling pathways. As these results strengthen the relevance of our approach for the design of innovative therapeutic strategies, an obvious limitation is the availability of clinically useable drugs targeting identified proteins and/or pathways. However, the screening of chemical libraries remains a valid tool for the identification of targeted inhibitors and the design of new clinically developable drugs.

In summary, we are convinced that the precise characterization of selected miRNAs target networks may provide new insights of the regulatory events involved in cancer cells survival, and therefore suggest combinatorial drug action. Recently, an interesting study integrating molecular and phenotypic data to understand and target disease effectively has been described to “harness the immense power of systems biology to identify therapeutic strategies against cancer” [32]. The availability of an increasing number of molecules designed for targeted therapies might provide clinically useable tools to mimic the effect of miRNAs, in the absence of a safe and reliable way to directly use miRNAs into clinics. In addition, our strategy might help to characterize in a detailed and comprehensive way the regulatory networks of ovarian cancer cells. If proven successful, our strategy could be easily applied in other cancerous pathologies. The age of “miRNA-based network medicine” has clearly begun...

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Conflict of interest

The authors declare no conflict of interest.

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