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Research article

Sequence-based analysis of 5′UTR and coding regions of *CASP3* in terms of miRSNPs and SNPs in targetting miRNAs

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ABSTRACT

Apoptosis is described as a mechanism of cell death occurring after adequate cellular harm. Deregulation of apoptosis occurs in many human conditions such as autoimmune disorders, ischemic damage, neurodegenerative diseases and different cancer types. Information relating miRNAs to cancer is increasing. miRNAs can affect development of cancer via many different pathways, including apoptosis. Polymorphisms in miRNA genes or miRNA target sites (miRSNPs) can change miRNA activity. Although polymorphisms in miRNA genes are very uncommon, SNPs in miRNA-binding sites of target genes are quite common. Many researches have revealed that SNPs in miRNA target sites improve or decrease the efficacy of the interaction between miRNAs and their target genes. Our aim was to specify miRSNPs on *CASP3* gene (caspase-3) and SNPs in miRNA genes targeting 5′UTR and coding exons of *CASP3*, and evaluate the effect of these miRSNPs and SNPs of miRNA genes with respect to apoptosis. We detected 141 different miRNA binding sites (126 different miRNAs) and 7 different SNPs in binding sites of miRNA in 5′UTR and CDS of *CASP3* gene. Intriguingly, mi*R*-339-3p's binding site on *CASP3* has a SNP (rs35372903, G/A) on *CASP3* 5′UTR and its genomic sequence has a SNP (rs565188493, G/A) at the same nucleotide with rs35372903. Also, miR-339-3p has two other SNPs (rs373011663, C/T rs72631820, A/G) of which the first is positioned at the binding site. Here, miRSNP (rs35372903) at *CASP3* 5′UTR and SNP (rs565188493) at miR-339-3p genomic sequence cross-matches at the same site of binding region. Besides, miR-339-3p targets many apoptosis related genes (*ZNF346, TAOK2, PIM2, HIP1, BBC3, TNFRSF25*, CLCF1, IHPK2, NOL3) although it had no apoptosis related interaction proven before. This means that miR-339-3p may also have a critical effect on apoptosis via different pathways other than caspase-3. Hence, we can deduce that this is the first study demonstrating a powerful association between miR-339-3p and apoptosis upon computational analysis.

1. Introduction

A process including cell suicide known as apoptosis gets rid of distorted cells in the body. Inhibition of apoptosis is very crucial for tumor initiation and progression because apoptosis ordinarily eliminates cells with high malignant potential, such as those with distorted DNA or abnormal cell cycling. Fundamental cancer research has provided striking developments in our comprehension on cancer biology and genetics in the last decade. The most crucial advance is to realize that apoptosis and the genes controlling it have a deep impact on the malignant phenotype. For instance, it is currently evident that some oncogenic mutations destroy apoptosis, causing tumorigenesis. However, extensive evidence is also accumulating that other oncogenic alterations may trigger apoptosis, underlining the fact that apoptosis is suppressed by selective forces acting on a multi-pathway tumorigenesis process (Lowel and Lin, 2000).

Two main cell-based mechanisms have been defined for apoptosis initiation, one starting at the level of death receptors of cell surface and another one including mitochondria activation tracked by cytochrome c releasing. The initiation of these cascades induces the activation of caspase pathways. Up to now, 14 distinct caspases have been defined. Despite the fact that upregulation of any one of them can cause cell death via apoptosis, none of them is not ordinarily included in this operation. The main starter caspases are caspases-8 and caspase-9. Binding of death ligands to their related receptors induces caspase-8 activation, however caspase-9 is triggered by releasing of mitochondrial cytochrome c and the

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following apoptosome complex formation. The main effector caspases are caspases-3, −6 and −7 and they cut many proteins undergoing proteolysis in apoptotic cells when they are activated (Thyrell et al., 2002).

Caspases are considered as important intermediary elements in programmed cell death (apoptosis). As final mediator, caspase-3 is a mostly activated death protease, facilitating the particular cleavage of many crucial cellular proteins. But, the particular necessities of this caspase have stayed mainly mysterious in apoptosis by now. Cascades involved in caspase-3 activation have been described as dependent on or independent of mitochondrial cytochrome c release and caspase-9 function. Caspase-3 is necessary for regular brain development and is crucial or requisite in other apoptotic mechanisms in a considerable tissue-, cell type- or death signal-specific way. Caspase-3 is further needed for some ordinary characteristics of apoptosis, and is inevitable for DNA breakup and apoptotic chromatin condensation in all cell types studied. Therefore, caspase-3 is necessary for some functions related with the disassembly of the cell and the apoptotic body formation however it may further work before or at the step when dedication to loss of cell viability is done (Porter and Jänicke, 1999).

Caspase-3 is executioner caspase and has zymogen characteristics, that is, has to be activated. Apoptotic cell can use both extrinsic (death ligand) and intrinsic (mitochondrial) cascades in order to activate caspase-3. Until caspase-3 activation (during other caspases' functions), apoptosis can be reversed or suppressed. However, after it is activated, it works in an irreversible manner. Other caspases can be regulated back but caspase-3 cannot be. Caspase-3 is indispensable for apoptotic chromatin condensation and DNA fragmentation in all cell types. Also, other caspases taking role in apoptosis can be regulated by many different ways but caspase-3 is most prominently regulated by post-transcriptional manner (especially RNAi). (Ergun and Oztuzcu, 2014). For these reasons, caspase-3 was selected to study apoptosis.

MicroRNAs (miRNAs) are small non-coding RNAs regulating gene expression negatively which are about 18–24 nucleotides in length. miRNA can work as oncogenes and tumor suppressors. Dysregulation of miRNA expression has been declared in various human cancers containing prostate cancer, breast cancer, colon cancer, hepatocellular carcinoma, and osteosarcoma. Intensive studies during the last several years have identified numerous affected miRNAs in association with apoptosis, their target genes and biological functions, and possible drug interventions. For example, let-7a is related with apoptosis via targeting caspase-3 directly. Moreover, miR-34 family members targets p53 directly, and their overexpression triggers cell cycle arrest and apoptosis. Hereof, miR-34a has been depicted to regulate genes taking role in cell cycle control and apoptosis, containing *E2F3*, cyclin-dependent kinase 4 (*CDK4*), *CCND1*, *SIRT* and *CDK6*. Futhermore, miR-16 is concerned with triggering of apoptosis via targeting Bcl-2, and is included in regulation of the cell cycle via targeting cell division cycle protein 27 (*CDC27*), *CDK6*, cyclin E, the caspase recruitment domain-containing protein 10 (*CARD10*) and *CCND1* (Aranha et al., 2010).

It has been recently shown that mammalian miRNA targeting doesnot always limit itself to 3′ miRNA action. It now seems that in addition to the traditional 3′ UTR targeting, mammals are starting to look a bit more like plants as they also target a number of the 5′ untranslated region (5′ UTR) and amino acid coding sequence (CDS) sites (Zhou and Rigoutsos, 2014). An elevated percentage (25%) of the reads were mapped to open reading frames (ORFs), even though the most matched to 3′ UTRs and just 1% to the 5′ UTR, affirming that miRNAs rather bind their targets via the 3′ UTR but also emphasizing the significance of CDS-mediated interactions. Likewise, one of the largest restrictions of available databases is that the estimations are mostly limited to the 3′ UTRs, when the latest experimental throughputs show that most of the miRNA/mRNA interactions may happen via CDSs or even the 5′ UTR. Moreover, the binding rules for miRNA/target interactions via the 3′ UTR may differ via other mRNA regions, also decreasing the capacity of available databases to estimate these interactions (Martinez-Sanchez and Murphy, 2013).

The plethora of published reports in recent years demonstrate that miRNAs play fundamental roles in many biological processes, such as carcinogenesis, angiogenesis, programmed cell death, cell proliferation, invasion, migration, and differentiation by acting as tumor suppressor or oncogene, and aberrations in their expressions have been linked to onset and progression of various cancers. Furthermore, each miRNA is capable of regulating the expression of many genes, allowing them to simultaneously regulate multiple cellular signalling pathways. Hence, miRNAs have the potential to be used as biomarkers for cancer diagnosis and prognosis as well as therapeutic targets. Also, dysregulation of miRNA networks has been implicated in biological processes specified above. One of the reasons for disturbed miRNA-mediated regulation is polymorphism in miRNA-binding sites (miRSNPs), which alter the strength of miRNA interaction with target transcripts (Dzikiewicz-Krawczyk, 2014). Moreover, common genetic variants like single nucleotide polymorphisms (SNPs) in miRNA genes could change their expression or maturation ensuing changed functional outcomes in carcinogenesis (Bansal et al., 2014). Many studies have revelaed that SNPs in miRNA target sites strengthen or weaken the interaction between miRNA and its target transcripts and are related to cancers and other diseases (Melo and Esteller, 2011; Salzman and Weidhaas, 2013).

Considering that miRNAs have been shown to play an essential role in apoptosis and that SNPs in miRNA-binding sites in target genes have been related to many cancers, in this study, we took aim at defining miRSNPs on executioner caspase, *CASP3* gene (caspase-3) and SNPs in miRNAs genes targeting 5′UTR and coding regions of *CASP3* and evaluating the effect of these miRSNPs and SNPs of miRNA genes targeting 5′UTR and coding regions of *CASP3*in terms of apoptosis.

2. Materials and methods

2.1. Screening of miRNA targeting 5′UTR and CDS of CASP3 gene

Two different online databases, miRWalk and miRcode, were used in order to investigate which miRNAs targets 5′UTR and coding regions of *CASP3* gene. The common characteristic of these two databases is the fact that they take into account 5′UTR and coding regions of targeted genes. Also, miRó database was used to associate specific miRNAs with apoptosis-related genes. Three different databases were used instead of only one database because all these databases uses different algorithms and computational approaches so we considered that we could achieve to screen all miRNAs targets without skipping any of them by using different databases.

2.1.1. miRWalk

Information about miRNA-target interactions is generated through miRWalk algorithm on the total sequence (promoter, 5′ UTR, CDS and 3′ UTR) of all known genes of Human, Mouse and Rat containing all transcripts. Predicted miRNA-target interactions gives information on genes related with 449 human biological cascades and 2356 OMIM diseases. The information is provided on 3 total mitochonrial genomes i.e. Human, Mouse and Rat. MiRNA interactions related with genes, organs, pathways, transcription factors and diseases are experimentally verified or predicted. It further gives approved miRNA interactions information on cell

lines. Furhtermore, it further ensures the information on proteins taking role in miRNA processing (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/) (Dweep et al., 2011).

2.1.2. miRcode

miRcode gives miRNA target predictions about human "whole transcriptome" based on the universal GENCODE gene annotation, containing genes of more than 10,000 long non-coding RNAs. Coding genes are also included, containing uncommon regions such as 5′UTRs and CDS. MiRNA family descriptions and names are coherent with TargetScan. Site conservation is assessed upon 46 vertebrates species (http://www.mircode.org/mircode/) (Jeggari et al., 2012).

2.1.3. miRó

miRò is a web-based algorithm providing users with miRNA-phenotype relationships in humans. It combine data from different online databases, like databases of miRNAs, targets, ontologies and diseases, into a united algorithm accoutred with an instinctive and flexible inquiry interface and data mining feasibilities. The masteraim of miRò is the institution of an information base allowing significant analysis via complicated mining procedures and the introduction of a new stage of interactions between genes and phenotypes deduced based on miRNAs annotations. (http://ferrolab.dmi.unict.it/miro/) (Laganà et al., 2009).

2.2. Screening of miRSNPs

The sites targeted by miRNA in 5′UTR and CDS of *CASP3* gene were taken from these two databases. A target site list was constructed. SNPs on 5′UTR and CDS of *CASP3* are obtained from dbSNP141 database. SNPs matching and not matching with these pairing sites were specified and evaluated in terms of caspase-3 activity and apoptosis.

2.3. Screening of SNPs of miRNA genes targeting 5′UTR and CDS of CASP3

SNPs on miRNA gene targeting 5′UTR and CDS of *CASP3* were screened from NCBI database. SNPs on miRNA gene and miRSNPs on 5′UTR and CDS of *CASP3* were matched. SNPs cross-matching were searched. SNPs matching and not matching were shown and interpreted in terms of caspase-3 activity and apoptosis.

3. Results and discussion

Finding miRNA target sites experimentally is an expensive and lengthy procedure. Last estimates offer that more than 50% of human genes coding proteins could be regulated via miRNAs. Computational prediction of miRNA/gene interactions is a precious instrument for conducting wet-lab experiments. Moreover, it stays the single choice for systematic genome-wide reconstruction of the sophisticated combinative figure of miRNA-mediated target binding. Also, it is a hard activity due to the intimidating trouble of differentiating exact miRNA-mRNA interactions among the noisy background of millions of possible miRNA-gene combinations and, more usually, since the basic mechanisms of miRNA target prediction become mostly mysterious (Sturm et al., 2010). Moreover, miRNAs manage various biological functions by suppressing mRNAs, however their mild influences on direct targets, besides their joining to large-scale regulatory systems, make it difficult to represent miRNA-mediated influences (Gosline et al., 2016). While it is predominantly approved that the most influential MREs are placed in the 3′UTRs, it has been notified that many miRNAs can effectively and ofttimes target ORFs and the 5′UTRs with the similar frequency (Steinkraus et al., 2016). Likewise, one of the largest restrictions of available databases is that the estimations are mostly limited to the 3′ UTRs, when latest experimental throughputs show that most of the miRNA/mRNA interactions may happen via CDSs or even the 5′ UTR (Martinez-Sanchez and Murphy, 2013). Therefore, we used three computational prediction databases to detect and correlate miRSNPs on executioner caspase, *CASP3* gene (caspase-3) and SNPs in miRNAs genes targeting 5′UTR and CDSs of *CASP3* with apoptosis.

Databases used in this study are three high-quality miRNA-mRNA interaction prediction algorithms. These three databases widely used in many experimental and computational studies in a reliable manner. miRWalk algorithm uses validated databases as sources (NCBI, Ensembl, UCSC, miRBase, KEGG, Biocarta and OMIM databases). Moreover, Validated Target module of miRWalk database incremented the validated information more than 3-fold as compared to other databases (Dweep et al., 2014). miRcode database is a universal search machine for putative miRNA target sites throughout the complete GENCODE annotated transcriptome (Shafiee et al., 2016). miRó database is designed as an extendable and automatically updatable information algorithm with strong data searching capabilities. It uses validated miRNA search tools (TargetScan, PicTar and Miranda)(Laganà et al., 2009).

We identified 141 different miRNA binding sites in 5′UTR and CDS of *CASP3* gene. 126 different miRNAs constructed this list. These miRNAs had three, two or single targeting site on 5′UTR and CDS of *CASP3* (Supplementary material 1). SNPs which we detected on 5′UTR and CDS of *CASP3* provide meaningful explanation about apoptosis. Especially, if these SNPs are on miRNA binding sites, then this relationship with apoptosis becomes stronger. Therefore, we commented on miRSNPs (SNPs on binding sites) with respect to apoptosis.

MiR-498, of which binding site has a SNP (rs11722179, A/G) on *CASP3* 5′UTR, contributes to apoptosis induction by vitamin D. MiR-498 was first identified in a microarray analysis as the most induced miRNA by vitamin D in ovarian cancer cells. miR-498 sponges, either standard or bulged, diminished the apoptosis

Table 1
List of miRNAs having SNP in their mature miRNA genomic sequences at targetting site to 5′UTR of *CASP3*. According to miRWalk, miRcode and miRò databases, SNPs targetting 5′UTR of *CASP3* were screened. Some of these miRNAs had SNPs defined on their genomic sequences. Some of these SNPs were in binding sites, but some of these were not. Also, some other of them were co-localized with SNP in 5′UTR of *CASP3*. Herein, SNP sites were symbolized as: **X**: SNP was not in the binding site of miRNA, **X**: SNP was in binding site of miRNA, **X**: SNP in the binding site of miRNA was co-localized withSNP in the 5′UTR of *CASP3*.

miRNA	Sequence of targetting miRNA	SNP	Nucleotide change
miR-1283-1	AGAAAGCGCTTTCCTTTG**T**AGA	rs372931855	T/C
miR-661	TGCCTGGGTCTCTGGCCTGC**G**CGT	rs537959815	G/A
miR-661	TGCCTGGGTCTCTGGCCTGCG**C**GT	rs567334781	C/T
miR-1234	T**C**GGCCTGACCACCCACCCCAC	rs372659498	C/T
miR-339-3p	TGAGC**G**CCTCGACGACAGAGCCG	rs565188493	G/A
miR-339-3p	TGAGCGCCTCGA**C**GACAGAGCCG	rs373011663	C/T
miR-339-3p	TGAGCGCCTCGACG**A**CAGAGCCG	rs72631820	A/G

induced by vitamin D. MiR-498 contributes to *in vivo* ovarian tumor suppression by a synthetic vitamin D analog EB1089 (Kasiappan et al., 2012). In another study again on ovarian cancer, miR-498 increases *FOXO3* expression via targeting the *FOXO3* 3′UTR (Liu et al., 2015) and also Bim, FasL, caspase-3 and p27KIP1 expressions upregulate upon increased *FOXO3* expression in rat oocytes, expressing that *FOXO3* takes role in apoptosis (Sui et al., 2010). Thereby, *FOXO3* is another target for miR-498 to lead to apoptosis.

Considering a study investigating if the dormancy supporting impact of miRNAs is moderated via suppression of tumor cell proliferation directly or elevated apoptosis, miR-580, of which binding site has a SNP (rs11729634, G/A) on *CASP3* 5′UTR, expressing cells revealed a tendency toward increased proliferation and decreased apoptosis (Almog et al., 2012).

SNPs on genomic sequences of miRNAs of which binding sites on 5′UTR and CDS of *CASP3*have SNPs may give possible explanation about apoptosis. Particularly, if these SNPs are on the same nucleotides with miRSNPs, then the association between miRNA and apoptosis should become very potential (Table 1). So, we commented on SNPs on genomic sequences of miRNAs of which binding sites on 5′UTR and CDS of *CASP3* with respect to apoptosis.

miR-661's genomic sequence has two SNPs (rs537959815, G/A; rs567334781, C/T) but has no SNP on binding site in 5′UTR of *CASP3* gene. Still, these SNPs may have crucial effect on binding affinity of it on 5′UTR of *CASP3* gene. According to miRó database, miR-661 has many apoptosis-related targets as addition to *CASP3*, like *PPARD*, *BCL2L15*, *TNFSF12*, *E2F2* etc. Moreover, miR-661 down-regulates both Mdm4 and Mdm2 to trigger p53 and by this way, it can activate apoptosis cascade (Hoffman et al., 2014). So, this shows its possible role in apoptosis.

miR-1234's genomic sequence has a SNPs (rs372659498, C/T) but has no SNP on binding site in 5′UTR of *CASP3* gene. In the same manner like miR-661, this SNP can be effective on binding affinity of it on 5′UTR of *CASP3* gene. In miRó database, miR-1234 has many apoptosis-related targets as addition to *CASP3*, like *BBC3, BCL2, SGPL1, SHF, ABL1* etc. For example, BBC3 induces apoptosis.

According to a study, placenta-specific miRNAs may organize the apoptosis, proliferation and invasiveness of trophoblast cells, so influencing the pathogenesis of preeclampsia. miR-1283-1, which its binding site has a SNP (rs113420705, T/C) on *CASP3* 5′UTR and its genomic sequence has a SNP (rs372931855, T/C) at the different site of binding site, is one of the apoptosis-related miRNAs in this study (Wang et al., 2012). So, our findings support the apoptotic role of miR-1283-1 via this study upon sequence-based analysis.

miR-339-3p's binding site has a SNP (rs35372903, G/A) on *CASP3* 5′UTR and its genomic sequence has a SNP (rs565188493, G/A) at the same nucleotide with rs35372903, a SNP (rs373011663, C/T) at the binding site of it and another SNP (rs72631820, A/G). The most important site of this study is here since miRSNP (rs35372903) at *CASP3* 5′UTR and SNP (rs565188493) at miR-339-3p genomic sequence cross-matches at the same site of binding region (Fig. 1). Furthermore, there is no information about miR-339-3p with respect to apoptosis. Besides, miR-339-3p targets many apoptosis related genes;*ZNF346, TAOK2, PIM2, HIP1, BBC3, TNFRSF25*, CLCF1, IHPK2, NOL3 according to miRó database. Upon these targetings, it may have role in regulation of apoptosis, induction of apoptosis by extracellular signals, caspase activation, apoptotic mitochondrial changes and release of cytochrome c from mitochondria. Therefore, this novel finding upon SNP cross-match between miR-339-3p and *CASP3* 5′UTR may give information about all these apoptotic processes. Then, we can conclude that this is the first study proving a strong association between miR-339-3p and apoptosis (Table 2).

Consequently, this study revealed that a computational approach to predict miRNA targets enables the course of narrowing down potential target sites for experimental validation and proves the significance of SNPs on target sites and miRNA geneomic sequences at target sites, especially 5′UTR and CDSs. Also, the analysis of cross-match of SNPs at binding site has gained to us a strong and novel association of miR-339-3p and apoptosis for the first time.

Table 2
List of apoptosis-related genes (other than CASP3) targetted by miR-339-3p according to miRó database. Herein, we listed genes targetted by miR-339-3p and associated with apoptotic pathways in different manners. This information was supplied by the miRó-Ontology Database.

Gene	Relationship with apoptosis
ZNF346	Regulation of apoptosis
TAOK2	Regulation of apoptosis
PIM2	Apoptotic mitochondrial changes
HIP1	Caspase activation
BBC3	Caspase activation
TNFRSF25	İnduction of apoptosis by extracellular signals
CLCF1	Negative regulation of apoptosis
IHPK2	Positive regulation of apoptosis
NOL3	Regulation of apoptosis
HIP1	Regulation of apoptosis
BBC3	Release of cytochrome c from mitochondria

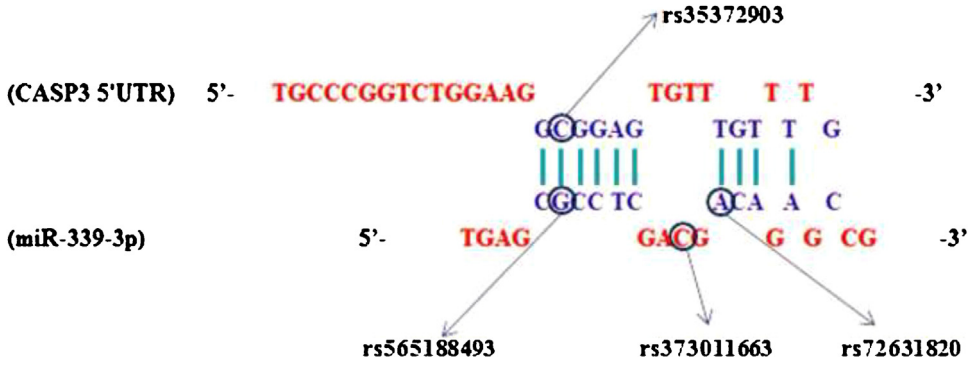

Fig. 1. Cross-matches of SNPs in the miR-339-3p and in the binding site with 5′UTR of *CASP3*. According to dbSNP 141 database, genomic locations of SNPs found in the miR-339-3p and in the binding site with 5′UTR of *CASP3* were matched with each other. Co-localizations were screened. In the binding site, miR-339-3p makes 10 base-pairings with *CASP3* 5′UTR. MiR-339-3p geneomic sequence has three SNPs. Two of them (rs565188493, G/A; rs72631820, A/G) were localized in the binding site on the *CASP3* 5′UTR and one (rs373011663, C/T) was not on the binding site on the *CASP3* 5′UTR. Also, *CASP3* 5′UTR had a SNP (rs35372903, G/A) on binding site of miR-339-3p and this miRSNP and SNP (rs565188493, G/A) at miR-339-3p genomic sequence co-localized at the same genomic location of binding region.

Conflict of interest

Authors have no conflict of interest regarding the subject of this manuscript.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.compbiolchem.2016.04.003.

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