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PathVisio Analysis: An Application Targeting the miRNA Network Associated with the p53 Signaling Pathway in Osteosarcoma

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Abstract: MicroRNAs (miRNAs) are small single-stranded, non-coding RNA molecules involved in the pathogenesis and progression of cancer, including osteosarcoma. We aimed to clarify the pathways involving miRNAs using new bioinformatics tools. We applied *WikiPathways* and *PathVisio*, two open-source platforms, to analyze miRNAs in osteosarcoma using miRTar and ONCO.IO as integration tools. We found 1298 records of osteosarcoma papers associated with the word "miRNA". In osteosarcoma patients with good response to chemotherapy, miR-92a, miR-99b, miR-193a-5p, and miR-422a expression is increased, while miR-132 is decreased. All identified miRNAs seem to be centered on the *TP53* network. This is the first application of *PathVisio* to determine miRNA pathways in osteosarcoma. MiRNAs have the potential to become a useful diagnostic and prognostic tool in the management of osteosarcoma. *PathVisio* is a full pathway editor with the potentiality to illustrate the biological events, augment graphical elements, and elucidate all the physical structures and interactions with standard external database identifiers.

Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor, comprising about 20% of primary bone sarcomas. It is a high-grade malignant tumor characterized by the cells forming immature bone or osteoid. The tumor is considered primary when the underlying bone is normal and secondary when it is altered by a pre-existing condition such as prior irradiation or Paget disease (Osasan *et al.*, 2016; Sergi and Zwerschke, 2008). OS is slightly more prevalent in males (male:female = 3:2) and has a bimodal age distribution with a preference for the adolescent and geriatric age groups, with most of the primary OS cases (60–70%) affecting adolescents and young adults (from 15 to 25 years of age). In the elderly, OS is usually associated with Paget disease of the bone, post-radiation sarcoma, and dedifferentiated chondrosarcomas (Sergi and Zwerschke, 2008). Primary OS may arise in any bone, generally in the long bones of the appendicular skeleton (80–90%), most commonly in the distal femur, proximal tibia, and proximal humerus. Within the long bones, the tumor is usually located in the metaphysis and arises as an enlarging and palpable mass, which results in progressive pain. OS originating in the mid-shaft of bones is uncommon. Conversely, tumors arise e often in the epiphysis where the growth plate is located. Less than 1% of OS is found in the bones of the hands and feet. There is an increase in osteosarcoma's relative incidence in non-long bones, including the jaws, pelvis, spine, and skull within the senior age group. The standard first-line treatment regimens for OS include surgery and multi-agent chemotherapy. Almost all patients receive a neoadjuvant intravenous combination of doxorubicin and cisplatin with or without methotrexate as the initial chemotherapy regimen. In cases where surgical resection is not feasible or the margins are inadequate, the use of radiation therapy may improve local control, but this is not considered a standard of care in pediatric and young adult patients. There has been a significant increase in the 5-year survival rates of patients with OS due to the advances

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in patients' clinical management. Most centers' survival rates now exceed 50%, but patients presenting with metastatic and recurrent disease have a survival rate of below 20%. The lung is the leading site of metastatic deposits (Abarrategi *et al.*, 2016; Chen *et al.*, 2016b).

Osteosarcoma and genetics

Some genetic syndromes are associated with an increased risk of OS. They include hereditary retinoblastoma (germline mutation of the *Rb* gene), Li-Fraumeni syndrome (germline mutation of the *TP53* gene), Bloom syndrome (germline mutation of the *RECQL2* gene), Werner syndrome (germline mutation of the *RECQL3* gene), and Rothmund-Thomson syndrome (germline mutation of the *RECQL4* gene) (Osasan *et al.*, 2016). The two most prominent genes that harbor germline mutations in patients with OS are the retinoblastoma (*Rb 1*) and the *TP53* tumor suppressor genes. Most OS demonstrate inactivation of both the retinoblastoma (Rb) and p53 pathways. OS has a disorganized genome characterized by complex, unbalanced karyotypes with varying patterns of abnormalities. The most consistent finding beyond the *TP53* and *RB* genes' dysregulation is significant aneuploidy with some evidence of chromothripsis. Chromothripsis is the phenomenon by which up to hundreds to thousands of clustered chromosomal rearrangements occur in a single event in localized and confined genomic regions in one or a few chromosomes (Ly and Cleveland, 2017; Poot, 2017; Smida *et al.*, 2017). These findings suggest an early defect in DNA repair/surveillance as a mechanism for the pathogenesis of OS (Behjati *et al.*, 2017). Tumor suppressor genes function to control cell growth by inhibiting cell proliferation and tumor development. Also, they play a role in cell repair and apoptosis. When tumor suppressors mutate, resulting in a loss or reduction in function, there is an increase in the likelihood of developing cancer. The retinoblastoma (*RB*) was the first tumor suppressor gene described and encodes a protein that functions as a negative regulator of the cell cycle (Ren and Gu, 2017). This protein stabilizes constitutive heterochromatin to maintain overall chromatin structure. RB1 is the checkpoint that binds the E2F family of transcription factors and inhibits cell cycle progression. Defects in this gene are associated with retinoblastoma, urinary bladder cancers, and OS. The *RB* gene is critical for the regulation of the G_1 to S cell cycle transition. In the absence of mitogenic stimuli, Rb remains dephosphorylated and binds to E2F family transcription factors, preventing their activation of the cell cycle. Mutations that result in the loss of function of the RB protein occur in approximately 70% of OS, mostly due to a loss of heterozygosity. Structural rearrangements and point mutations in the *RB* gene can also occur (Ren and Gu, 2017). The *TP53* gene functions as a tumor suppressor in essentially all tumors. It encodes a tumor suppressor protein, which contains transcriptional activation, DNA binding, and oligomerization domains. This protein plays a crucial role in maintaining genomic stability functioning as a transcription factor that regulates the expression of various genes involved in cell cycle arrest, DNA repair, changes in metabolism, and apoptosis. Mutations in this gene are associated with a wide variety of cancers, including OS. The function of p53 can be affected by mutations in the gene itself or by mutations to up- or downstream mediators of its activity. Mutations that result in the loss of function of the p53 gene occur in approximately 75% of OS cases. The mutations in the *TP53* gene include allelic loss (75–80%), rearrangements (10–20%), and point mutations (20–30%) (Braithwaite *et al.*, 2017; Duffy *et al.*, 2017; Gold, 2017; Guha and Malkin, 2017; Kastenhuber and Lowe, 2017; Merkel *et al.*, 2017). In the RB pathway setting, E2F3 and CDK4, both of which counteract RB control of cell cycle progression, are estimated to possess gain of function mutations. E2F3 is found in 60% of tumors, while CDK4 is found in 10% of tumors (Sampson *et al.*, 2015). Within the p53 pathway, MDM2 is an E3 ubiquitin ligase that acts as a negative regulator of p53. The *MDM2* gene is amplified in 3–25% of OS. COPS3 promotes the proteasomal degradation of p53, and COPS3 amplification is seen in 20–80% of OS cases. In the c-Myc pathway, the *c-Myc* gene is a key transcription factor that functions as a general amplifier of gene expression (Iaccarino, 2017). It enhances the transcription of essentially all genes with active promoters within the cell. This gene is amplified in 7–67% of OS cases and over-expressed in at least 30% of tumors (Morrow and Khanna, 2015; Sampson *et al.*, 2015).

Role of MiRNA in osteosarcoma

MicroRNAs (miRNAs) are a small single-stranded, non-coding RNA molecule (from 18 to 25 nucleotides in length), which are usually found in eukaryotic cells. They are involved in various biological processes that regulate differentiation, apoptosis, and proliferation of numerous non-neoplastic and neoplastic diseases (Dong *et al.*, 2016; Hashimoto and Tanaka, 2017; Leichter *et al.*, 2017; Nugent, 2014; Ram Kumar *et al.*, 2016; Sampson *et al.*, 2015; Sergi *et al.*, 2017a; Sergi *et al.*, 2017b; Zhao *et al.*, 2013). This process is achieved by complementarily pairing with the 3' untranslated region (3' UTR) or 5' untranslated region (5' UTR) of target genes, thus inhibiting the mRNA translation of these genes. In 1993, miRNA was first discovered in the nematode species *C. elegans*, and the first molecule was named *lin-4*. Since this discovery, it has been estimated that as many as 1000 miRNAs exist in the human genome, with more than 30% of the human genome regulated by miRNAs that simultaneously target multiple genes. In the last decade, it became clear that miRNAs are implicated in the pathogenesis of cancer, including OS (Ram Kumar *et al.*, 2016). This aspect was demonstrated by the differences in the miRNA expression profiles detected between normal and cancer cells. The expression of many different types of miRNA was found to be altered (either over-expressed or reduced) in malignancy. MiRNAs can function as tumor suppressors, oncogenes, or both. The dysregulation of miRNA expression may contribute to cancer development through the loss of controls of biological processes. These natural and physical properties can make miRNAs useful diagnostic and prognostic tools in the management of various cancers, including OS and non-oncological diseases (Agarwal *et al.*, 2015; Chen *et al.*, 2016a; Chen *et al.*, 2013; Dong *et al.*, 2016; Hsu *et al.*, 2011; Jones *et al.*, 2012;

Kobayashi *et al*., 2012; Leichter *et al*., 2017; Lin *et al*., 2016; Nugent, 2014; Ram Kumar *et al*., 2016; Sampson *et al*., 2015; Zhao *et al*., 2013; Zhou *et al*., 2016). There is increasing evidence that multiple miRNAs may play a role in determining the response to chemotherapy in the treatment of OS (Ram Kumar *et al*., 2016; Sampson *et al*., 2015).

Bioinformatics

In the last two decades, numerous bioinformatics tools have been developed to manage the increasing abundance of data. The massive flow of miRNA data can be handled effectively and efficiently using specific bioinformatics tools. In targeting miRNAs, we can address the identification, expression, and analysis of explicit and multiple miRNAs, establish miRNA regulatory networks, miRNA metabolic and signaling pathways, and miRNA-transcription factor interplay, thereby linking miRNAs to particular diseases or status of the disease. *WikiPathways* is an open, collaborative platform for drawing, editing, and sharing biological pathways, built using the same software underlying Wikipedia. This platform can be used to integrate, visualize, and analyze system-wide transcriptomics, proteomics, and metabolomics data. Several studies have demonstrated miRNAs' involvement in the pathogenesis, diagnostic potential, and therapeutics of OS. As indicated above, these miRNAs have been re-emphasized most recently because they intrinsically regulate the expression of different genes that play essential roles in tumorigenesis, cell invasion, migration, and metastasis. In this review, we aimed to discuss the current knowledge of miRNAs' role and their target genes in OS and attempt to develop an OS pathway involving miRNA integrating *WikiPathways* and other bioinformatic tools.

Materials and Methods

PubMed, Scopus, and Google Scholar were used to systematically search for reviewed publications that investigated the functions of miRNA in the pathogenesis, treatment, and prognosis of osteosarcoma. Publications in the time frame "2008-2018" and targeting "miRNA" and "Osteosarcoma" were retrieved from the archives. The findings from these publications were used to compile a list of miRNAs that are associated with OS. This study relies on a systematic search, but it does not comply with PRISMA eligibility criteria (Preferred Reporting Items for Systematic Reviews and Meta-Analyses).

PathVisio, a no-cost open-source pathway editor, visualization, and analysis software, has significantly enhanced the capacity to explore large scale data. It provides an invaluable tool for investigating genes, proteins, and metabolites in both the healthy and diseased states of complex tissues and related diseases, including OS. We used *PathVisio* as a pathway editor, visualization, and analysis software. Since the first publication of *PathVisio* in 2008, the software has been cited more than 170 times and used in many different biological studies. As an online editor, *PathVisio* is also integrated into the community curated pathway database *WikiPathways*. *Wikipathways* is one of the most popular freely available databases for assessing biological pathways. It is an open, collaborative platform used to create and share paths and is known as a plugin for *PathVisio*. *PathVisio 3* is a free open-source pathway editor, visualization, and analysis toolbox implemented in Java, a class-based, object-oriented programming language able to run on all major operating systems (Bhat *et al*., 2018; Kutmon *et al*., 2015). The *miRTar* bioanalysis tool was used to determine miRNAs' interaction with genes in the *TP53* pathway (Hsu *et al*., 2011). In particular, the *miRTar* tool adopts seven scenarios to identify putative miRNA target sites of the gene transcripts. It illustrates the biological functions of miRNAs concerning their targets in metabolic pathways. The prediction system helps biologists to quickly identify the regulatory relationships between crucial miRNAs and their targets.

The results were used in assembling the pathway for OS. Common miRNAs that have been previously identified in studies to have a role in the development and progression of OS were selected from the literature and imputed into this tool to identify the targeted genes. A pathway network was constructed using the ONCO.IO micro-analysis tool. A pathway for miRNAs linked to OS was then built using *PathVisio* and the *Wikipathways* plugin. The URLs of the website platforms we used are https://onco.io/, http://mirtar.mbc.nctu.edu.tw/human/, https://www.pathvisio.org/, and https://www.wikipathways.org/index.php/WikiPathways.

Results

There is a significant number of miRNAs that we found to be associated with OS. We found 1298 records of osteosarcoma papers associated with the word "miRNA". Three studies were substantially selected from which miRNAs associated with osteosarcoma were used for further detailed analysis (Chen *et al*., 2016a; Kobayashi *et al*., 2012; Nugent, 2014). In these studies,

A total of 6 miRNAs were found on chromosome 1, making chromosome 1 the most popular miRNA location. Chromosomes X and 11 were the second most frequent miRNA locations, with each chromosome being responsible for five miRNAs. The third most common chromosomal location for miRNAs is chromosome 19, responsible for four miRNAs. In addition, miRNAs are also located on chromosomes 3, 4, 5, 6, 7, 9, 13, 14, 15, 16, 17, 18, 20, and 21. All types of cellular pathways from development to oncogenesis are affected by miRNAs. Tab. 1 highlights the miRNAs associated with OS. Tabs. 2 and 3 recapitulate the roles and target genes of miRNAs in OS, with Tab. 2 displaying those with increased expressions and Tab. 3 displaying those with decreased expressions. A careful perusal of the literature showed that OS has increased expression of miR-21, miR-93, miR-135b, miR-150, miR-210, miR-221, miR-199b-5p, miR-218, miR-542-5p, and miR-652. While target genes are known for each of these miRNAs, the role in which they play is only known for miR-21, miR-93, miR-221, and miR-199b-5p. Conversely, there was decreased expression of miR-16, miR-24, miR-29a, miR-29b, miR-31, miR-34a, miR-34b, miR-34c, miR-125b, miR-132, miR-133a, miR-143, miR145, miR-183,

TABLE 1

Experimental groups highlighting the MiRNAs associated with osteosarcoma (Kutmon *et al.*, 2015)

miRNA	Expression	Chromosome	Start	End
miR-16-1	Decreased	13	49521110	46521198
miR-16-2	Decreased	3	1.62E+08	1.62E+08
miR-21	Increased	17	55273409	55273480
miR-24-1	Decreased	9	6888124	6888191
miR-24-2	Decreased	19	13808101	13808173
miR-29a	Decreased	7	1.3E+08	1.3E+08
miR-29b-1	Decreased	7	1.3E+08	1.3E+08
miR-29b-2	Decreased	1	2.06E+08	2.06E+08
miR-31	Decreased	9	21502114	21502184
miR-34a	Decreased	1	9134314	9134423
miR-34b	Decreased	11	1.11E+08	11088956
miR-34c	Decreased	11	1.11E+08	1.11E+08
miR-92a-1	Increased	13	90801569	90801646
miR-92a-2	Increased	X	1.33E+08	1.33E+08
miR-93	Increased	7	99529327	99529406
miR-99b	Increased	19	56887677	56887746
miR-125b-1	Decreased	11	1.21E+08	1.21E+08
miR-125b-2	Decreased	21	16884428	16884516
miR-132	Decreased	17	1899952	1900052
miR-133a-1	Decreased	18	17659657	17659744
miR-133a-2	Decreased	20	60572564	60572665
miR-135b	Increased	1	2.04E+08	2.04E+08
miR-136		14	1E+08	1E+08
miR-140	Increased	16	68524485	68524584
miR-143	Decreased	5	1.49E+08	1.49E+08
miR-145	Decreased	5	1.49E+08	1.49E+08
miR-150	Increased	19	54695854	54695937
miR-183	Decreased	7	1.29E+08	1.29E+08
miR-192		11	64415185	64415294
miR-199a-3p	Decreased	19	10789102	10789172
miR-199b-5p	Increased	9	1.3E+08	1.3E+08
miR-200a	Decreased	1	1093106	1093195
miR-200b	Decreased	1	1092347	1092441
miR-200c	Decreased	12	6943123	6943190
miR-206	Decreased	6	52117106	52117191
miR-210	Increased	11	558089	558198
miR-215		1	2.18E+08	2.18E+08
miR-218-1	Increased	4	20138996	20139105
miR-218-2	Increased	5	1.68E+08	1.68E+08
miR-221	Increased	X	45490529	45490638
miR-335	Decreased	7	1.3E+08	1.3E+08
miR-340	Decreased	5	1.79E+08	1.79E+08
miR-422a	Increased	15	61950182	61950271
miR-424	Decreased	X	1.34E+08	1.34E+08
miR-542-5p	Increased	X	1.34E+08	1.34E+08
miR-652	Increased	X	1.09E+08	1.09E+08

TABLE 2

MiRNAs with increased expression in osteosarcoma (Hsu *et al.*, 2011; Nugent, 2014)

miRNA	Role	Target gene
miR-21	Cell invasion and migration via regulation of RECK	SERPINB5, THBS1
miR-93	Increased cell proliferation and invasion	ATM, CASP8, CCND2, CD82, CYCS, SERPINE1, TP53, ZMAT3
miR-135b		CCND2, PPM1D
miR-150		CCND1, RPRM, TP53
miR-210		ATR, IGFBP3
miR-221	Induces cell survival via inhibition of PTEN	CCND2, CYCS,
miR-199b-5p	Involved in Notch signaling	BBC3, CDKN1A, IGFBP3, SESN1,TP53
miR-218		CYCS, CCND2
miR-542-5p		CCND3, CDKN2A, SHISA5, TP73
miR-652		CASP3, CYCS

TABLE 3

MiRNAs with decreased expression in osteosarcoma

miRNA	Role	Target gene
miR-16	Inhibition of cell proliferation via IGFIR	*CCND2, CDK6, CDKN2A, CHEK1, PPM1D, SESN2, SIAH1*
	Inhibition of osteosarcoma cell proliferation via LPAATβ downregulation	
miR-24		*BBC3, CASP8, CASP3, CCND2, CDK2, CDKN2A, CCYS, PPM1D, SESN1, TNFRSF10B*
miR-29a	Induces apoptosis	*CASP8, CASP9, CDKN2A, CYCS, IGF1, PPM1D, TNFRSF10B*
miR-29b	Osteogenic differentiation of mesenchymal stem cells via regulation of bone	*CCND3*
miR-31	transcription factor Osterix	
miR-34a	Inhibition of cell proliferation via Notch- 1 inhibition	*BID, CASP9, CCNE2, CDK6, E124, IGF1, LRDD, SERPINE1, THBS1*
miR-34b	Suppresses proliferation of osteoclasts	*GTSE1*
miR-34c	by downregulation of Runx2	
miR-125b	Suppresses Proliferation via down- regulation of STAT3	
miR-132	Facilitates angiogenesis	
miR-133a	Promotes apoptosis by targeting Bcl-xL and Mcl-l	*CCND2, SHISA5, TNFRSF10B*
miR-143	Inhibition of cell proliferation	
miR-145	via Notch- 1 inhibition	
miR-183	Suppresses Ezrin-linked migration and invasion	
miR-199a-3p	Regulates cell proliferation	
miR-200	Inhibition of cell proliferation via Notch- 1 inhibition	
miR-206	Involved in apoptosis and inhibition cell invasion and migration	*CCND2, IGF1*
miR-335	Suppresses migration and invasion by targeting ROCK1	
miR-340	Suppresses proliferation, migration, and invasion by targeting ROCK1	
miR-424	Inhibits migration and invasion via fatty acid synthase	

miR-199a-3p, miR-200, miR-206, miR-335, miR-340 and miR-424. Roles are defined for all the miRNAs except miR-29b, miR-34b, and miR143. Target genes have been identified for miR-16, miR-29a, miR-29b, miR-31, miR-34a, miR-34b, miR-133a and miR-206. The interconnection of these miRNAs with signaling pathways was the next step in our analysis and the miRNAs with OS intrinsic regulation are key and displayed in Tab. 4.

Fig. 1 shows the *TP53* Network built by ONCO.IO, a bioinformatic tool on *PathVisio* software, and the miRNA-regulation of TP53 in OS, respectively. The weight of miR34 for transcriptional regulation is prominent.

The purpose of this study was to attempt to construct a pathway involving the miRNA regulation of the p53 signaling pathway for OS. No unique, perpetual, and solid pathway involving miRNAs for OS was found, but there are multiple pathways related to the *TP53* gene which are associated with different conditions. Data regarding miRNA and target genes involved in the development and progression of OS corresponded to information that was published in previous studies. This data was imputed into onco.io to generate a signaling pathway for p53 that shows miRNA regulation. Fig. 1 is exclusively an example of multiple genetic interactions that can be revealed using *PathVisio*. It does not mean that it is comprehensive of all genes-miRNAs interaction as networks. The gray shadow of the left corner of Fig. 1 is supposed to polarize the attention on some molecules of interest, but it can be displayed in other locations according to different research questions.

Discussion

The mechanism of action of miRNAs in OS remains not clearly understood. However, the *TP53* gene is mutated in more than 20% of OS, with mutations demonstrated to be involved in tumorigenesis. MiRNAs are involved in the control of many cellular processes, and the dysregulation of miRNA expression can influence carcinogenesis once tumor suppressor genes or oncogenes encode the relevant target mRNAs. Even a small variation can have significant implications for the cell since each miRNA can have many targets. In humans, it has been established that many miRNA genes are located in cancer-associated regions or at the fragile sites of chromosomes, which are prone to deletion, amplification, and mutations in cancer cells. Since miRNAs can function as negative regulators of gene expression, an over-expression of oncogenic miRNAs can contribute to tumor development by promoting cellular proliferation and evasion of apoptosis. A similar effect will occur if there is a reduction in the expression of tumor-suppressive miRNAs. Research has demonstrated both increases and decreases in the expression of specific miRNAs in cancer. These appear to vary depending on the particular tissue and the cancer type (He *et al.*, 2007; Kao *et al.*, 2012; Kobayashi *et al.*, 2012). Several miRNAs have been identified as direct targets of p53.

The miR-34 family (miR-34a, miR-34b, and miR-34c) has been an important component of the p53 tumor suppressor pathway. P53 induces the expression of these miRNAs in response to DNA damage and oncogenic stress in many cancers. He *et al.* (2007) reported that the miR-34 family induces G1 arrest and apoptosis via their targets CDK6, E2F3, Cyclin E2, and BCL2 in a p53-dependent manner in OS cells (Bhat *et al.*, 2018). The expression of miR-34 is decreased in OS, and miR-34 enhances p53 mediated cell cycle arrest and apoptosis. Also, p53 induces the upregulation of miR-192, miR-194, and miR215 in U2OS cells, which carry the wild-type p53. The loss of

TABLE 4

MiRNAs with intrinsic regulation in osteosarcoma

miRNA	Function in OS	Expression	MiRNA target in OS
miR-34a, *miR-34b*, *miR-34c*	P53 related G1 arrest and apoptosis	Decreased	CDK6, E2F3, Cyclin E2, BCL2
miR-31	Cell proliferation	Increased	E2F3
miR-192, *miR-215*	P53 related cell cycle arrest	Increased	CDKN1A/p21
miR-140	Chemoresistance to MTX and 5- FU	Increased	HDAC4
miR-215	Chemoresistance to MTX	Increased	DTL
miR-92a, *miR-99b*, *miR- 193a-5p*, *miR-422a*	Discriminate good from poor responders	Increased	
miR-132		Decreased	
miR-21	Cell invasion and migration	Increased	RECK, MET, mTOR, STAT3, MCL-1
miR-199a-3p	Cell proliferation and migration	Decreased	BCL-X
miR-143	Pulmonary metastasis	Increased	MMP-13

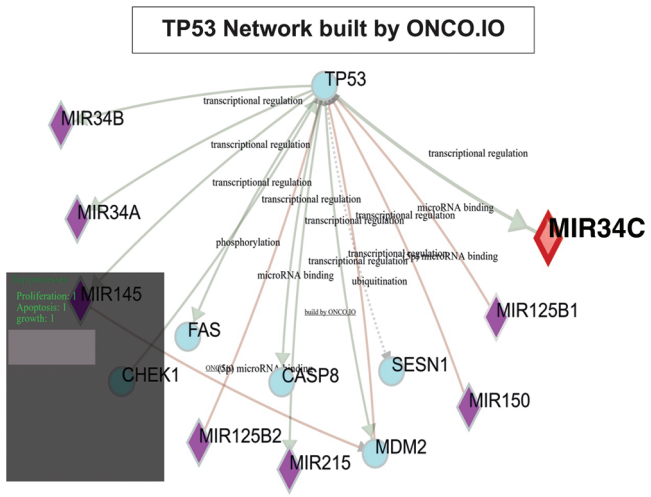

FIGURE 1. *TP53* results are intimately linked to MiR34A, MiR34B, MiR125B1, MiR125B2, MiR145, MiR150, and MiR215.
Some genes, which are intrinsically modulating *TP53* gene expression, are depicted (*CASP8*, *CHEK1*, *FAS*, *MDM2*, *SESN1*). The *CASP8* gene is responsible for the production of a member of the cysteine-aspartic acid protease family. The sequential activation of caspases is critical in the execution-phase of the programmed cell death or apoptosis. *CHEK1* is the gene for the serine/threonine-specific protein kinase, which coordinates the DNA damage response and cell cycle checkpoint response preventing damaged cells from progressing through the cell cycle. *FAS* forms the death-inducing signaling complex upon ligand binding, and, in several settings, there is evidence for crosstalk between the extrinsic and intrinsic pathways of apoptosis. Mouse double minute 2 (MDM2) homolog is a protein that in humans is encoded by the *MDM2* gene. MDM2 is an essential negative regulator of the p53 tumor suppressor. SESN1 or Sestrin 1, p53-regulated protein PA26, is a protein encoded by the *SESN1* gene. The p53 tumor suppressor protein induces Sestrins, which play significant roles in the cellular response to DNA damage and oxidative stress.

miR-31 is associated with defects in the p53 pathway, while overexpression of miR-31 significantly inhibits OS cells' proliferation. Moreover, miR-31 seems to have the potential to prevent disease progression or the development of pulmonary metastasis in OS (Kao *et al.*, 2012; Kobayashi *et al.*, 2012).

Biological pathways are descriptive. Sometimes complex diagrams are used to summarize and describe physical processes. These pathways show the potential interaction among genes, proteins, and metabolites. Path diagrams are a common way to graph the wealth of information available on these biological processes. To the best of our knowledge, no established pathways involving miRNAs for OS has been confirmed so far. However, there are multiple pathways related to *TP53*, which are associated with different disease conditions. The purpose of this study was to construct a path involving the miRNA regulation of the p53 signaling pathway for OS using *PathVisio*. Data regarding miRNA and target genes involved in the development and progression of OS corresponds to information that is available in the biomedical research literature. There is significant involvement of miRNAs in the development, progression, and metastasis of OS. The involvement spans from gene expression to epigenetics.

MiRNAs and their identified target genes are associated with multiple biological pathways and functions related to bone biology and cancer development and progression. Dysregulation of miRNAs is thereby associated with tumorigenesis in OS. A study by Andersen *et al.* (2018) investigated the miRNA expression in 101 OS samples (Andersen *et al.*, 2018). A total of 752 miRNAs were profiled, with 33 of these being identified as deregulated in OS. Andersen *et al.* (2018) found a significant role of miRNAs in the tumorigenesis of OS and that 29 deregulated miRNAs were strongly correlated with cancer development and progression. MiR-221 and miR-222 are associated significantly with time to metastasis. Significant downregulated miRNAs were identified as miR-100-5p, miR-125b-5p, miR-127-3p, miR-370-3p, miR-335-5p and miR-411-5p. Scott *et al.* (2007) and Sempere *et al.* (2004) showed that miR-125b is an important regulator of both proliferation and differentiation of different cell types. At the same time, Mizuno *et al.* (2008) indicated that miR-125b inhibits normal OB proliferation in mouse cells and plays a role in bone development and OS tumorigenesis. Andersen *et al.* (2018) also identified miR-181a-5p, miR-181c-5p, miR-223-3p and miR-342-3p as being significantly upregulated in OS.

Our study was done to summarise and further increase our understanding of the roles played by various miRNAs at various stages of the signaling pathway regulated by *TP53* in OS. Improved knowledge would allow for the development of specific miRNAs as biomarkers for diagnosis, disease monitoring, and OS progression. The possibility exists that miRNAs may have a therapeutic role in managing OS in the nearest future, particularly with the adoption of protocols of personalized medicine, renewed gene technologies, and digital pathology (Burnett *et al.*, 2020; Jin *et al.*, 2020; Sergi, 2019). MiRNA-directed gene regulation will pave the way for improving traditional gene therapy approaches to cancer, including OS. Presently, validation of miRNA pathways and targets in metastatic osteosarcoma has not been determined. Still, miRNA plays a role in the progression of OS by regulating proliferation, invasion, adhesion, metastasis, apoptosis, and angiogenesis. Identifying dysregulated miRNAs in patients with OS may contribute to the development of biomarkers for diagnosis and prognosis. There are challenges faced in identifying all the targets of miRNAs and establishing their contribution towards malignancy. Circulating miRNAs are considered predictive biomarkers for various types of cancers. They can be used as non-invasive disease biomarkers in cancer since they exist in human serum and plasma in remarkably stable forms. Comprehensive screening of miRNA profiles would allow for earlier detection of OS, as well as nullify the need for the collection of tissue samples through invasive procedures such as biopsies. Despite the clinical potential for the use of miRNAs as diagnostic biomarkers, several limitations are present. In most studies, the cohort of patients used has been relatively small, and therefore evaluations of large, long-term sample sizes with long-term follow-up are required. There is a lack of standardized approaches in the methodology of the normalization of circulating miRNAs. A refined approach is needed in future

studies to establish miRNAs as circulating biomarkers for clinical use. The role of miRNAs in OS has been studied in detail, but it is not clear whether it can be utilized to treat patients with OS. The involvement of miRNA function in the progression of OS has raised the possibility of the utilization of miRNA as a novel therapy. Extensive toxicity studies and preclinical safety trials would need to be conducted before considering a miRNA-based therapeutic approach. A greater understanding of the roles that different miRNAs play in the development and progression of OS could ultimately improve this tumor (Abarrategi *et al*., 2016; Bhat *et al*., 2018; He *et al*., 2007; Jones *et al*., 2012; Kao *et al*., 2012; Kobayashi *et al*., 2012; Kutmon *et al*., 2015; Leichter *et al*., 2017; Nugent, 2014; Ram Kumar *et al*., 2016).

Moreover, the EIMMO, MicroInspector, miRU, MMIA, RNA22, StarMir, and MMIA are additional tools with variable data from biology scientists. They are web-based and specific for identifying miRNA binding sites (Hsu *et al*., 2011).

There are a few additional limitations to our study. First, the most common weakness of bioinformatics tools is the generation of large amounts of false-positive data. We considered the other open-source tools, such as DIANA, TARGETSCAN, and MIRANDA, but we chose to use miRTar because of the familiarity with this tool. Although based on available scientific data, many of the proposed gene interactions in these databases may be speculative. Second, the current method of pathway analysis depends on existing databases. Not all the miRNAs and genes linked to OS were found in the ONCO.IO miRNA analysis tool database, which was used to construct the pathway network. Third, the interpretation of results based on pathway analysis tools needs to be interpreted with caution because the miRNA field is an evolving platform spanning from genomics to proteomics.

In conclusion, although the field of miRNA research is still relatively new, its rapid expansion has the potential to use these small molecules in the management of cancer. The *PathVisio* analysis of *Wikipathways* may be a useful bioinformatic tool for cancer research. Several miRNAs have been involved in OS, with some demonstrated to be overexpressed while others are downregulated. Our analysis indicates that there is indeed potential for miRNAs to play a critical role in the management of OS both as promising diagnostic biomarkers and either predictive or prognostic indicators. Bioinformatics speed has increased daily, and we expect that the *PathVisio* analysis on *Wikipathways* may be a useful tool for cancer research readily available for cancer research investigators worldwide. The miRTar bioanalysis tool can be used to determine the interaction of miRNAs with genes in the *TP53* pathway, and the ONCO.IO miRNA analysis tool database was used to identify miRNAs and OS. In OS patients considered good responders to chemotherapy, miR-92a, miR-99b, miR-193a-5p, and miR-422a expression increased, while miR-132 decreased. This is the first application of PathVisio to determine miRNA pathways in osteosarcoma to the best of our knowledge. *PathVisio* is a full pathway editor with the potentiality to illustrate the biological events, augment graphical elements, and elucidate all the biological structures and interactions with standard external database identifiers. MiRNAs have the potential to become a useful diagnostic and prognostic tool in the management of OS.

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Availability of Data and Materials: Project Name: *PathVisio* Analysis: An Application Targeting the miRNA Network in Osteosarcoma and Review on its Tumorigenesis; Project Home Page: https://www.pathvisio.org/ Operating System: Platform Independent; Programming Language: Java; Other Requirements: Java 7 (update 51); License: Apache License, Version 2.0. The data that support the findings of this study are available from the open-sources platforms described in this paper. We would like to foster and extend our availability to any research cooperation useful to strengthen bone cancer research and improve therapy protocols against osteosarcoma.

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References

Abarrategi A, Tornin J, Martinez-Cruzado L, Hamilton A, Martinez-Campos E, Rodrigo JP, González M, Baldini N, Garcia-Castro J, Rodriguez R (2016). Osteosarcoma: Cells-of-origin, cancer

stem cells, and targeted therapies. *Stem Cells International* **2016**: 3631764. DOI 10.1155/2016/3631764.

Agarwal V, Bell G, Nam J, Bartel D (2015). Predicting effective microRNA target sites in mammalian mRNAs. *eLife* **4**: e05005. DOI 10.7554/eLife.05005.

Andersen GB, Knudsen A, Hager H, Hansen LL, Tost J (2018). mi RNA profiling identifies deregulated mi RNA s associated with osteosarcoma development and time to metastasis in two large cohorts. *Molecular Oncology* **12**: 114–131. DOI 10.1002/1878-0261.12154.

Behjati S, Tarpey PS, Haase K, Ye H, Young MD, Alexandrov LB, Farndon SJ, Collord G, Wedge DC, Martincorena I (2017). Recurrent mutation of IGF signalling genes and distinct patterns of genomic rearrangement in osteosarcoma. *Nature Communications* **8**: 15936. DOI 10.1038/ncomms15936.

Bhat MY, Solanki HS, Advani J, Khan AA, Prasad TK, Gowda H, Thiyagarajan S, Chatterjee A (2018). Comprehensive network map of interferon gamma signaling. *Journal of Cell Communication and Signaling* **12**: 745–751. DOI 10.1007/s12079-018-0486-y.

Braithwaite A, Ballinger M, Baran-Marszak F, Bond GL, Concin N, Donehower L, El-Deiry W (2017). Recommended guidelines for validation, quality control, and reporting of TP53 variants in clinical practice. *Cancer Research* **77**: 1250–1260.

Burnett M, Abuetabh Y, Wronski A, Shen F, Persad S, Leng R, Eisenstat D, Sergi C (2020). Graphene oxide nanoparticles induce apoptosis in wild-type and CRISPR/Cas9-IGF/IGFBP3 knocked-out osteosarcoma cells. *Journal of Cancer* **11**: 5007–5023. DOI 10.7150/jca.46464.

Chen G, Fang T, Huang Z, Qi Y, Du S, Di T, Lei Z, Zhang X, Yan W (2016a). MicroRNA-133a inhibits osteosarcoma cells proliferation and invasion via targeting IGF-1R. *Cellular Physiology and Biochemistry* **38**: 598–608. DOI 10.1159/000438653.

Chen L, Wang Q, Wang GD, Wang H-S, Huang Y, Liu XM, Cai XH (2013). miR-16 inhibits cell proliferation by targeting IGF1R and the Raf1-MEK1/2-ERK1/2 pathway in osteosarcoma. *FEBS Letters* **587**: 1366–1372. DOI 10.1016/j.febslet.2013.03.007.

Chen Y, Yu XC, Xu SF, Xu M, Song RX (2016b). Impacts of tumor location, nature and bone destruction of extremity osteosarcoma on selection of limb salvage operative procedure. *Orthopaedic Surgery* **8**: 139–149. DOI 10.1111/os.12237.

Dong J, Liu Y, Liao W, Liu R, Shi P, Wang L (2016). miRNA-223 is a potential diagnostic and prognostic marker for osteosarcoma. *Journal of Bone Oncology* **5**: 74–79. DOI 10.1016/j.jbo.2016.05.001.

Duffy MJ, Synnott NC, Crown J (2017). Mutant p53 as a target for cancer treatment. *European Journal of Cancer* **83**: 258–265. DOI 10.1016/j.ejca.2017.06.023.

Gold B (2017). Somatic mutations in cancer: Stochastic versus predictable. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis* **814**: 37–46. DOI 10.1016/j.mrgentox.2016.12.006.

Guha T, Malkin D (2017). Inherited TP53 mutations and the Li-Fraumeni syndrome. *Cold Spring Harbor Perspectives in Medicine* **7**: a026187. DOI 10.1101/cshperspect.a026187.

Hashimoto N, Tanaka T (2017). Role of miRNAs in the pathogenesis and susceptibility of diabetes mellitus. *Journal of Human Genetics* **62**: 141–150. DOI 10.1038/jhg.2016.150.

He L, He X, Lowe SW, Hannon GJ (2007). microRNAs join the p53 network — another piece in the tumour-suppression puzzle. *Nature Reviews Cancer* **7**: 819–822. DOI 10.1038/nrc2232.

Hsu JBK, Chiu CM, Hsu SD, Huang WY, Chien CH, Lee TY, Huang HD (2011). miRTar: An integrated system for identifying miRNA-target interactions in human. *BMC Bioinformatics* **12**: 300. DOI 10.1186/1471-2105-12-300.

Iaccarino I (2017). lncRNAs and MYC: An intricate relationship. *International Journal of Molecular Sciences* **18**: 1497. DOI 10.3390/ijms18071497.

Jin L, Shen F, Weinfeld M, Sergi C (2020). Insulin Growth Factor Binding Protein 7 (IGFBP7)-related cancer and IGFBP3 and IGFBP7 crosstalk. *Frontiers in Oncology* **10**: 727. DOI 10.3389/fonc.2020.00727.

Jones KB, Salah Z, Del Mare S, Galasso M, Gaudio E, Nuovo GJ, Lovat F, Leblanc K, Palatini J, Randall RL (2012). miRNA signatures associate with pathogenesis and progression of osteosarcoma. *Cancer Research* **72**: 1865–1877. DOI 10.1158/0008-5472.CAN-11-2663.

Kao S, Shiau CK, Gu DL, Ho CM, Su WH, Chen CF, Lin CH, Jou YS (2012). IGDB. NSCLC: Integrated genomic database of non-small cell lung cancer. *Nucleic Acids Research* **40**: D972–D977. DOI 10.1093/nar/gkr1183.

Kastenhuber ER, Lowe SW (2017). Putting p53 in context. *Cell* **170**: 1062–1078. DOI 10.1016/j.cell.2017.08.028.

Kobayashi E, Hornicek FJ, Duan Z (2012). MicroRNA involvement in osteosarcoma. *Sarcoma* **2012**: 359739. DOI 10.1155/2012/359739.

Kutmon M, Van Iersel MP, Bohler A, Kelder T, Nunes N, Pico AR, Evelo CT (2015). PathVisio 3: An extendable pathway analysis toolbox. *PLoS Computational Biology* **11**: e1004085. DOI 10.1371/journal.pcbi.1004085.

Leichter AL, Sullivan MJ, Eccles MR, Chatterjee A (2017). MicroRNA expression patterns and signalling pathways in the development and progression of childhood solid tumours. *Molecular Cancer* **16**: 15. DOI 10.1186/s12943-017-0584-0.

Lin Z, Song D, Wei H, Yang X, Liu T, Yan W, Xiao J (2016). TGF-β1-induced miR-202 mediates drug resistance by inhibiting apoptosis in human osteosarcoma. *Journal of Cancer Research and Clinical Oncology* **142**: 239–246. DOI 10.1007/s00432-015-2028-9.

Ly P, Cleveland DW (2017). Rebuilding chromosomes after catastrophe: Emerging mechanisms of chromothripsis. *Trends in Cell Biology* **27**: 917–930. DOI 10.1016/j.tcb.2017.08.005.

Merkel O, Taylor N, Prutsch N, Staber PB, Moriggl R, Turner SD, Kenner L (2017). When the guardian sleeps: Reactivation of the p53 pathway in cancer. *Mutation Research/Reviews in Mutation Research* **773**: 1–13. DOI 10.1016/j.mrrev.2017.02.003.

Mizuno Y, Yagi K, Tokuzawa Y, Kanesaki-Yatsuka Y, Suda T, Katagiri T, Fukuda T, Maruyama M, Okuda A, Amemiya T (2008). miR-125b inhibits osteoblastic differentiation by down-regulation of cell proliferation. *Biochemical and Biophysical Research Communications* **368**: 267–272. DOI 10.1016/j.bbrc.2008.01.073.

Morrow JJ, Khanna C (2015). Osteosarcoma genetics and epigenetics: Emerging biology and candidate therapies. *Critical Reviews in Oncogenesis* **20**: 173–197. DOI 10.1615/CritRevOncog.2015013713.

Nugent M (2014). MicroRNA function and dysregulation in bone tumors: The evidence to date. *Cancer Management and Research* **6**: 15. DOI 10.2147/CMAR.S53928.

Osasan S, Zhang M, Shen F, Paul PJ, Persad S, Sergi C (2016). Osteogenic sarcoma: A 21st century review. *Anticancer Research* **36**: 4391–4398. DOI 10.21873/anticanres.10982.

Poot M (2017). Of simple and complex genome rearrangements, chromothripsis, chromoanasynthesis, and chromosome chaos. *Molecular Syndromology* **8**: 115–117. DOI 10.1159/000454964.

Ram Kumar RM, Boro A, Fuchs B (2016). Involvement and clinical aspects of microRNA in osteosarcoma. *International Journal of Molecular Sciences* **17**: 877. DOI 10.3390/ijms17060877.

Ren W, Gu G (2017). Prognostic implications of *RB1* tumour suppressor gene alterations in the clinical outcome of human osteosarcoma: A meta-analysis. *European Journal of Cancer Care* **26**: e12401. DOI 10.1111/ecc.12401.

Sampson VB, Yoo S, Kumar A, Vetter NS, Kolb EA (2015). MicroRNAs and potential targets in osteosarcoma: Review. *Frontiers in Pediatrics* **3**: 69. DOI 10.3389/fped.2015.00069.

Scott G, Goga A, Bhaumik D, Berger C, Sullivan C, Benz C (2007). Coordinate suppression of erBB2 and erBB3 by enforced expression of micro-rna mir-125a or mir-125b. *Journal of Biological Chemistry* **282**: 1479–1486. DOI 10.1074/jbc.M609383200.

Sempere LF, Freemantle S, Pitha-Rowe I, Moss E, Dmitrovsky E, Ambros V (2004). Expression profiling of mammalian microRNAs uncovers a subset of brain-expressed microRNAs with possible roles in murine and human neuronal differentiation. *Genome Biology* **5**: R13. DOI 10.1186/gb-2004-5-3-r13.

Sergi C, Shen F, Lim DW, Liu W, Zhang M, Chiu B, Anand V, Sun Z (2017a). Cardiovascular dysfunction in sepsis at the dawn of emerging mediators. *Biomedicine & Pharmacotherapy* **95**: 153–160.

Sergi C, Zwerschke W (2008). Osteogenic sarcoma (osteosarcoma) in the elderly: Tumor delineation and predisposing conditions. *Experimental Gerontology* **43**: 1039–1043. DOI 10.1016/j.exger.2008.09.009.

Sergi CM (2019). Digital pathology: The time is now to bridge the gap between medicine and technological singularity. In: Cvetković D, eds. *Interactive Multimedia-Multimedia Production and Digital Storytelling*, IntechOpen. DOI 10.5772/intechopen.84329.

Sergi CM, Caluseriu O, Mccoll H, Eisenstat DD (2017b). Hirschsprung's disease: Clinical dysmorphology, genes, micro-RNAs, and future perspectives. *Pediatric Research* **81**: 177–191. DOI 10.1038/pr.2016.202.

Smida J, Xu H, Zhang Y, Baumhoer D, Ribi S, Kovac M, Von Luettichau I, Bielack S, O'leary VB, Leib-Mösch C (2017). Genome-wide analysis of somatic copy number alterations and chromosomal breakages in osteosarcoma. *International Journal of Cancer* **141**: 816–828. DOI 10.1002/ijc.30778.

Zhao H, Li M, Li L, Yang X, Lan G, Zhang Y (2013). MiR-133b is down-regulated in human osteosarcoma and inhibits osteosarcoma cells proliferation, migration and invasion, and promotes apoptosis. *PLoS One* **8**: e83571. DOI 10.1371/journal.pone.0083571.

Zhou C, Tan W, Lv H, Gao F, Sun J (2016). Hypoxia-inducible microRNA-488 regulates apoptosis by targeting Bim in osteosarcoma. *Cellular Oncology* **39**: 463–471. DOI 10.1007/s13402-016-0288-2.