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A miR302 mRNA Up-regulation Associated with Soft Tissue Sarcoma Grade

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Abstract

There is still a difficulty of how to diagnose and to treat the soft tissue sarcomas because its comprise of highly heterogeneous of group tumors in terms of primary site, histopathology, histological grade, and molecular signature. Histopathologic grading in adult soft tissue sarcomas is the most significant tools to predict tumor progression and metastatic risk. Tumor grading can be considered as a morphologic translation of molecular events that determine tumor aggressiveness. Therefore, molecular parameters could eventually complement or even replace histologic parameters. microRNAs (miRNAs) are small, endogenous RNAs of about 22 nucleotides that play important roles in regulation of cell proliferation, differentiation, and maintenance of "stemness". miRNAs are negatively regulating gene expression, mainly by translational repression and play a particularly prominent role in development. In this study, we investigated profile of miR302b gene expression at level of RNA from Formalin Fixed Paraffin Embedded of low grade, intermediate grade and high grade soft tissue sarcoma using quantitative Real Time PCR (qRT-PCR). We identified 23 samples of STS cases in Jambi city, from 2007 till 2013 consist of 6 cases low grade, 9 cases intermediate grade and 8 cases high grade. miR302b gene expression was normalized to internal control by using GAPDH gene expression, and we analyzed the relative gene expression using the $2^{-\Delta\Delta Ct}$ method. Statistical tests were performed on groups identified to find discriminating each target gene expression. There were no significant difference averages of ΔCt miR302b expression at low-, intermediate- and high-grade STS (p=0.041/ANNOVA) and also no significant with negative correlation between miR302b expression with a difference grading of STS (p>0.05, Spearman).The status of mRNA miR302bof low grade soft tissue tumor was relatively decreased expression by more than 3 fold, and relatively increased in intermediate grade and the status of high grade soft tissue sarcoma is increasing by one fold than GAPDH gene expression. It means that there is a relative change in mRNA genes expression of miR302b which is higher the histological grade more expressed the gene expression. We concluded that there was a miR302 mRNA up-regulation associated with tumor grade even no significantly but its relationship suggests that the higher grade the more expressed the miR302 gene expression.

Keywords: miR302b, Soft tissue sarcoma, Histologic grading.

Introduction

Soft-tissue sarcomas are rare, involving of 1% of all cancer diagnoses. Sarcomas represent a heterogeneous group of diseases of a variety of recognized histologic types and primary site. The WHO reported that the diversity of histological subtypes is impressive with >100 benign and malignant soft-tissue tumor entities defined. The advances in classification and concept the pathogenesis of tumor genesis based on the correlation of histologic and genetic findings have been particularly significant in the field of daily practice importantly the progression of tumor and risk of metastasis[1-3].

Tumor grading by histopathologic examination is the most crucial thing to predict the prognostic factor and the indicator of metastatic risk in adult soft tissue sarcomas and its main value for therapeutic management is the most efficient use of chemotherapy. The most commonly used systems are 3-grade systems which it's based on tumor necrosis, histologic type and subtype, and mitotic activity. This well-known system was used by the FNCLLC (*Fédération Nationale des Centres de Lutte le Cancer*) grading and the NCI (National Cancer Institute) grading[2]. Genes associated with cell-cycle, proliferation, adhesion, motility, protein degradation, homeostasis and immune response showed to play an important role of soft tissus sarcomapleomorphic subtypes.

microRNAs (miRNAs) are short (20-24 nt) non-coding RNAs that are transcribed by RNA polymerase II as part of capped and polyadenylated primary transcripts (pri-miRNAs) that can be either protein-coding or non-coding. miRNA is implicated as an essential post-transcriptional regulation of gene expression in multicellular organisms through affecting both the stability and translation of mRNAs. miRNA was known as an important epigenetic regulatory molecule[4]. The miR302b is a polycistronic miRNA cluster, that play a critical role to regulate the G1-to-S transition balance and it was verified that this cluster target is CDKN1A (cyclin-dependent kinase inhibitor 1, also known as p21), which was referred to as an inhibitor to Cyclin E/CDK2 complex[5-7]. It was reported that miR302 down regulated AKT1,

P27, cyclin A, cyclin D1, CDK2, CDK4, and E2F-1 [4]. The miR-302/367 cluster gene, which consists of miR-302a, -302a*, -302b, -302b*, -302c, -302c*, -302d, -367, and -367*, was found to be located in an intron on the 4q25 region of human chromosome 4.

It was reported that miR-302 members also have an important role to regulate the cell cycle via targeting the transcripts of C–C motif ligand (CCL5), C–C chemokine receptor type 5 (CCR5), C–X–C chemokine receptor type 4 (CXCR4) and epidermal growth factor receptor (EGFR)[8,9]. Further studies confirmed that miR302 binding siteregulate the ESC-specific transcription factors Oct4, Sox2, Nanog and Rex-1[10]. Lin and colleagues demonstrated miR302 have the tumor suppressive activity in the G1-S cell cycle transition through cyclin E-Cdk2 and cyclin D-Cdk4/6 pathways in human pluripotent stem cells[11]. More importantly, miR-302 was found to target Bmi-1, thus promoting the tumor suppressor functions of p16Ink4a and p14/p19Arf directed against Cdk4/6–mediated cell proliferation[12]. More recently, miR-302 has been reported to be a new tumor marker used to predict the malignant behavior of different types of tumor[13].

Very few similar studies, whether there has relationship between the expressions of miR302b with pathological features of soft tissue sarcoma. There is no research in this area yet at home and abroad. In this study, RT-PCR are used to detect the levels of miR302b in low grade, intermediate grade and high grade of soft tissue sarcoma.

Material and Methods

Patient Characteristic: The specimen was collected using total sampling method including all soft tissue sarcoma in Department of Pathology of Abdul Manap Hospital Jambi City, Indonesia during 2007-2013. All cases were diagnosed as soft tissue tumor with pathological biopsy. The patient characteristic of patient is summarized in Table-1.

Samples: All FFPE samples were taken through total sampling technique and diagnosed of each tumor was confirmed by histological examination as soft tissue sarcoma. There were 23 samples, based on NCI classification, consist of 6 samples of low grade, 9 samples of intermediate grade and 8 samples of high grade soft tissue sarcoma. This study was approved by the Research Ethics Board of the Faculty of Medicine, Padjadjaran University, Bandung, Indonesia.

RNA preparation: RNA was extracted from FFPE material using the paraffin block, RNA isolation kit. Firstly, 20-µm paraffin sections were cut, and performed deparaffinization by incubation in xylene for 20 minutes and continued with centrifuged at room temperature, and finished by washing three times in 100% ethanol. After the final wash, the material was air-dried and then incubated in digestion buffer with proteinase K. After that the homogenized residue was incubated overnight at 55°C. RNA was purified by the addition of RNA extraction buffer and continues with adding the chloroform and followed by additional incubation and centrifugation. This final centrifugation followed by air-dried the pellet and re-suspended in 10 µl of RNA storage solution. All samples underwent DNase treatment to remove genomic DNA according to the manufacturer. The homogenized solution was centrifuged according to the manufacturer's instructions at 14,000 g using column tube. At a later stage, phenol-chloroform extraction and ethanol precipitation to purify the RNA pellet is done, then the results are diluted by using TE buffer at pH 8.0 so that the solution RNA ready for analysis by Real Time PCR (qRT-PCR) (Kappa Biosystem, USA).

Table-1
Patient Characteristic

Variable	Low Grade (n=6)	Intermediate Grade (n=9)	High Grade (n=8)
Age			
Average ± Std Dev.	45,16± 4,665	46,55± 16,98	58,87± 21,64
Median	44,50	44,00	65,00
Range (min-max)	3800-5200	1900-7500	2700-8000
Sex			
Male	3 (50,0%)	7 (77,8%)	3 (37,5%)
Female	3 (50,0%)	2 (22,2%)	5 (62,5%)
Site			
Femur	2 (33,3%)	6 (66,7%)	0 (0,0%)
Pectoralis	1 (16,7%)	0 (0,0%)	1 (12,5%)
Scapula	0 (0,0%%)	0 (0,0%)	1 (12,5%)
Cruris	0 (0,0%)	0 (0,0%)	1 (12,5%)
Parietoccipital	0 (0,0%)	1 (11,1%)	0 (0,0%)
Others	3 (50,0%)	2 (22,2%)	5 (62,5%)
Diagnose			
Fibrosarcoma	2 (33,3%)	5 (55,6%)	3 (37,5%)
Lyposarcoma	2 (33,3%)	0 (0,0%)	1 (12,5%)
Dermafibrosarcoma	2 (33,3%)	2 (22,2%)	0 (0,0%)
Others	0 (0,0%)	2 (22,2%)	4 (50,0%)

Quantitative Real time PCR (qRT-PCR): Determination of expression levels of mRNA and microRNA done using one-step qRT-PCR kit in accordance with the provisions of steps from the factory (Kapa Biosystem, USA).

miR-302b	*Forward*: 5′-GATAAGTGCT TCCATGT-3′
	Reverse: 5′-CGCTTCACGAATTTGCGTGTCAT-3′
GAPDH	*Forward:* 5'-TGCACCACCAACTGCTTAGC-3'
	Reverse: 5'-GGCATGGACTGTGGTCATGAG-3'

The qRT-PCR reaction was performed using the 1-step qRT-PCR kit according the manufacture's instruction (Kappa Bio systems, USA). This procedure undergone to reverse transcription (RT) for 5 minutes at 42°C, continued by inactivation of the RT enzyme at 95°C for 3 minutes and the PCR cycle for 40 cycles. The circle comprised of 95°C of denature for 30 sec; 60°C of annealing for 20 sec and after that 72°C of extensions for 20 sec. The ΔCt was equal to the difference in threshold cycles for target and GAPDH (Ct_{target}–Ct_{GAPDH}). Gene expressions was then analyzing relatively by calculated using $2^{-\Delta\Delta Ct}$ method[14].

Statistical Analysis: Annova analysis of variance were used for PCR data statistics, and Spearman test was used for correlation analysis. P<0.05 was considered to have statistical significance. Statistical Program for Social Sciences (SPSS) software version 17 (SPSS Inc., Chicago, IL, USA) were utilized for statistical analysis of real time PCR data.

Results and Discussion

Even soft tissue sarcomas (STS) are a rare case, it represents a distinct group of mesenchymal tumors which is still demanding in the clinical management, and requires the combined information from clinical investigations, imaging, histopathology, and cytogenetic and molecular genetics analyses into account[1,15]. From Table-1 showed that in our study just found 23 cases of soft tissue sarcoma for 5 years, consist of 13 cases male and 10 cases of female, it means that as mention in literature that the soft tissue sarcoma is a rare case. Most of case was found in extremity.

It was reported that almost 50% cases of soft tissue sarcoma found in the extremity[1]. In our study the most case was diagnosed as fibrosarcoma, consist of 2 cases of low grade, 5 cases of intermediate cases and 3 cases of high grade. Prognosis of this malignancy is dominated by local recurrence and distant metastasis. Recently in general about 50% of soft tissue sarcoma patient owing to the fact that partly to the high potential of metastatic. It is also admitted that grading should certainly be adapted to the modern management of patients with combined by radiologic and molecular parameters. It was realized that histologic classification and grading is currently the most important prognostic factor and the best indicator of metastatic risk but it has some limitation. Therefore, it given the recent strides forward in molecular genetics through gene expression studies. Nowadays gene expression research have disclosed profiles of diagnostic and upregulation of specific pathways in sarcomas with type-specific genetic defects, e.g. Ewing sarcoma, gastrointestinal stromal tumors (GIST), rhabdomyosarcoma, dermatofibrosarcoma protuberans, and have allowed potential targets for novel therapies[15,16].

Expression of miR302b Gene in Soft Tissue Sarcoma: The mean *Δ*CT value of *miR302b* in low grade, intermediate grade and high grade soft tissue sarcoma were 2.153±2.411, 1.351±3.874 and -0,076+2,722 (p>0.05) Table-2.

We analyzed the relative gene expressions using the $2^{-\Delta\Delta Ct}$ method by using GAPDH as a reference gene[14]. There is a relative change in mRNA genes expression of miR302b which is higher the histological grade more expressed the gene expression. The status of mRNA miR302b of low grade soft tissue tumor was relatively decreased expression by more than 3 fold, and relatively increasing in intermediate grade and the status of high grade soft tissue sarcoma increased by one fold than GAPDH gene expression (Table-3).

Table-2
The mean *Δ*CT value of *miR302b* in soft tissue sarcoma

Variable		Tumor grade			P value
		Low Grade	Intermediate Grade	High Grade	
		n = 6	n = 9	n = 8	
ΔmiR-302B	Average ± Std Devitation	2,153±2,411	1,351±3,874	-0,076±2,722	**0,419**
	Median	1,430	1,540	0,090	
	Range (min-max)	-0,55-6,11	-4,48-8,18	-4,38-3,54	
ΔCt GAPDH		28.09+2.22	28.29±1.96	29.22±2.75	

Relationship between Aurka Gene Expresssion and Tumor Grade of Soft tissue Sarcoma: According to Spearman correlation coefficient, there is a negative correlation with R=0.182 and no significant relationship was found between miR30b and histopatologic grade characteristics (Table-3).

Table-3
The relative fold changes of gene expressions normalized to GAPDH based on $2^{-\Delta\Delta Ct}$

Target Gene	Tumor Grade	Ct GAPDH	Ct gene	ΔCt gen*)		ΔΔCt	*Normalized target gene relative to GAPDH* $2^{-\Delta\Delta Ct}$
				+ΔCt	-ΔCt		
miR-302b	Low	28.09±2.22	29.88+2.96	2,153+2.411	-	2,153	0.22
	I'mediat	28.29±1.96	29.65+3.81	1.351±3.874	-	1.351	0.39
	High	29.25±1.13	29.22±2.75	-	0.076+2.72	-0.076	0.95
GAPDH	Low	28.09±2.22	28.09±2.22	0.00	-	0.00	1.00
	I'mediat	28.29±1.96	28.29±1.96	0.00	-	0.00	1.00
	High	29.25±1.13	29.25±1.13	0.00	-	0.00	1.00

*=$\Delta Ct=Ct_{target}-Ct_{GAPDH.}$ +$\Delta Ct=Ct_{target}>Ct_{GAPDH}$. -$\Delta Ct=Ct_{target}<Ct_{GAPDH}$. $\Delta\Delta Ct=\Delta Ct_{target}-\Delta Ct_{GAPDH}$

Table-4
Relationship between miR302b Gene Expresssion and Tumor Grade of Soft tissue Sarcoma

Variable	R	P value
Correlation miR302b Gene Expression with Histologic grade	**-0,288**	**0,182**

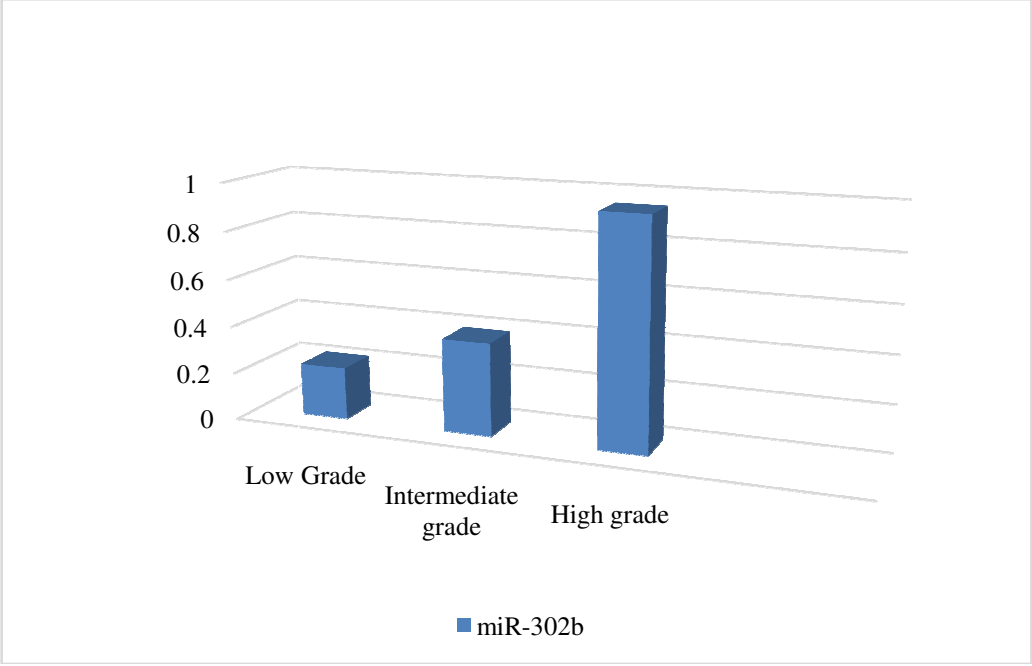

Figure-1
The relative fold Changes of miR302 b Gene Expressions Normalized to GAPDH in Low Grade, Intermediate Grade and High Grade Soft Tissue Sarcoma

Based on our result, the status of mRNA miR302b of low grade soft tissue tumor was relatively decreased expression by more than 3 fold, and relatively increasing in intermediate grade and the status of high grade soft tissue sarcoma increased by one fold than GAPDH gene expression (Table-3). In Figure-1, we showed the up-regulation of miR302 gene expression correlated with histological grade of soft tissue sarcoma, with negative correlation even not significant ($p>0.05$, Spearman) (Table-4). It is contrary with other investigation in esophageal squamous cell carcinoma that miR302b was significantly down-regulated and also correlated with tumor differentiation[17].

We suggested that this interesting finding of increasing miR302b gene expression in higher histologic grade of soft tissue sarcoma showed their role in cell cycle regulation, signaling and in addition we speculated their role in epigenetic regulation. It has identified a number of potential miR-302 targets which are cell cycle regulators, and many of these are intricately involved in the regulation of G1 phase, such as Cdk2, Cdk6, E2F1, Rb, and p130. It was reported the expression of miR-302a promotes an increase in S-phase and a decrease in G1-phase cells profile[6,9-13,17]. Further investigation showed that members of miR-302 resist BMI-1 to provoke CDKN2A (cyclin-dependent kinase inhibitor 2A, or p16) expression resulting ease in the output of CDK4/6 and CyclinD complex and finally diminish the G1-to-S transition. Additionally, several study have investigated that miR-302 members also participated in the cellular G1 phase arrest and distinguishing to failed expression of cell cycle genes by directly inhibit the Cyclin D1/2, CDK2 and RBP1, which depress the pRB phosphorylation[8,9].

In this report, we also speculated that the upregulation of miR302 in soft tissue sarcoma grade potentially caused by the function of this gene in regulation cellular signalling pathways. miR302 b is regarded as a currently acceptable function for the signaling pathway to coordinate different biological processes through several pathways. A recent study showed that miR-302 members can fine-tune stem cell self renewal through promotion of BMP signalling[9,18]. Based on our result Table-2, there are no significantly difference ΔCt of miR302b gene expression between low, intermediate and high grade soft tissue sarcoma probably associated with miR302 role of epigenetics regulation. Several studies have demonstrated miR-302 members associated with global demethylation in target cells caused by their function of targeting different epigenetic factors[7,9].

Conclusion

From our study, it appears that miR302 mRNA up-regulation associated with tumor grade even no significantly but by using $2^{-\Delta\Delta Ct}$ method[19] showed an increasing gene expression due to progression of tumor. Further investigation is required to understanding and seeking a significant role of miR302b in the procession of the formation and development in soft tissue sarcoma in vitro so hopely could use it to be a prognostic indicator of soft tissue sarcoma.

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