

Prognostic significance of miR-34a in Ewing sarcoma is associated with cyclin D1 and ki-67 expression

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Received 18 March 2014; revised 26 May 2014; accepted 20 June 2014

Background: At diagnosis, identification of reliable biological indicators of prognosis to allow stratification of patients according to different risks is an important but still unresolved aspect in the treatment of Ewing sarcoma (EWS) patients. This study aimed to explore the role of miR-34A expression on prognosis of EWS patients.

Patients and methods: Specimens from 109 patients with non-metastatic EWS treated at the Rizzoli Institute with neoadjuvant chemotherapy (protocols ISG/SSGIII, EW-1, EW-2, EW-REN2, EW-REN3, EW-PILOT) and 17 metastases were studied. Sixty-eight patients (62%) remained disease-free and 41 (38%) relapsed (median follow-up: 67 months, range 9–241 months). Expression of miR-34a and of some of its targets (cyclin D1, bcl-2, SIRT1 and YY1) was evaluated by qRT-PCR using TaqMan MicroRNA Assays and/or by immunohistochemistry on tissue microarrays from the same patients.

Results: High expression of miR-34a in localized tumors was significantly related to better event-free and overall survival ($P = 0.004$). Relevance of miR-34a was confirmed by using different calibrators (normal mesenchymal stem cells and different normal tissues). By multivariate Cox regression analysis, low miR-34a expression as well as nontotal necrosis and high levels of lactate dehydrogenase were all confirmed as independent risk factors associated with poor outcome. Expression of miR-34a was lower in metastases than in primary tumors. It inversely correlated with expression of cyclin D1 and Ki-67.

Conclusions: By demonstrating its relationship with clinical outcome, we propose evaluation of miR-34a at diagnosis of EWS patients to allow early risk stratification. Validation of these results would nonetheless ultimately need a prospective assessment.

Key words: Ewing sarcoma, miR-34a, cyclin D1, Ki-67, prognostic biomarkers

Introduction

Ewing sarcoma (EWS) is the second most common tumor of bone and soft tissue. It occurs mainly in children and young adults and is characterized by a very aggressive nature with rapid growth and a marked tendency to form distant metastases [1]. Treatment of EWS is based on a combined approach of local therapy with surgery and/or radiotherapy of the primary tumor and systemic chemotherapy based on doxorubicin, vincristine, actinomycin D, ifosfamide and etoposide [2]. Intensified chemotherapy regimens with high doses have been used in patients with poor response to standard chemotherapy leading to some improvements [3]. However, prolonged and intensive chemotherapy treatments are

characterized by toxicity and long-term adverse effects [4]. Moreover, cost-effectiveness of treatments is another important problem for EWS patients [4], where cure mainly relies on public investments. These two aspects render extremely important identification of reliable indicators of prognosis, which may allow stratification of patients according to different risks at diagnosis. At present, only clinico-pathological features, such as presence of clinically evident metastases at diagnosis and poor response to neoadjuvant chemotherapy are widely accepted as prognostic and predictive factors in EWS [4]. In fact, despite many biological and molecular markers have been proposed in past years [5, 6], their clinical value still needs validation and experimental explanation.

We recently reported that high levels of miR-34a expression in this tumor were associated with a more favorable clinical history likely due to better responses to chemotherapy [7]. In order to further assess its prognostic significance, miR-34a expression was analyzed in a larger series of 109 primary localized tumors

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from patients treated at a single institution from 1991 to 2009 and in 17 metastases. Analysis of some validated targets of miR-34a (cyclin D1, bcl-2, SIRT1 and YY1) was also carried out.

materials and methods

patient selection

Patients with localized EWS who were enrolled in prospective studies and treated at the Rizzoli Institute were included in the present analysis.

The ethical committee of the Rizzoli Institute approved the studies, and informed consent was obtained. Details about patients' selection are provided in supplementary Material and Methods, available at *Annals of Oncology* online.

sample processing and total RNA isolation

Total RNA from tumor samples was isolated from snap-frozen tissue material using Trizol LS Reagent (Invitrogen, Carlsbad, CA). RNA quality and quantity were assessed by NanoDrop analysis (NanoDrop ND1000 ThermoFisher Scientific, Waltham, MA) and/or by electrophoresis analysis (1% agarose gel). To check whether extracted RNA was representative of EWS cells, the tissue was morphologically analyzed after hematoxylin–eosin staining (supplementary Figure S1, available at *Annals of Oncology* online) before any processing and patients with nonrepresentative samples (33/142) were excluded.

real time PCR

qRT-PCR analysis of miR-34a was carried out using TaqMan® MicroRNA Assays and TaqMan® Universal PCR Master Mix, no AmpErase® UNG (Applied Biosystems, Foster City, CA, USA) according to manufacturer's

instructions. Primer sequences are available in supplementary Table S1, available at *Annals of Oncology* online. Further details are provided in supplementary Material and Methods, available at *Annals of Oncology* online.

Ki-67 staining

Avidin–biotin–peroxidase procedure was used. MAb (dilution 1:50) anti-Ki-67 (MIB-1, Calbiochem-Novabiochem, San Diego, CA) was used as primary antibody on tissue microarray (TMA) containing 58 EWS samples. Sections (5 μ m) from formalin-fixed, paraffin-embedded tumor xenografts patients' tumor blocks were placed on poly-L-lysine-coated slides (Sigma). Further details are provided in supplementary Material and Methods, available at *Annals of Oncology* online.

statistical methods

Details about statistical methods are provided in supplementary Material and Methods, available at *Annals of Oncology* online.

results

expression of miR-34a in primary tumors predicts Ewing sarcoma patient outcome

In keeping with its oncosuppressor role [8], miR-34a expression was found to be generally lower in EWS with respect to several normal controls, including human bone-marrow-, corion- or dental pulp-derived mesenchymal stem cells, osteoblast precursors as well as muscle and skin (supplementary Figure S2, available at *Annals of Oncology* online). Human mesenchymal stem cell (hMSC) primary culture hMSC163 was used as representative (Figure 1A): only a minority of patients (15/109, 14%)

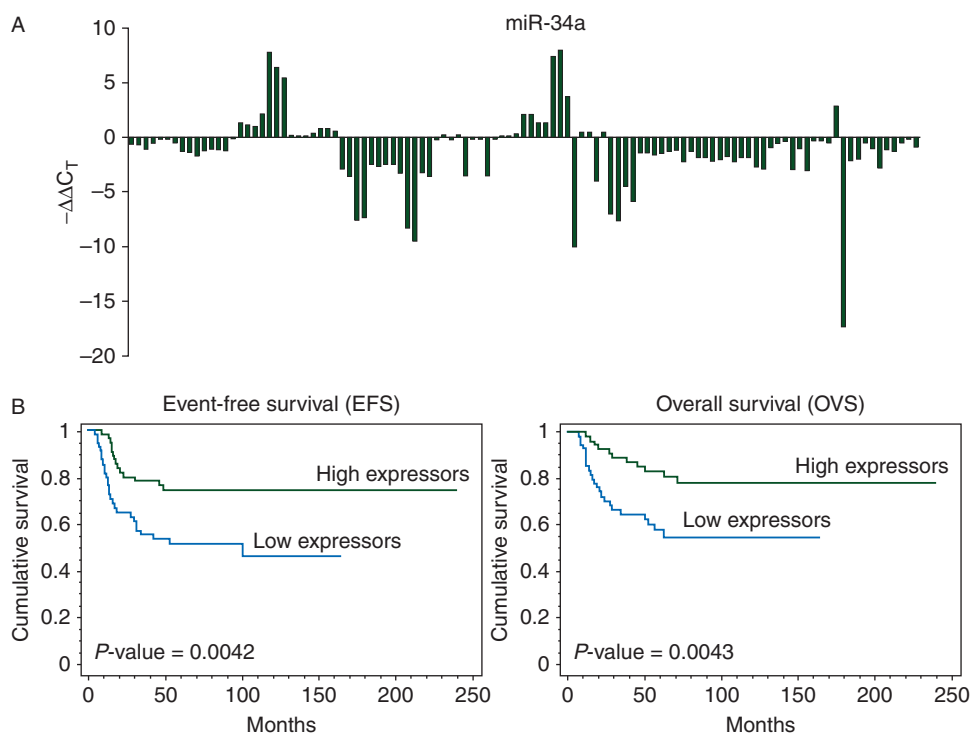


Figure 1. (A) Relative expression of miR-34a in 109 localized EWS primary tumors by using qRT-PCR. Human mesenchymal stem cell (hMSC) primary culture hMSC163 was used as calibrator. (B) Prognostic impact of miR-34a expression according to Kaplan–Meier curves and log-rank test. High expressors and low expressors were defined according to the median value of $-\Delta\Delta C_T$. Event-free survival (EFS) or overall survival (OVS) was considered.

had miR-34a expression levels remarkably higher (fold change >2) than the normal control, while the others showed comparable or definitely lower levels. To establish clinical relevance of miR-34a expression in EWS, the median was used as cutoff value to stratify patients and define two categories of high or low expressors, as previously reported [7]. Median follow-up of the

109 EWS patients was 67 months (range 9–241 months). Sixty-eight patients (62%) remained continuously free of disease and 41 (38%) relapsed. First relapse pattern consisted of metastases in 35 cases (lungs 17 cases, bones 8, lungs and bones 10), local recurrence in 5, local recurrence and bone metastasis in 1. The clinico-pathological characteristics are reported in Table 1.

Table 1. Clinico-pathological features of EWS patients evaluated for miR-34a expression by qRT-PCR in 109 tissue samples

Characteristics	RT-PCR (N = 109)		Association with prognosis EFS ^a	Association with survival OVS ^b
	N	%		
Gender				
Female	31	28.4	<i>P</i> = 0.3	<i>P</i> = 0.4
Male	78	71.5		
Age (years)				
≤14	43	39.4	<i>P</i> = 0.9	<i>P</i> = 0.9
>14	66	60.6		
Location				
Extremity	84	77.1	<i>P</i> = 0.06	<i>P</i> = 0.2
Central	8	7.3		
Pelvis	17	15.6		
LDH ^c				
Normal	72	73.5	<i>P</i> = 0.03	<i>P</i> = 0.0084
High	26	26.5		
Surgery				
YES	95	87.2	<i>P</i> = 0.08	<i>P</i> = 0.06
NO	14	12.8		
Local treatment				
RxT	14	12.8	<i>P</i> = 0.4	<i>P</i> = 0.2
RxT + surgery	17	15.6		
Surgery	78	71.6		
Chemo protocol				
ISG/SSG III	85	78	<i>P</i> = 0.25	<i>P</i> = 0.35
Others	24	22		
Response to chemotherapy ^d				
Good	40	41.2	<i>P</i> = 0.0183	<i>P</i> = 0.018
Poor	57	58.8		
Type of translocation				
EWS/Fli-1 type I	56	51.4	<i>P</i> = 0.642	<i>P</i> = 0.304
EWS/Fli-1 nontype I	53	48.6		
miR-34a				
High	55	50.5	<i>P</i> = 0.0042	<i>P</i> = 0.0043
Low	54	49.5		
EFS (status)				
NED	68	62.4		
REL	41	37.6		
OVS (status)				
Alive	75	68.8		
Dead	34	31.2		

Associations with prognosis were calculated by univariate analysis using the log-rank test. Results in bold are significant at $P \leq 0.05$.

^aEFS (median follow-up: 57 months; range 6–241 months).

^bOVS (median follow-up: 67 months; range 9–241 months).

^cData available for 98 patients in qRT-PCR.

^dData available for 97 patients in qRT-PCR.

EWS, Ewing sarcoma; LDH, lactate dehydrogenase; RxT, radiotherapy; NED, no evidence of disease; REL, relapsed; EFS, event-free survival; OVS, overall survival.

Adverse events, occurred in 13 of 55 (24%) patients with high expression of miR-34a but in 28 of 54 (52%) patients with low expression ($P = 0.003$, Fisher's exact test). Accordingly, tumor-related deaths occurred in 11 of 55 (20%) high expressors but in 23 of 54 (43%) low expressors ($P = 0.01$, Fisher's exact test). Kaplan–Meier curves (Figure 1B) confirmed that miR-34a expression in localized tumors is associated with a significantly different risk of recurrence: 5-year event-free survival (EFS) was 74% for the 55 patients with high expression of miR-34a [95% confidence interval (CI) 62–82] while it was 51% for the 54 patients with low expression (95% CI 37–64) ($P = 0.0071$); 5-year overall survival (OVS) was 83% in high expressors (95% CI 73–93) but it was 58% in low expressors (95% CI 44–71) ($P = 0.0027$).

Considering the predictive significance of tumor response to induction chemotherapy, data were analyzed focusing on this topic. miR-34a expression was similar in good and poor responders (data not shown). In good responder patients, the probability of EFS and OVS were slightly better in case of high miR-34a [5-year EFS: high miR-34a 86% (95% CI 70–100), low miR-34a 71% (95% CI 50–93), 5-year OVS: high miR-34a 90% (95% CI 77–100), low miR-34a 77% (95% CI 57–97)], without statistical differences (5-year EFS: $P = 0.2$, 5-year OVS: $P = 0.2$). In poor responder patients, the probability of EFS and OVS again were better in case of high- miR-34a [5-year EFS: high miR-34a 67%

(95% CI 45–84), low miR-34a 45% (95% CI 27–64), 5-year OVS: high miR-34a 90% (95% CI 66–96), low miR-34a 54% (95% CI 35–73)], and the difference reached statistical significance (5-year EFS: $P = 0.09$, 5-year OVS: $P = 0.05$) in spite of the relatively low number of patients.

To verify the reliability of this type of analysis, we also evaluated whether the impact of miR-34a expression may vary by changing the type of calibrator that was used to define the levels of miR-34a expression. Despite the relative expression of miR-34a in individual EWS patients differed when various normal cells or tissues were used as calibrators (Figure 2A) (statistical variability across the controls for expression of miR-34a was: $-\Delta C_T$ median = -2.740 , standard deviation = 0.882), patients defined with high expression of miR-34a according to the different median values (supplementary Table S2, available at *Annals of Oncology* online) always maintained a significantly lower probability to face adverse events. Representative Kaplan–Meier survival curves obtained by using corion-derived hMSCs as calibrator are shown in Figure 2B.

Low miR-34a expression, poor response of tumors after neoadjuvant chemotherapy and high LDH values, which resulted significantly associated with worse clinical outcome in univariate analysis (Table 1), were all confirmed as independent risk factors associated with poor outcome by multivariate Cox's proportional hazards regression analysis (Table 2). Fisher's

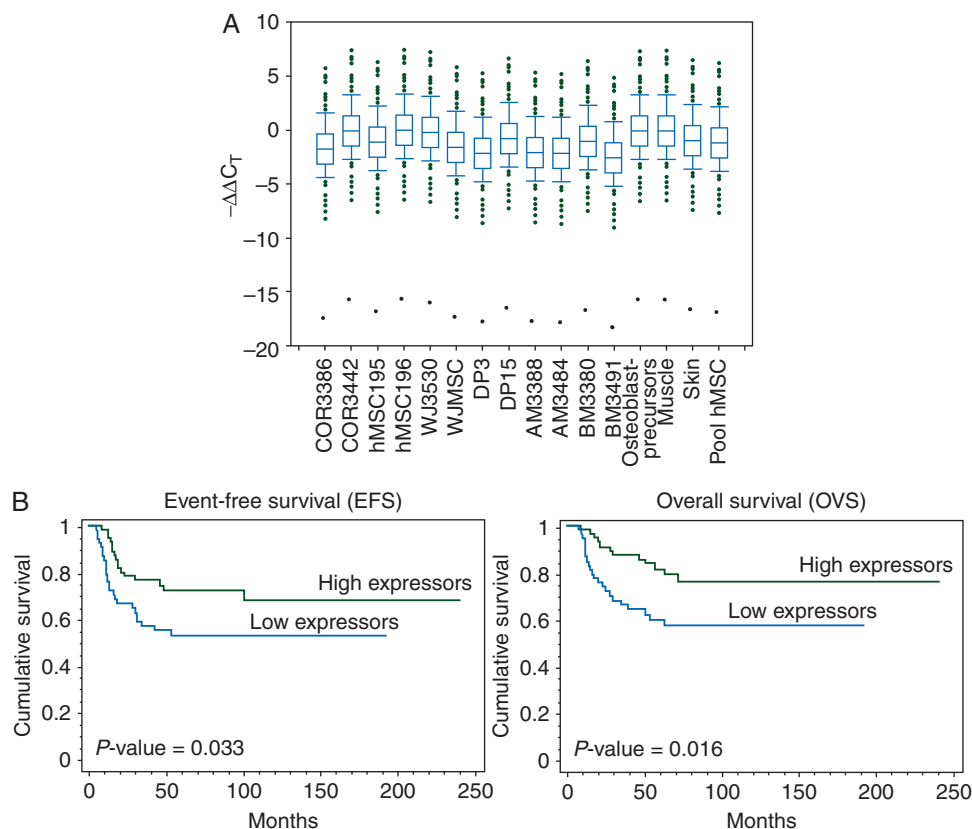


Figure 2. (A) Relative expression of miR-34a in 109 primary EWS samples compared with 16 different calibrators (COR3386; COR3442; hMSC195; hMSC196; WJ3530; WJMSC; DP3; DP15; AM3388; AM3484; BM3380; BM3491; osteoblast precursors; muscle; skin; pool hMSC). (B) Prognostic impact of miR-34a expression according to Kaplan–Meier curves and log-rank test using corion-derived hMSCs (COR3386) as calibrator. High expressors and low expressors were defined according to the median value of $-\Delta C_T$. Event-free survival (EFS) or overall survival (OVS) was considered.

Table 2. Multivariate analysis using Cox's proportional hazards regression analysis

	RR	95% CI	P value
Variables associated with better EFS			
miR-34a: high expression	0.406	0.244–1.033	0.0177
LDH: normal expression	0.389	0.184–0.821	0.0132
Necrosis: total	0.086	0.012–0.639	0.0165
Variables associated with better OVS			
miR-34a: high expression	0.372	0.159–0.873	0.023
LDH: normal expression	0.316	0.140–0.714	0.006
Necrosis: total	0.001	0.001–1000	0.962

Adjusted risk-rate (RR) ratio of relapse was estimated for the variables that resulted to be significantly associated with prognosis by univariate analysis.

exact test analysis confirmed that miR-34a expression did not correlate with any of the clinico-pathological characteristics of EWS patients at study entry (supplementary Table S3, available at *Annals of Oncology* online).

expression of miR-34a in Ewing sarcoma metastases

RNAs from 5 bone and 12 lung metastases were available and used for miR-34a analysis. miR-34a expression was significantly lower in metastases compared with primary tumors (supplementary Figure S3, available at *Annals of Oncology* online) ($P = 0.022$, Mann–Whitney U -test).

expression of miR-34a correlates with proliferation rate of EWS samples

To further validate the clinical relevance of miR-34a, we also analyzed by qRT-PCR the expression of some genes that were reported to be targeted by miR-34a [9, 10], including the evaluation of (i) cyclin D1, a cell cycle regulator [11], (ii) bcl-2, a major antiapoptotic driver [12], (iii) the transcription factor Yin Yang 1 (YY1), which controls several divergent cellular processes, including cell proliferation and apoptosis [13] and promotes p53 degradation [13], (iv) SIRT1, a NAD-dependent deacetylase that deactivates p53 [14, 15]. Statistically significant inverse correlations (supplementary Table S4, available at *Annals of Oncology* online) were observed between miR-34a levels and expression of cyclin D1 ($r = -0.242$, $P = 0.0114$, Spearman's test), bcl-2 ($r = -0.182$, $P = 0.0589$) and YY1 ($r = -0.200$, $P = 0.0369$), which indeed appeared as molecular hubs of the putative complex network of genes regulated by miR-34a (supplementary Figure S4, available at *Annals of Oncology* online, GeneGo network analysis). However, at protein level, when cyclin D1, bcl-2 and YY1 were evaluated on TMA created with matching tumor samples (58 cases), the expression of miR-34a was found to maintain statistically significant inverse correlation only with cyclin D1 expression ($r = -0.27$, $P < 0.05$, Pearson's correlation test, supplementary Table S5, available at

Annals of Oncology online). Accordingly, miR-34a expression was evaluated in paired samples and found to inversely correlate with Ki-67 staining ($r = -0.31$, $P = 0.02$, Pearson's correlation test).

discussion

A large number of studies have demonstrated that altered expression of specific miRNAs plays an important role in human tumorigenesis [16]. Detection of aberrant miRNA expression levels either in tumors or more recently in blood could be used for prediction of prognosis because miRNAs are stable, resistant to several harsh conditions, and can be detected easily and cheaply by reliable and quantitative methods. Our previous miRNA array data in EWS tissue samples have indicated the possible prognostic relevance of miR-34a. High expression of miR-34a at diagnosis was found to be associated with better outcome [7]. Although interesting, this first evidence warrants further examination in larger cohorts in order to obtain a more general acknowledgement from pathologists and oncologists of the clinical value of miR-34a. Validation of experimental results is always a difficult task in rare tumors. Combination of samples from different institutions may have significant drawbacks in terms of local treatments and/or sample preservation. In contrast, analysis of larger series from single institutions implicates analysis of samples from different decades.

In this study, we analyzed the expression of miR-34a in a retrospective series of 109 tumors, strictly controlled either in terms of clinical or technical parameters and fitting the standards for reporting prognostic biomarkers (REMARK guidelines) [17]. In line with its role as a master driver of tumor suppression, miR-34a expression was found to be generally downregulated in EWS compared with normal tissue or mesenchymal stem cells, which very likely represent the cell of origin of EWS [18]. Expression of miR-34a was lower in metastases compared with primary tumors. In addition, when miR-34a levels were evaluated at diagnosis in localized tumors, they were associated with a significantly different outcome: higher levels correlated with a lower risk to develop secondary events. This evidence was confirmed also when different types of normal calibrators were considered. This is an important point because it indicates that, despite the quantification of miR-34a obtained by quantitative PCR is relative, the use of different normal samples do not alter net results: patients with a higher expression of miRNA have a more favorable outcome. For perspective studies, the use of digital, which provides absolute rather than relative quantification of nucleic acids PCR [19], may be useful to solve the problem of the definition of a threshold level for miR-34a.

MiR-34a can antagonize many different oncogenic processes by regulating genes that function in various cellular pathways [20]. A major function of miR-34a is the control of cellular proliferation: ectopic expression of miR-34a in cancer cells decreases cell doubling times and leads to G1/G2 arrest. Accordingly, transcripts validated for their interactions with miR-34a include cyclin D1 and E2 as well as cyclin-dependent kinases 4 and 6, all genes involved in promoting cell cycle. Moreover, miR-34a is also involved in regulation of apoptosis: it is transcriptionally induced by p53 and is an important effector

in the execution of p53 signaling because it represses Bcl-2, SIRT1 and YY1. Although the ability of miR-34a to impact these cellular processes may suggest that it can act in synergism with conventional cytotoxic therapies, as previously confirmed in experimental conditions in various tumors including EWS [7], no relation was found between miR-34a expression and pathological response of the primary tumor to induction chemotherapy in our series of patients. The expression of miR-34a better discriminates prognosis in patients with poor rather than good responses to chemotherapy. Considering that these patients were treated with high-dose chemotherapy, high miR-34a expression very likely characterizes patients whose tumors have a lower biological aggressiveness, in particular a lower propensity to metastasize rather than a higher chemosensitivity.

In our samples, it appears that miR-34a function in cell cycle and proliferation is prevalent with respect to apoptosis control. Significant correlations with cyclin D1 and with Ki-67 staining were indeed observed both at mRNA and protein level. At clinical level, the relevance of miR-34a may thus be complemented by Ki-67 expression, which was previously shown to constitute a valuable indicator of poor prognosis in localized EWS [21]. At biological level, however, these evidences may highlight a more complex relationship, which deserves further investigations. Cyclin D1 is expressed at much higher levels in EWS when compared with cancers devoid of EWS fusions and bone-marrow mesenchymal stem cells from which Ewing tumors are thought to originate [22]. EWS-FLI1, the genetic hallmark of EWS, is known to affect transcription and alternative processing of cyclin D1 transcripts, both of which favor EWS cell transformation [22]. Besides its well-defined role in regulating cell cycle, cyclin D1 is indeed involved in several other crucial processes, including induction of cellular migration and invasion, enhancement of angiogenesis, inhibition of mitochondrial metabolism, enhancement of DNA damage sensing and DNA damage repair [11]. Our data showed a correlation between miR-34a and cyclin D1 expression. We can speculate that miR-34a may counteract the action of EWS-FLI1 by reducing the expression of a critical driver of EWS transformation also in presence of the oncogene. In addition, cyclin D1 has been recently found to induce expression of Dicer, a central regulator of miRNA maturation [23], and thereby promotes maturation of miRNA [23]. This new evidence indicates the existence of a complex crosstalk between cyclin D1 and expression of the noncoding genome. Likely, when expression of miR-34a is higher in EWS tumors, cyclin D1 could not evade negative feedback from the noncoding genome and its expression is reduced.

Hence, although we acknowledge the limits that may be related to a retrospective although well-controlled study, we consider that miR-34a constitutes a valuable indicator of good prognosis in localized EWS and strongly recommend its detection in a prospective series of patients. Upregulation of miR-34a in tumors that do not express it could be of great therapeutic interest. Nanotechnology-based approaches and in particular stable nucleic acid lipid particles, which are characterized by good transfection efficiency and stability in serum, have been recently proposed as effective agents to deliver miR-34a *in vivo* [24], thus representing a promising tool for miRNA-therapeutics against tumors.

acknowledgements

We thank Enrico Lucarelli (Rizzoli Institute) and Laura Bonisi (University of Bologna) for sharing primary mesenchymal stem cell cultures; Alba Ballardelli and Cristina Ghinelli for helping us in editing the manuscript.

funding

This work was supported by grants from the Italian Association for Cancer Research–AIRC [IG2013_14049 to KS] and by Italian Ministry of Health–TRANSCAN_Provabes to PP and KS; SV is in receipt of the fellowship ‘Guglielmina Lucatello e Gino Mazzega’ granted by Fondazione Italiana per la Ricerca sul Cancro–FIRC; RSP is a recipient of a fellowship from the Associazione Onlus ‘il Pensatore: Matteo Amitrano’ and ‘Liberi di Vivere Luca Righi.’

disclosure

The authors have declared no conflicts of interest.

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Annals of Oncology 25: 2086–2091, 2014
doi:10.1093/annonc/mdu266
Published online 23 July 2014

On demand Gamma-Knife strategy can be safely combined with BRAF inhibitors for the treatment of melanoma brain metastases

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Received 2 December 2013; revised 7 July 2014; accepted 11 July 2014

Background: Both Gamma-Knife radiosurgery (GKRS) and BRAF inhibitors (BRAF-I) have been shown to be useful in melanoma patients with brain metastases (BMs), thus suggesting that it could be interesting to combine their respective advantages. However, cases of radiosensitization following conventional radiation therapy in BRAF-I treated patients have raised serious concerns about the real feasibility and risk/benefit ratio of this combination.

Patients and methods: Review by two independent observers of brain magnetic resonance imaging (MRI) follow-up pictures, and volume and edema quantifications, and survival assessment in all patients who had been treated by GKRS and BRAF-I at a single institution.

Results: Among 53 GKRS carried out in 30 patients who ever received BRAF-I and GKRS, 33 GKRS were carried out in 24 patients while under BRAF-I treatment, from which only 4 with an interruption of BRAF-I. The 20 other GKRS were carried out in 15 patients (including 9 of the 24) before initiation of BRAF-I treatment. No case of radiation-induced necrosis and no scalp radiation dermatitis occurred. A >20% increase in volume was observed in 35 of the 263 BM treated by GKRS (13.3%), but only 3 clear-cut edemas and 3 hemorrhages were detected within 2 months after GKRS, and 4 edemas and 7 hemorrhages later. Neither the MRI features nor the incidence of the volume changes, hemorrhage and edema were deemed unexpected for melanoma BM treated by GKRS. Median survival from first GKRS under BRAF-I and first dose of BRAF-I were 24.8 and 48.8 weeks, respectively.

Conclusion: This series does not show immediate radiotoxicity nor radiation recall, in melanoma patients with BRAF-I whose BMs are treated by GKRS. Interrupting BRAF-I for stereotactic radiosurgery (SRS) of BM seems useless, although it is still advised for other radiation therapies. The potential benefit of combining SRS and BRAF-I can be safely tested.

Key words: BRAF inhibitors, Gamma-Knife radiosurgery, radiosensitization, brain metastasis, metastatic melanoma

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