Characteristic *miR-24* Expression in Gastric Cancers among Atomic Bomb Survivors

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Key Words

Gastric cancer · Radiation carcinogenesis · Atomic bomb · microRNA · *miR-24*

Abstract

Objective: To elucidate the mechanism of radiation-induced cancers, we analyzed the expression profiles of microRNAs extracted from formalin-fixed paraffin-embedded (FFPE) gastric cancer (GC) tissue samples from atomic bomb survivors. **Methods:** The expression levels of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* were measured by quantitative reverse transcription polymerase chain reaction (qRT-PCR). **Results:** The expression of microRNAs was measured by qRT-PCR in a Hiroshima University Hospital cohort comprising 32 patients in the high-dose-exposed group and 18 patients in the low-dose-exposed group who developed GC after the bombing. The GC cases showing high expression of *miR-24*, *miR-143*, and *miR-145* were more frequently found in the high-dose-exposed group than in the low-dose-exposed group. We next performed qRT-PCR of *miR-24*, *miR-143*, and *miR-145* in a cohort from the Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital

comprising 122 patients in the high-dose-exposed group and 48 patients in the low-dose-exposed group who developed GC after the bombing. High expressions of *miR-24* and *miR-143* were more frequently found in the high-dose-exposed group than in the low-dose-exposed group. Multivariate analysis demonstrated that only high expression of *miR-24* was an independent predictor for the exposure status. **Conclusion:** These results suggest that the measurement of *miR-24* expression from FFPE samples is useful to identify radiation-associated GC.

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Introduction

Gastric cancer (GC) is the fourth most common malignancy worldwide, with approximately 870,000 new cases occurring each year (World Health Organization). The effect of radiation on GC development has been estimated on the basis of the Life Span Study (LSS), in which both mortality and incidence were used as end points. The excess relative risks per gray (Gy) were 1.20 for mortality [1] and 1.32 for incidence [2]. Cancer develops as a

result of multiple genetic and epigenetic alterations [3]. Although several genetic alterations, including mutations in TP53 and BRAF, have been reported in several cancers from atomic bomb survivors [4–6], little is known about the changes in gene expression in radiation-associated GC. We previously showed that versican and osteonectin are expressed at much lower levels in tumor-associated stroma of atomic-bomb-exposed patients than in non-atomic-bomb-exposed patients [7]. However, little is known about changes in gene expression in radiation-associated GC.

MicroRNAs are 18- to 25-nucleotide, noncoding RNA molecules that regulate the translation of many genes [8]. Previous studies have shown that the expression levels of microRNAs are altered in most types of human cancers [9–11]. However, the expression of microRNA has not been investigated in atomic bomb survivors.

Biomarkers measured by RNA-based techniques require freshly frozen tissues. However, formalin-fixed paraffin-embedded (FFPE) tissue samples have been collected through decades of routine histopathologic examination and are the most widely available materials in clinical use. Unfortunately, formaldehyde-containing fixatives cause cross-linkage between nucleic acids and proteins, making the subsequent extraction and quantification of RNA challenging [12]. A major obstacle to the RNA expression analysis of FFPE tissues has been the uncertainty about whether gene expression analyses from routinely archived tissues accurately reflect the expression before fixation, and this is likely due to high fragmentation [13]. Because fragmentation does not cause further loss of quality when naturally occurring small RNAs are targeted, microRNAs are more ideal for the analysis of RNA extracted from FFPE samples.

In the present study, we aimed to identify potential molecular markers for radiation-associated GC by analyzing the expression profiles of microRNAs from FFPE GC tissue samples from atomic bomb survivors.

Material and Methods

Tissue Samples

In a retrospective study design, we used archival FFPE tissues from 239 patients who had undergone surgical excision of GC. The patients were treated at the Hiroshima University Hospital (HUH; Hiroshima, Japan) or at the Hiroshima Red Cross Hospital and Atomic-Bomb Survivors Hospital (HRCHABSH; Hiroshima, Japan).

An initial test cohort of 19 GC samples was used for TaqMan MicroRNA Array (Applied Biosystems, Austin, Tex., USA) analysis. All 19 patients were atomic bomb survivors in Hiroshima and

were treated at the HUH. The second cohort (n = 50) was also an HUH cohort and was used as a validation cohort for quantitative reverse transcription polymerase chain reaction (qRT-PCR). All 50 patients were atomic bomb survivors in Hiroshima and were treated at the HUH. The initial test cohort of 19 GC patients and the second cohort of 50 GC patients were LSS cohort members, and atomic bomb radiation doses were estimated with the DS02 system [14]. They were further classified into 2 groups according to the levels of exposed radiation dose received: the high-dose-exposed group (≥ 5 mGy) and the low-dose-exposed group (< 5 mGy).

The third cohort (n = 170) was the HRCHABSH cohort, which was used as a validation cohort for qRT-PCR. All 170 patients were atomic bomb survivors in Hiroshima and were treated at the HRCHABSH. Because these patients were not LSS cohort members, the atomic bomb radiation doses were not estimated. The HRCHABSH cohort patients included directly exposed patients and those not present in Hiroshima city at the time of bombing but who entered the city soon after the bombing (within 2 weeks). They were likewise classified into a high-dose-exposed group (directly exposed patients, exposure distance from the hypocenter of ≤ 4 km) and a low-dose-exposed group (patients not present in Hiroshima city at the time of bombing but who entered the region ≤ 4 km from the hypocenter within 2 weeks after the explosion).

Tumor staging was performed according to the TNM classification system [15]. Histologic classification of GC was carried out according to the Lauren classification system [16].

Ethical Considerations

This study was approved by the Ethics Committee for Human Genome Research of Hiroshima University (Hiroshima, Japan).

RNA Extraction from FFPE Samples

The FFPE samples were sectioned (10 μ m), deparaffinized, and stained with hematoxylin and eosin to ensure that the sectioned block contained tumor cells. The tumor areas in the adjacent sections were marked under a light microscope. The tumor areas were macrodissected with sterile disposable scalpels and subjected to RNA isolation using the Recover All™ Total Nucleic Acid Isolation kit (Ambion, Austin, Tex., USA), according to the manufacturer's instructions. Total RNA was quantified using the NanoDrop ND-1000 spectrometer (NanoDrop, Wilmington, Del., USA), and both optical density 260/280 and 260/230 ratios were used for quality control.

TaqMan MicroRNA Array Analysis and qRT-PCR

MicroRNA expression profiling was carried out with the TaqMan Array Human MicroRNA A Card v2.0 (Applied Biosystems) using the 7900 HT-Fast Real-Time PCR System (Applied Biosystems). The TaqMan Array Human MicroRNA A Card v2.0 is designed with 384 unique assays of human microRNAs. The microRNAs were amplified after specific reverse transcription and pre-amplification using Megaplex Assay Performance (Megaplex RT Primer Pools and Megaplex PreAmp Pools, Applied Biosystems) according to the manufacturer's instructions.

The expression levels of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, *miR-145*, and *RNU6B* were measured using TaqMan MicroRNA Assays (Applied Biosystems). The cDNA was synthesized using microRNA-specific primers and a TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems) according to the manufacturer's instructions. Briefly, 40 ng of RNA were reverse

Table 1. Six microRNAs with higher expression in GCs from the high-dose-exposed group than those from the low-dose-exposed group

Name	Exposure status	mRNA expression	p value
<i>miR-21</i>	High-dose exposed	1.81 (0.21–13.01)	0.0437
	Low-dose exposed	0.11 (0.01–11.39)	
<i>miR-24</i>	High-dose exposed	5.68 (1.52–10.56)	0.0194
	Low-dose exposed	1.35 (0.03–6.97)	
<i>miR-34a</i>	High-dose exposed	0.83 (0.26–4.95)	0.0258
	Low-dose exposed	0.11 (0.01–6.33)	
<i>miR-106a</i>	High-dose exposed	5.71 (0.78–10.43)	0.0437
	Low-dose exposed	0.56 (0.02–8.51)	
<i>miR-143</i>	High-dose exposed	6.71 (0.17–33.07)	0.0437
	Low-dose exposed	0.20 (0.01–9.77)	
<i>miR-145</i>	High-dose exposed	44.258 (0.76–78.26)	0.0339
	Low-dose exposed	1.10 (0.02–202.77)	

Values represent medians (ranges) unless otherwise stated. The p values were assessed using the Mann-Whitney U test.

transcribed in a 20- μ l reaction with gene-specific RT probes. qRT-PCR was performed using the 7900 HT-Fast Real-Time PCR System (Applied Biosystems). RNU6B was used as an endogenous normalization control. The relative target microRNA expression levels were calculated by the formula $2^{-\Delta\Delta CT}$, where $\Delta\Delta CT = \Delta CT$ target microRNA – ΔCT RNU6B, and a ≥ 32 CT value was interpreted as an amplification too low to quantify.

In situ Hybridization

The biotin 3'-labeled locked nucleic acid-incorporated microRNA probe (*miR-CURY* LNA detection probe, Exiqon, Woburn, Mass., USA) was used for the visualization of *miR-24*. Scrambled probes were incubated as negative controls (Exiqon). The FFPE samples were sectioned, deparaffinized, and rehydrated. Sample slides were treated with proteinase K (Dako Cytomation, Carpinteria, Calif., USA) for 20 min. A 20-nM probe was hybridized with the 1 $\times$ Enzo in situ hybridization buffer (Exiqon). The sample slides were heated to 50°C for 5 min and incubated at 37°C for 14 h. Immunological detection was performed using the Dako GenPoint™ Tyramide Signal Amplification System for Biotin-labeled Probes (Dako Cytomation). The sections were then exposed to a streptavidin-peroxidase reaction system and developed with DAB.

Statistical Methods

The differences in microRNA expression levels between 2 samples were tested by the Mann-Whitney U test for individual genes. Univariate and multivariate logistic regression analysis was carried out to assess the relation among clinicopathologic characteristics, expression levels of microRNAs, and radiation exposure status. A p value of <0.05 was considered statistically significant.

Table 2. Clinicopathologic characteristics of the patients by radiation exposure status in the HUH cohort

Variables	High-dose-exposed group (n = 32)	Low-dose-exposed group (n = 18)	p value
Median radiation dose, mGy	72 (5–2,601)	0 (0–4)	
Sex			
Male	17	10	0.8685
Female	15	8	
Age at diagnosis, years			
≤ 65	6	2	0.4794
> 65	26	16	
Tumor stage			
Stage I/II	17	12	0.3517
Stage III/IV	15	6	
Lauren classification			
Intestinal	16	14	0.0742
Diffuse	16	4	

Values represent numbers (ranges) unless otherwise stated. The p values were assessed using Fisher's exact test.

Results

MicroRNA Expression Profile

To identify potential molecular markers for radiation-associated GC, we analyzed the microRNA expression profiles of the initial test cohort of 19 GC samples using the TaqMan MicroRNA Array. Among the 19 GC tissue samples, 5 samples were included in the high-dose-exposed patient group (1,062, 314, 192, 113, and 102 mGy), and 14 samples were in the low-dose-exposed patient group (<5 mGy). To determine whether there is a microRNA expression profile characteristic to exposed patients, we compared the expression levels of individual microRNAs between high-dose-exposed and low-dose-exposed patient groups. The expression levels of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* were significantly higher in GC from high-dose-exposed patients than in GC from low-dose-exposed patients (table 1). No microRNA showed a lower expression in GC from high-dose-exposed patient group than in GC from low-dose-exposed patient group.

Expression of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* in the HUH Cohort

To validate the high expression of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* in the high-

Table 3. Univariate and multivariate Cox logistic analysis of *miR-24* expression and clinicopathologic characteristics in the HUH cohort

Characteristics	Univariate analysis		Multivariate analysis	
	odds ratio (95% CI)	p value	odds ratio (95% CI)	p value
Sex				
Male	1 (ref.)	0.4099	1 (ref.)	0.3567
Female	1.66 (0.49–5.61)		1.85 (0.49–7.26)	
Age at diagnosis, years				
≤65	1 (ref.)	0.6448	1 (ref.)	0.8091
>65	0.67 (0.12–3.75)		0.77 (0.09–6.41)	
Tumor stage				
Stage I/II	1 (ref.)	0.2943	1 (ref.)	0.7705
Stage III/IV	1.96 (0.56–6.85)		1.28 (0.25–6.67)	
Lauren classification				
Intestinal	1 (ref.)	0.1443	1 (ref.)	0.4880
Diffuse	2.67 (0.71–10.00)		1.80 (0.34–9.43)	
Exposure status				
High-dose group	1 (ref.)	0.0100	1 (ref.)	0.0262
Low-dose group	0.18 (0.05–0.66)		0.21 (0.05–0.83)	

dose-exposed patient group, the second cohort was analyzed. The second cohort (n = 50) was the HUH cohort and was used as a validation cohort for qRT-PCR. All 50 patients were atomic bomb survivors (LSS cohort members) in Hiroshima, and the cohort comprised 32 high-dose-exposed and 18 low-dose-exposed patients who developed GC after the bombing. The patient characteristics including sex, age at diagnosis, tumor stage, and Lauren classification are summarized in table 2. The clinicopathologic characteristics of the patients did not statistically differ between the high-dose-exposed and low-dose-exposed patient groups.

We performed qRT-PCR of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* in the 50 cases and divided the cases into high-microRNA expression and low-microRNA expression cases. When low expression was classified according to the lowest tertile, the number of high-microRNA expression cases was 34, and the number of low-microRNA expression cases was 16. We performed univariate and multivariate logistic regression analyses to determine which variables are independent markers for radiation exposure status (table 3; see online suppl. tables 1–5; for all online suppl. material, see www.karger.com/doi/10.1159/000398809). In the univariate analysis, high expression of *miR-24*, *miR-143*, and *miR-145* was correlated with exposure status; however, high expression of *miR-21*, *miR-34a*, and *miR-106a* was not correlated with exposure status. In the multivariate analysis, only high expression of *miR-24* was correlated with exposure status (table 3), and high expression of *miR-143*

or *miR-145* was not correlated with exposure status (online suppl. tables 4, 5).

Expression of miR-21, miR-24, miR-34a, miR-106a, miR-143, and miR-145 in the HRCHABSH Cohort

Univariate analysis in the HUH cohort revealed that GC cases showing high expression of *miR-24*, *miR-143*, and *miR-145* were more frequently found in the high-dose-exposed group than in the low-dose-exposed group. To further validate the high expression of *miR-24*, *miR-143*, and *miR-145* in atomic bomb survivors, a third cohort was analyzed. The third cohort (n = 170) was the HRCHABSH cohort, in which atomic bomb radiation doses were not estimated because these patients were not LSS cohort members. The patient characteristics are summarized in table 4. The clinicopathologic characteristics of the patients did not statistically differ between the high-dose-exposed and low-dose-exposed groups.

We performed qRT-PCR of *miR-24*, *miR-143*, and *miR-145* in the HRCHABSH cohort. These 170 GC cases were divided into high-microRNA expression cases and low-microRNA expression cases. When low expression was classified according to the lowest tertile, the number of high-microRNA expression cases was 113, and the number of low-microRNA expression cases was 57. We performed univariate and multivariate logistic regression analyses to determine which variables might be independent markers for radiation exposure status. In the univariate analysis, high expression of both *miR-24* (table 5)

Table 4. Clinicopathologic characteristics of patients by radiation exposure status in the HRCHABSH cohort

Variables	High-dose-exposed group (n = 122)	Low-dose-exposed group (n = 48)	p value
Median distance from the hypocenter, km	2.1 (0.1–4.1)	Located far from the hypocenter at the time of the atomic bomb explosion and entered the exposed area later	
Sex			
Male	80	28	0.3773
Female	42	20	
Age at diagnosis, years			
≤65	30	7	0.1547
>65	92	41	
Tumor stage			
Stage I/II	79	29	0.5969
Stage III/IV	43	19	
Lauren classification			
Intestinal	58	31	0.0602
Diffuse	64	17	

Values represent numbers (range) unless otherwise stated. The p values were assessed using Fisher's exact test.

Table 5. Univariate and multivariate Cox logistic analysis of *miR-24* expression and clinicopathologic characteristics in the HRCHABSH cohort

Characteristics	Univariate analysis		Multivariate analysis	
	odds ratio (95% CI)	p value	odds ratio (95% CI)	p value
Sex				
Male	1 (ref.)	0.9430	1 (ref.)	0.9742
Female	0.97 (0.50–1.89)		0.99 (0.49–1.98)	
Age at diagnosis, years				
≤65	1 (ref.)	0.5308	1 (ref.)	0.3463
>65	1.27 (0.60–2.72)		1.46 (0.66–3.23)	
Tumor stage				
Stage I/II	1 (ref.)	0.4558	1 (ref.)	0.5293
Stage III/IV	0.78 (0.40–1.50)		0.80 (0.41–1.59)	
Lauren classification				
Intestinal	1 (ref.)	0.7063	1 (ref.)	0.8647
Diffuse	1.13 (0.59–2.14)		1.06 (0.54–2.08)	
Exposure status				
High-dose group	1 (ref.)	0.0050	1 (ref.)	0.0042
Low-dose group	0.37 (0.19–0.74)		0.35 (0.17–0.72)	

and *miR-143* (online suppl. table 6) correlated with exposure status; however, high expression of *miR-145* (online suppl. table 7) did not correlate with exposure status. In the multivariate analysis, only high expression of *miR-24* (table 5) was correlated with exposure status, and high expression of *miR-143* or *miR-145* (online suppl. tables 6, 7) was not correlated with exposure status.

Expression and Localization of miR-24 in GC

We found that high expression of *miR-24* was an independent marker for exposure status by multivariate analysis in both the HUH cohort and the HRCHABSH cohort. Because high expression of *miR-24* was analyzed by qRT-PCR analysis of bulk GC tissues, we next determined which cells expressed *miR-24* by in situ hybridization. We

Fig. 1. In situ hybridization analysis of *miR-24* in GC tissue samples. **a** In situ hybridization analysis of *miR-24* in a GC case showing high *miR-24* expression. Original magnification, $\times 400$. **b** In situ hybridization analysis of *miR-24* in a GC case showing low *miR-24* expression. Original magnification, $\times 400$.

performed in situ hybridization in 5 GC cases showing high *miR-24* expression and in 5 GC cases showing low *miR-24* expression. Expression of *miR-24* was detected in GC cells from cases showing high *miR-24* expression (fig. 1a). In contrast, expression of *miR-24* was not detected in stromal cells, such as inflammatory cells and fibroblasts. In GC cases showing low *miR-24* expression, few *miR-24*-positive GC cells were observed (fig. 1b).

Discussion

To identify potential molecular markers for radiation-associated GC and to better understand the development of radiation-associated GC, we here analyzed the microRNA expression profiles of GC from atomic bomb survivors. From the microRNA expression profiles of the initial test cohort by TaqMan MicroRNA Array, we found that the expression levels of *miR-21*, *miR-24*, *miR-34a*, *miR-106a*, *miR-143*, and *miR-145* were significantly higher in GC from high-exposed patients than in GC from low-exposed patients. Among the examined microRNAs, only high expression of *miR-24* was an independent marker for exposure status by multivariate analysis in both the HUH cohort and the HRCHABSH cohort. GC cases showing high *miR-24* expression were more frequently found in the high-dose-exposed patient group than in the low-dose-exposed patient group in both the

HUH cohort and the HRCHABSH cohort. These results suggest that the measurement of the *miR-24* expression from FFPE samples is useful to identify radiation-associated GC.

We found that GC showing high-*miR-24* expression was frequently detected in high-dose-exposed patients. A previous study [17] showed that *miR-24* is downregulated in GC tissues compared with matched nontumor tissues and that ectopic expression of *miR-24* in GC cells suppressed cell proliferation, migration, and invasion in vitro as well as tumorigenicity in vivo. Furthermore, upregulation of *miR-24* in terminally differentiated cells has been demonstrated [18]. In contrast, in breast cancer, *miR-24* is overexpressed, and the ectopic expression of *miR-24* promotes breast cancer cell invasion and migration [19, 20]. Although downregulation of *miR-24* has been described in GC, the relationship with the radiation exposure history of patients has not been previously studied. Our study provides the first evidence of high *miR-24* expression levels in GC tissue samples from atomic bomb survivors.

MicroRNAs mediate sequence-specific posttranscriptional gene expression. One of the targets of *miR-24* is a histone H2AX, a key DNA double-strand break (DSB) repair protein [18]. DSBs are caused by the deleterious effects of ionizing radiation [21] and can induce chromosomal aberrations that cause cells to malfunction, resulting in cell death or tumorigenesis [22]. One of the earliest

steps in the cellular response to DSBs is the phosphorylation of histone H2AX at serine 139 (γ H2AX). The number of γ H2AX foci has been correlated directly with the number of DSBs produced by ionizing radiation [23]. Therefore, the number of γ H2AX foci is a significant marker for DSBs. Because in the high-dose-exposed patient group GC cases showing high *miR-24* expression were frequently detected, the expression of H2AX could be downregulated, and chromosomal instability could frequently occur in GC. In fact, we previously showed that the number of γ H2AX foci in high-dose-exposed patients is significantly higher than that in low-dose-exposed patients [24]. Analysis of chromosomal instability including gene amplification or deletion should be performed.

Previous reports showed that *miR-24* was downregulated in GC tissues compared with matched nontumor tissues. Ectopic expression of *miR-24* in GC cells suppressed cell proliferation, migration, and invasion in vitro as well as tumorigenicity in vivo [17]. The mechanisms of downregulation of *miR-24* in GC have not yet been completely investigated. At least, TGF- β 1 represses *miR-24* expression. Because TGF- β 1 is upregulated in GC [25], TGF- β 1 could suppress *miR-24* expression in low-dose-exposed patients, and activation of TGF- β 1 signaling pathway may be absent in GC from the high-dose patient group. We previously showed that versican and osteonectin were expressed at much lower levels in the tumor-associated stroma of high-dose exposed patients than in low-dose-exposed patients, and both versican and osteonectin were targets of the TGF- β 1 signaling pathway [7, 26, 27]. Taken together, these data suggest that the TGF- β 1 signaling pathway may not be activated in GC from high-dose-exposed patients. Other pathways that are activated in GC from high-dose patients should be elucidated.

In the HUH cohort and the HRCHABSH cohort, GC cases showing high *miR-143* expression were frequently

found in the high-dose-exposed patient group. In contrast, multivariate analysis revealed that the expression of *miR-143* was not correlated with the exposure status. *miR-143* has been shown to control smooth muscle cell phenotypes [28] and plays an important role in the pathogenesis of diffuse-type GC [29]. In the present study, in the HUH cohort and the HRCHABSH cohort, diffuse-type GC was found more frequently in the high-dose-exposed patient group than in the low-dose-exposed patient group, although the difference was not statistically significant. Therefore, the correlation between *miR-143* expression and exposure status is attributable to the high frequency of diffuse-type GC in the high-dose-exposed patient group. In fact, high expression of *miR-143* was frequently found in diffuse-type GC in the HRCHABSH cohort.

In summary, we found frequent high expression of *miR-24* in GC in high-dose-exposed atomic bomb survivors. Because the present study is a retrospective study investigating gene expression patterns, without any functional assay, the study design does not allow elucidating any mechanisms of irradiation-induced cancers. Therefore, further investigation is required to examine the correlation between *miR-24* expression and radiation-induced cancer.

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