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ARTICLE

Molecular pathogenesis of interstitial cystitis based on microRNA expression signature: *miR-320* family-regulated molecular pathways and targets

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Abstract

Interstitial cystitis (IC), also known as bladder pain syndrome, is a chronic inflammatory disease that affects the bladder. The symptoms of IC vary, including feeling an urgent need for immediate urination and of needing to urinate often, as well as bladder or pelvic pain. Despite its high incidence, no molecular diagnostic methods are available for IC, and the molecular pathogenesis is unknown. microRNAs (miRNA) can regulate expression of RNA transcripts in cells and aberrant expression of miRNAs is associated with several human diseases. Here, we investigated the molecular pathogenesis of IC based on miRNA expression signatures. RNA sequencing of miRNA levels in IC tissues and comparison with levels in normal bladder tissue and bladder cancer revealed dysregulated expression of 366 miRNAs (203 and 163 down- and upregulated miRNAs, respectively). In particular, *miR-320* family miRNAs(*miR-320a*, *miR-320b*, *miR-320c*, *miR-320d* and *miR-320e*) had downregulated expression in IC tissues. Genome-wide gene expression analyses and in silico database analyses showed that three transcription factors, *E2F-1*, *E2F-2* and *TUB*, are regulated by *miR-320* family miRNAs. Immunostaining of IC tissues confirmed that these transcription factors are overexpressed in IC tissues. Novel approaches that identify aberrantly expressed miRNA regulatory networks in IC could provide new prognostic markers and therapeutic targets for this disease.

Introduction

Interstitial cystitis (IC) is a non-specific chronic inflammatory bladder disease characterized by urinary frequency, urinary urgency, and pelvic pain induced by bladder filling [1, 2]. Patients with IC suffer significant declines in quality of life (QOL). The regional morbidity rate of IC varies, with 3–4 per 10 million in Japan, 60–70 per 10 million in the United States, and 18 per 10 million in Europe [3]. Based on cystoscopic findings, IC can be classified into 2 groups: Hunner type with ulcerative lesions (Hunner's lesion), and non-Hunner type with bladder petechial oozing (glomerulation) after hydrodistension. Diagnosis and classification of IC is determined by cystoscopic findings and exclusion of other diseases based on subjective symptoms. Although several studies searching for diagnostic markers IC have been conducted, no definitive markers are known [4]. Moreover, no symptomatic treatments for IC such as bladder hydrodistension, drug therapy and food therapy, or curative therapy have been established [5]. Given the lack of consensus about IC etiology, a genomic approach to elucidate molecular mechanisms underlying IC pathogenesis is needed.

microRNA (miRNA) are small (19 to 22 nucleotide) non-coding RNAs that can finely control expression of protein-coding or non-coding RNAs [6]. A single miRNA can control sequence-dependent expression of an extremely large number of RNA transcripts [7]. Therefore, aberrantly expressed miRNAs can lead to

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dysregulation of intracellular RNA networks. Indeed, a large number of studies have shown aberrant miRNA expression in several diseases and cancers, thus highlighting the important role for miRNAs in human disease pathogenesis [8–13].

We have successfully identified several cancer networks that are regulated by antitumor miRNAs in various cancer types using genome-wide gene expression analyses and in silico database searches [14–19]. Here we adapted our miRNA analysis strategy to characterize the molecular pathogenesis of IC. First, we analyzed the miRNA expression signature of IC based on RNA sequencing of samples from clinical specimens of Hunner-type IC and non-Hunner type IC. We then examined dysregulated miRNA expression in IC tissues to identify novel molecular networks involved in IC pathogenesis.

Analyses of miRNA signature revealed that expression of 366 miRNAs (203 and 163 downregulated and upregulated, respectively) was dysregulated in IC tissues. Moreover, we identified several genes that are targeted by the dysregulated miRNAs in IC tissues. Novel approaches that identify aberrantly expressed miRNA regulatory networks in IC could provide new prognostic markers and therapeutic targets for this disease.

Materials and methods

Patients and IC clinical specimens

IC clinical specimens were obtained from patients who were diagnosed at Dokkyo Medical University Hospital and Chiba University Hospital. Written informed consent was obtained from each patient in advance of sample collection. All patients underwent bladder hydrodistension, and IC tissues were collected by bladder biopsy. Hunner type IC patients were diagnosed by the presence of lesions, whereas non-Hunner type IC was classified based on the presence of glomerulation during bladder hydrodistension. Patient characteristics and cystoscopic findings are summarized in Supplementary Table 1 and Fig. 1, respectively.

RNA extraction and cell culture

We extracted total RNA using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol, and RNA quality was assessed as previously described [15, 17, 20, 21]. The human bladder cancer (BC) cell line T24 was obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained as previously described [15, 17, 20, 21].

Construction of miRNA expression signature for IC

To create a miRNA expression signature for IC, we performed small RNA sequencing using a HiSeq 2000 (Illumina, San Diego, CA, USA) for 8 IC samples (Supplementary Table 1). Samples from 5 normal and 5 cancer tissues of bladder that we described in a previous study were used as comparative specimens [17]. We carried out small RNA sequencing and data mining procedures as described previously [16, 17, 19, 22]. A false discovery rate (FDR) of less than 0.05 was considered to be significant.

Transfection with mature miRNA

We used Ambion Pre-miR miRNA precursor for *hsa-miR-320b* (assay ID: PM13132; Applied Biosystems, Foster City, CA, USA) and *hsa-miR-320c* (assay ID: PM13133; Applied Biosystems)as mature miRNA species. miRNA transfection of T24 cells and analysis of transfection efficiencies were conducted as described previously [15, 17, 20, 21].

Identification of putative genes regulated by the *miR-320* family in T24 cells

To identify genes regulated by the *miR-320*-family, we combined in silico and genome-wide gene expression analyses as previously described [15, 17, 20, 21]. We used the TargetScanHuman 7.1 (June, 2016 release, http://www.targetscan.org/vert_71) database and an oligo microarray (Agilent Technologies; Human Ge 60 K) for gene expression analyses. Microarray data were deposited into the GEO database (https://www.ncbi.nlm.nih.gov/geo/).

Immunohistochemistry

We incubated tissue specimens overnight at 4 °C with anti-E2F-1 antibodies (1:50 dilution; Cat. no. sc-251; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-E2F-2 antibodies (1:50; Cat. no. sc-9967; Santa Cruz Biotechnology) and anti-TUB antibodies (1:50; product no. HPA049019; Sigma-Aldrich, St. Louis, MO, USA). The procedures were conducted as described previously [15, 17, 20, 21].

Results

Small RNA sequences of specimens from normal bladder, bladder cancer and IC specimens

We performed deep sequencing of 18 small RNA libraries from specimens from 5 normal bladders, 5 bladder cancer cases, and 8 IC cases. Patient backgrounds for the

Table 1 Annotation of reads aligned to small RNAs

IC samples	#IC1 Count	(%)	#IC2 Count	(%)	#IC3 Count	(%)	#IC4 Count	(%)	#IC5 Count	(%)	#IC6 Count	(%)	#IC7 Count	(%)	#IC8 Count	(%)
Total	2,01,73,819	100	2,08,69,996	100	2,01,26,993	100	2,09,50,175	100	2,23,67,982	100	2,27,47,525	100	1,97,81,545	100	1,74,50,727	100
exon	50,619	0.25	96,993	0.46	2,08,000	1.03	78,823	0.38	2,45,920	1.10	1,41,999	0.62	61,449	0.31	35,290	0.20
exon_antisense	2	0.00	1	0.00	1	0.00	2	0.00	1	0.00	12	0.00	0	0.00	1	0.00
miRNA	21,82,531	10.82	56,92,405	27.28	59,32,462	29.48	26,87,212	12.83	76,94,621	34.40	1,78,93,426	78.66	27,03,027	13.66	28,00,833	16.05
rRNA	18,558	0.09	17,660	0.08	24,482	0.12	14,068	0.07	51,886	0.23	41,177	0.18	10,877	0.05	11,746	0.07
tRNA	21,60,022	10.71	15,84,616	7.59	13,08,127	6.50	22,46,789	10.72	17,00,832	7.60	3,55,922	1.56	23,83,040	12.05	18,06,166	10.35
snRNA	1,040	0.01	1,651	0.01	2,553	0.01	2,132	0.01	2,756	0.01	1,820	0.01	2,180	0.01	780	0.00
snoRNA	1,88,472	0.93	3,07,437	1.47	2,43,483	1.21	2,07,905	0.99	3,54,014	1.58	1,87,423	0.82	2,25,064	1.14	1,10,663	0.63
lcnRNA	2	0.00	23	0.00	64	0.00	30	0.00	77	0.00	37	0.00	6	0.00	3	0.00
ribozyme	3,136	0.02	2,116	0.01	2,531	0.01	5,611	0.03	2,091	0.01	586	0.00	3,680	0.02	2,500	0.01
sRNA	6	0.00	35	0.00	23	0.00	8	0.00	14	0.00	23	0.00	14	0.00	9	0.00
Unannotated	2,32,908	1.15	3,80,430	1.82	5,83,055	2.90	3,77,159	1.80	10,41,733	4.66	5,95,777	2.62	2,29,500	1.16	1,75,569	1.01
Unmapped	1,53,36,523	76.02	1,27,86,629	61.27	1,18,22,212	58.74	1,53,30,436	73.18	1,12,74,037	50.40	35,29,323	15.52	1,41,62,708	71.60	1,25,07,167	71.67
Normal bladder samples	#N1 Count	(%)	#N2 Count	(%)	#N3 Count	(%)	#N4 Count	(%)	#N5 Count	(%)						
Total	1,62,57,146	100	1,72,38,485	100	1,71,61,980	100	1,71,46,596	100	1,87,94,948	100						
exon	66,604	0.41	58,462	0.34	64,833	0.38	46,367	0.27	76,042	0.40						
exon_antisense	8	0.00	15	0.00	12	0.00	8	0.00	17	0.00						
miRNA	1,50,45,439	92.55	1,62,55,082	94.30	1,59,71,718	93.06	1,62,38,530	94.70	1,74,59,082	92.89						
rRNA	72,490	0.45	30,690	0.18	44,693	0.26	29,571	0.17	58,653	0.31						
tRNA	70,766	0.44	68,461	0.40	60,860	0.35	66,395	0.39	85,384	0.45						
snRNA	1,723	0.01	2,165	0.01	3,017	0.02	2,268	0.01	4,366	0.02						
snoRNA	45,942	0.28	51,534	0.30	51,259	0.30	49,350	0.29	68,347	0.36						
lcnRNA	11	0.00	3	0.00	16	0.00	3	0.00	12	0.00						
ribozyme	137	0.00	168	0.00	161	0.00	192	0.00	679	0.00						
sRNA	5	0.00	4	0.00	1	0.00	1	0.00	4	0.00						
Unannotated	5,08,136	3.13	3,80,346	2.21	5,19,914	3.03	3,40,750	1.99	4,99,262	2.66						
Unmapped	4,45,885	2.74	3,91,555	2.27	4,45,496	2.60	3,73,161	2.18	5,43,100	2.89						
BC samples	#T1 Count	(%)	#T2 Count	(%)	#T3 Count	(%)	#T4 Count	(%)	#T5 Count	(%)						
Total	1,55,95,761	100	1,47,55,694	100	1,36,63,048	100	1,38,66,298	100	1,35,54,054	100						
exon	2,74,922	1.76	4,00,241	2.71	2,34,310	1.71	2,25,867	1.63	2,58,728	1.91						
exon_antisense	336	0.00	18	0.00	35	0.00	81	0.00	28	0.00						

Table 1 (continued)

BC samples	#T1 Count	(%)	#T2 Count	(%)	#T3 Count	(%)	#T4 Count	(%)	#T5 Count	(%)
miRNA	1,27,17,389	81.54	94,07,032	63.75	1,00,32,602	73.43	1,01,14,382	72.94	93,92,970	69.30
rRNA	2,63,248	1.69	5,62,716	3.81	4,41,707	3.23	3,40,354	2.45	4,32,583	3.19
tRNA	1,25,786	0.81	1,43,750	0.97	1,11,058	0.81	1,14,524	0.83	1,82,630	1.35
snRNA	9,174	0.06	16,202	0.11	8,612	0.06	8,418	0.06	10,193	0.08
snoRNA	2,61,553	1.68	6,23,810	4.23	1,51,884	1.11	2,22,487	1.60	2,09,765	1.55
lcnRNA	117	0.00	96	0.00	38	0.00	74	0.00	96	0.00
ribozyme	1,706	0.01	3,433	0.02	1,141	0.01	1,830	0.01	2,843	0.02
sRNA	9	0.00	3	0.00	0	0.00	6	0.00	3	0.00
Unannotated	8,69,123	5.57	16,21,155	10.99	12,71,323	9.30	14,58,899	10.52	14,51,317	10.71
Unmapped	10,72,398	6.88	19,77,238	13.40	14,10,338	10.32	13,79,376	9.95	16,12,898	11.90

specimens from normal and bladder cancer cases are described in our previous report [17], and those for IC specimens are summarized in Supplementary Table 1. BC and normal tissues were reanalyzed for this study. We obtained between 13,554,054 and 22,747,525 total reads and 4,837,296–19,218,202 mapped reads (Table 1). We focused on previously annotated miRNAs and detected 745–1,417 miRNAs for each sample. From comparisons of normal bladder samples, BC samples, and IC samples, we constructed miRNA expression signatures that included miRNAs exhibiting significantly up- or downregulated expression (Tables 2–5; Supplementary Tables 2–7; ($|\log_2 FC| > 1$ and FDR < 0.05).

To extract miRNAs that had marked downregulation in IC tissues, we downselected candidate miRNAs according to the strategy shown in Supplementary Figure 1. Among the 203 miRNAs that were significantly downregulated in IC tissues compared to normal tissues, we excluded miRNAs that were also markedly upregulated or downregulated in comparisons of normal tissues with BC tissues. Of the 151 remaining miRNAs, we focused on the *miR-320* family (*miR-320a/b/c/d/e*), which is composed of 5 miRNAs (*miR-320a/b/c/d/e*) having the same seed sequence, given the high possibility that these miRNAs will target similar genes (Supplementary Figure 2). In our profile, expression of all *miR-320* family members was markedly downregulated in IC tissues.

Identification of molecular targets regulated by the *miR-320* family

We hypothesized that genes regulated by miRNAs that have decreased expression in IC tissues could contribute to IC pathogenesis. Using the TargetScanHuman 7.1 database for initial analyses, we found 5158 genes that had candidate target sites for the *miR-320* family. Next, genome-wide gene expression analyses were performed to narrow down candidate genes. This time, by transfection with *miR-320b* and *miR-320c*, which were mostly downregulated in IC, genes whose expression decreased in T24 cells were extracted. The microarray data were deposited into the GEO database (GEO accession number: GSE106791). These analyses identified 162 and 222 genes as candidates for regulation by *miR-320b* and *miR-320c*, respectively. Finally, we integrated these results, 85 genes remained as candidate genes for regulation by the *miR-320* family (Table 6). The strategy for selection of target genes is shown in Supplementary Figure 3. Among putative targets of *miR-320* family, several genes, e.g., *E2F1*, *RAB23*, *ITGB3*, and *TERT*, have been reported to be associated with bladder cancer [23–26]. However, as far as the PubMed database was searched, no reports were found about the relationship between these genes and IC. Additionally, we assigned genes to KEGG (Kyoto Encyclopedia of Genes

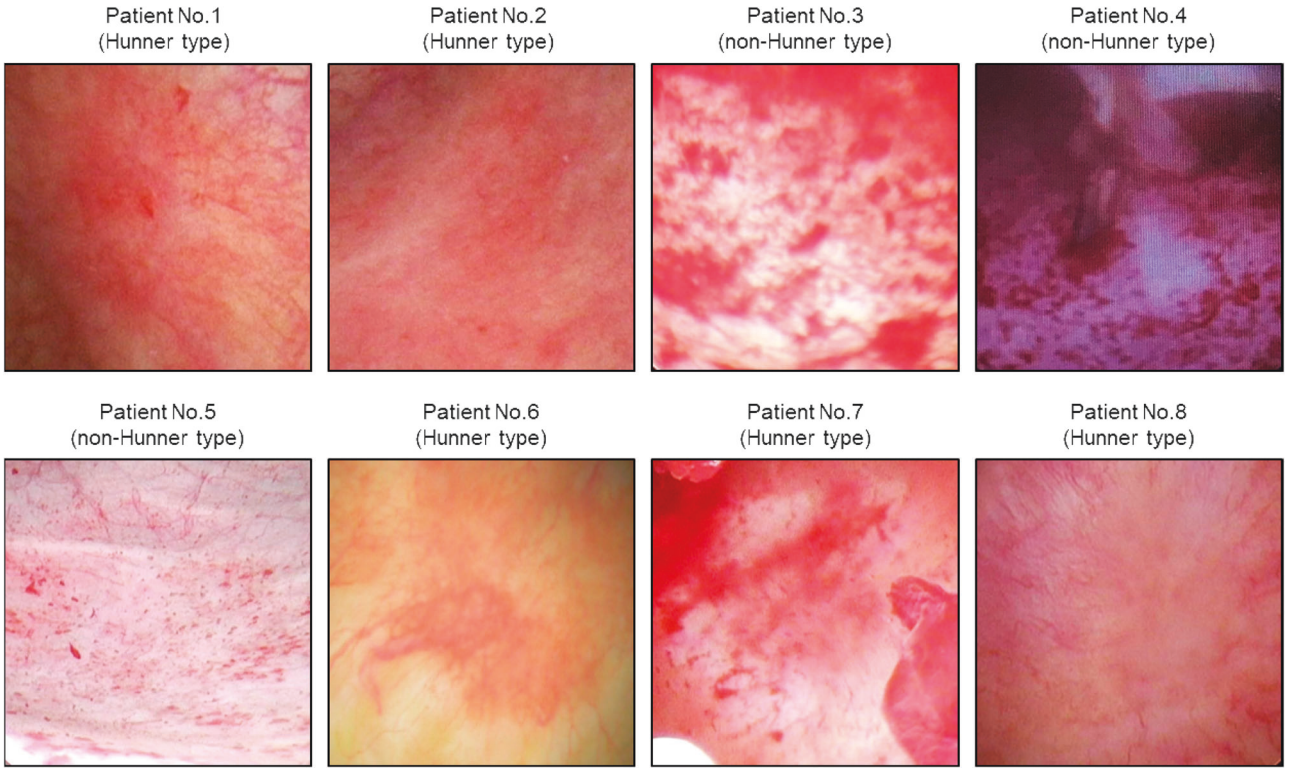

Fig. 1 Cystoscopic findings of IC patients. Cystoscopic findings of IC patients used in deep sequencing analysis. No.1, 2, 6, 7 and 8 are Hunner type IC patients' findings, indicating Hunner's lesions. No. 3, 4 and 5 are non-Hunner type IC patients' findings, showing bladder petechial oozing (glomerulation) after hydrodistension

and Genomes) pathways using the GENECODIS program and identified 6 significantly enriched pathways (Supplementary Table 8).

E2F-1, E2F-2 and TUB expression in IC clinical specimens

We focused on 3 transcription factors (*E2F-1*, *E2F-2* and *TUB*) and further analyses were performed. Previous studies showed that dysregulated expression or mutation of several transcription factors were deeply involved in human diseases and syndromes [27]. Function of E2F family is the transcriptional activation or repression of its target genes and pivotal role of cell proliferation, differentiation and apoptosis [28, 29]. The involvement of these genes and IC is obscure. We performed immunohistochemistry to assess protein expression of E2F-1, E2F-2, and TUB in IC clinical specimens. All three proteins were strongly expressed in IC urothelial cells compared with normal bladder and BC tissues (Fig. 2).

Discussion

Recently developed RNA sequencing technology is suitable for compiling miRNA expression signatures. We previously described several miRNA expression signatures of human cancers based on RNA sequencing and identified novel RNA networks regulated by antitumor miRNAs [14–19]. In this study, we presented the miRNA signature of IC determined by RNA sequencing. Identification of dysregulated miRNAs in IC tissues compared with normal bladder tissues or bladder cancer tissues will be useful for exploring molecular pathogenesis in IC cells.

Among the downregulated miRNAs in IC tissues, we focused on the *miR-320* family because all members of this family showed downregulated expression of *miR-320* in IC tissues compared with normal bladder tissues. In contrast to our study, a miRNA expression profile of IC generated by PCR-based microarray analysis in a previous study showed that several miRNAs (*miR-449b*, *miR-500*, *miR-328* and *miR-320*) were upregulated in IC tissues [30]. As a factor that causes different results from our research, analysis methods and differences in patient background may be considered. This report also showed that IC patients had notably decreased expression of neurokinin-1 (tachykinin receptors) that was presumed to be due to upregulated *miR-320* expression in IC tissues [30]. Downregulation of the *miR-320* family and the antitumor activity of these miRNAs have been reported for several cancers [31–35]. In bladder

Table 2 Top 20 miRNAs with downregulated expression in IC compared to normal bladder

miRNA	Locus	Log_2FC	*P*-value	FDR
hsa-miR-1	chr18:19408976-19408997 chr20:61151558-61151579	−11.613	5.01E−35	2.31E−33
hsa-miR-320b	chr1:117214409-117214430 chr1:224444751-224444772	−9.751	3.13E−185	2.01E−182
hsa-miR-206	chr6:52009199-52009220	−8.805	1.30E−33	5.87E−32
hsa-miR-320c	chr18:19263520-19263539 chr18:21901680-21901699	−8.463	3.99E−184	2.06E−181
hsa-miR-23b-5p	chr9:97847509-97847530	−8.434	7.59E−141	2.17E−138
hsa-miR-185-5p	chr22:20020676-20020697	−7.539	1.36E−205	1.17E−202
hsa-miR-107	chr10:91352513-91352535	−7.206	0	0
hsa-miR-320a	chr8:22102488-22102509	−6.960	9.58E−150	3.08E−147
hsa-miR-4732-5p	chr17:27188718-27188740	−6.843	1.20E−28	4.53E−27
hsa-miR-378g	chr1:95211436-95211455	−6.772	1.18E−23	3.74E−22
hsa-miR-193b-5p	chr16:14397837-14397858	−6.693	5.00E−80	9.90E−78
hsa-let-7b-5p	chr22:46509571-46509592	−6.687	3.43E−285	4.41E−282
hsa-let-7c-5p	chr21:17912158-17912179	−6.595	4.81E−184	2.07E−181
hsa-miR-139-3p	chr11:72326110-72326132	−6.532	1.34E−95	3.14E−93
hsa-miR-5010-5p	chr17:40666226-40666247	−6.510	3.60E−19	9.29E−18
hsa-let-7e-5p	chr19:52196046-52196067	−6.432	3.17E−57	3.03E−55
hsa-miR-92b-5p	chr1:155164987-155165008	−6.125	6.59E−38	3.40E−36
hsa-miR-320e	chr19:47212551-47212568	−5.962	1.35E−17	3.21E−16
hsa-let-7f-5p	chr9:96938635-96938656 chrX:53584207-53584228	−5.902	1.80E−94	3.85E−92
hsa-miR-140-3p	chr16:69967045-69967065	−5.814	4.32E−157	1.59E−154

Table 3 Top 20 miRNAs with upregulated expression in IC compared to normal bladder

miRNA	Locus	Log_2FC	*P*-value	FDR
hsa-miR-141-3p	chr12:7073318-7073339	9.324	4.08E−31	1.72E−29
hsa-miR-10b-5p	chr2:177015057-177015079	8.512	3.84E−67	4.95E−65
hsa-miR-126-3p	chr9:139565105-139565126	8.201	2.00E−49	1.56E−47
hsa-miR-4485	chr11:10529824-10529843	7.986	1.34E−27	5.00E−26
hsa-miR-3609	chr7:98479323-98479346	7.724	1.69E−11	2.71E−10
hsa-miR-19a-3p	chr13:92003193-92003215	7.224	7.85E−24	2.53E−22
hsa-miR-6723-5p	chr1:567762-567783	6.755	5.39E−13	1.01E−11
hsa-miR-335-3p	chr7:130136003-130136024	6.513	2.13E−20	5.91E−19
hsa-miR-141-5p	chr12:7073276-7073297	6.398	3.89E−08	5.06E−07
hsa-miR-126-5p	chr9:139565068-139565088	6.370	7.89E−61	8.47E−59
hsa-miR-454-3p	chr17:57215148-57215170	6.307	1.31E−25	4.56E−24
hsa-let-7f-2-3p	chrX:53584157-53584178	6.272	3.14E−12	5.40E−11
hsa-miR-151a-5p	chr8:141742722-141742742	6.221	8.99E−40	5.04E−38
hsa-miR-381-3p	chr14:101512305-101512326	6.145	1.06E−29	4.25E−28
hsa-miR-301a-3p	chr17:57228510-57228532	6.122	2.95E−23	9.27E−22
hsa-miR-148a-5p	chr7:25989580-25989601	5.966	8.36E−21	2.34E−19
hsa-miR-501-3p	chrX:49774380-49774401	5.951	3.76E−46	2.49E−44
hsa-miR-3529-3p	chr15:89155087-89155110	5.847	2.43E−08	3.26E−07
hsa-miR-3065-5p	chr17:79099686-79099708	5.841	2.56E−12	4.52E−11
hsa-miR-577	chr4:115577930-115577950	5.772	1.01E−06	1.14E−05

Table 4 Top 20 miRNAs with downregulated expression in IC compared to BC

miRNA	Locus	Log_2FC	*P*-value	FDR
hsa-miR-1323	chr19:54175232-54175253	−12.361	9.19E−17	3.76E−15
hsa-miR-516b-5p	chr19:54228711-54228732	−10.504	4.71E−13	1.35E−11
	chr19:54240114-54240135			
hsa-miR-1269a	chr4:67142608-67142629	−8.677	2.73E−11	6.21E−10
hsa-miR-320b	chr1:117214409-117214430	−8.661	3.78E−85	4.86E−82
	chr1:224444751-224444772			
hsa-miR-371b-3p	chr19:54290932-54290954	−8.657	3.65E−08	5.40E−07
hsa-miR-7977	chr3:176232891-176232908	−8.435	2.12E−23	1.40E−21
hsa-miR-518a-3p	chr19:54234310-54234331	−8.319	6.16E−09	1.06E−07
	chr19:54242639-54242660			
hsa-miR-320c	chr18:19263520-19263539	−8.266	4.39E−64	2.83E−61
	chr18:21901680-21901699			
hsa-miR-107	chr10:91352513-91352535	−8.211	1.34E−98	3.45E−95
hsa-miR-518e-3p	chr19:54233145-54233165	−8.112	2.59E−08	4.00E−07
hsa-miR-519a-3p	chr19:54255703-54255724	−8.038	2.96E−10	6.15E−09
	chr19:54265651-54265672			
hsa-miR-512-5p	chr19:54169946-54169968	−8.037	1.63E−10	3.50E−09
	chr19:54172430-54172452			
hsa-miR-483-5p	chr11:2155411-2155432	−7.862	1.35E−13	4.08E−12
hsa-miR-523-3p	chr19:54201691-54201713	−7.839	9.93E−09	1.67E−07
hsa-miR-519b-3p	chr19:54198517-54198538	−7.456	1.71E−09	3.27E−08
hsa-miR-200b-5p	chr1:1102504-1102525	−7.349	5.57E−25	4.63E−23
hsa-miR-372-3p	chr19:54291185-54291207	−7.244	1.75E−07	2.24E−06
hsa-miR-185-5p	chr22:20020676-20020697	−7.193	5.96E−68	5.12E−65
hsa-miR-517a-3p	chr19:54215575-54215596	−7.166	2.80E−09	5.23E−08
hsa-miR-517b-3p	chr19:54224372-54224393	−7.166	2.80E−09	5.23E−08

cancer, previous studies showed that *miR-320a* and *miR-320c* acted as antitumor miRNAs by targeting integrin beta-3 (*ITGB3*) and cyclin-dependent kinase 6 (*CDK6*), respectively [25, 36].

To identify possible genes involved in IC pathogenesis, we searched molecular pathways containing genes targeted by the *miR-320* family using genome-wide gene expression analyses and in silico database analyses. Using these approaches, we identified 85 genes as putative targets of *miR-320* family regulation. Among these genes, we focused on transcription factors due to their potential to significantly affect downstream RNA networks. Several studies indicated that dysregulation of transcription factor activity is associated with various human diseases [37, 38]. More recently, the urothelial master transcription factors *TP63*, *SHH* and *FOXA1* were reported to act as novel diagnostic markers and to have potential involvement in the molecular pathology of IC [39]. In this study, we focused on 3 transcription factors: E2F transcription factor 1 (*E2F-1*), E2F transcription factor 2 (*E2F-2*) and tubby bipartite transcription factor (*TUB*) and confirmed upregulated protein expression of all three in IC lesions.

The E2F family of transcription factors is involved in cell cycle regulation and synthesis of DNA in higher eukaryotes, and plays a pivotal role during the G1/S transition in mammalian cells [28, 29]. *E2F-1*, *E2F-2*, and *E2F-3a* function as transcription activators, whereas *E2F-3b*, *E2F-4*, *E2F-5*, *E2F-6*, *E2F-7*, and *E2F-8* act as transcription suppressors [28]. E2Fs are associated with cancer and are major target genes for the Rb tumor suppressor protein (pRb). Inactivation of pRb causes overexpression of E2Fs that can act as a cancer promoter [40]. Another report described the involvement of *E2F-1* and *E2F-2* overexpression in inflammation associated with traumatic spinal cord injury (SCI) in a mouse model. Results from that report suggested that SCI-induced activation of *E2F-1* and *E2F-2* expression may result in movement disorder and hypersensitivity after trauma. Furthermore, *E2F-1* and *E2F-2* knockout significantly reduced the amount of neuron death, neuroinflammation and tissue damage in the SCI mouse

Table 5 Top 20 miRNAs with upregulated expression in IC compared to BC

miRNA	Locus	Log_2FC	*P* Value	FDR
hsa-miR-10b-5p	chr2:177015057-177015079	9.502	1.83E−53	7.85E−51
hsa-miR-126-3p	chr9:139565105-139565126	9.202	1.50E−38	3.88E−36
hsa-miR-100-5p	chr11:122022983-122023004	8.195	3.05E−21	1.64E−19
hsa-miR-126-5p	chr9:139565068-139565088	8.109	1.90E−44	5.44E−42
hsa-miR-204-5p	chr9:73424947-73424968	7.411	6.30E−32	1.01E−29
hsa-miR-381-3p	chr14:101512305-101512326	7.339	5.42E−25	4.63E−23
hsa-miR-490-3p	chr7:136587989-136588010	7.335	1.01E−10	2.19E−09
hsa-miR-136-3p	chr14:101351087-101351108	6.994	1.75E−29	1.96E−27
hsa-miR-214-5p	chr1:172107997-172108018	6.902	2.98E−15	1.04E−13
hsa-miR-19a-3p	chr13:92003193-92003215	6.874	2.46E−23	1.58E−21
hsa-miR-125b-2-3p	chr21:17962610-17962631	6.619	1.61E−27	1.66E−25
hsa-miR-153-3p	chr2:220158848-220158869	6.568	1.47E−18	6.99E−17
	chr7:157367041-157367062			
hsa-miR-548ba	chr2:49286742-49286763	6.412	3.94E−05	0.000320332
hsa-miR-486-5p	chr8:41518002-41518023	6.369	6.28E−16	2.31E−14
	chr8:41517962-41517983			
hsa-miR-99a-5p	chr21:17911421-17911442	6.321	1.47E−17	6.66E−16
hsa-miR-561-5p	chr2:189162244-189162265	6.284	1.31E−12	3.48E−11
hsa-miR-145-3p	chr5:148810262-148810283	6.235	1.05E−22	6.32E−21
hsa-miR-100-3p	chr11:122022948-122022969	6.161	2.79E−10	5.85E−09
hsa-miR-143-3p	chr5:148808541-148808561	6.137	4.44E−13	1.30E−11
hsa-miR-133a-3p	chr18:19405673-19405694	6.077	1.79E−12	4.51E−11
	chr20:61162177-61162198			

Table 6 Candidate target genes commonly regulated by the *miR-320* family

Gene Symbol	Gene name	Location	Log_2 ratio *miR-320b* transfectant (IC/Normal)	Log_2 ratio *miR-320c* transfectant (IC/Normal)
MZT1	Mitotic spindle organizing protein 1	13q21.33	−2.99	−3.59
NXT2	Nuclear transport factor 2-like export factor 2	Xq23	−2.52	−2.60
CDCA3	Cell division cycle associated 3	12p13.31	−2.32	−2.12
SLC48A1	Solute carrier family 48 (heme transporter), member 1	12q13.11	−2.18	−1.41
HADH	Hydroxyacyl-CoA dehydrogenase	4q25	−2.17	−2.20
LSM11	LSM11, U7 small nuclear RNA associated	5q33.3	−2.11	−2.05
CPD	Carboxypeptidase D	17q11.2	−2.11	−2.54
TGOLN2	Trans-golgi network protein 2	2p11.2	−1.88	−1.74
LIN28A	Lin-28 homolog A (C. elegans)	1p36.11	−1.87	−1.40
TNFSF18	Tumor necrosis factor (ligand) superfamily, member 18	1q25.1	−1.81	−1.28
SLC26A2	Solute carrier family 26 (anion exchanger), member 2	5q32	−1.81	−2.12
LEF1	Lymphoid enhancer-binding factor 1	4q25	−1.80	−1.87
TUB	Tubby bipartite transcription factor	11p15.4	−1.79	−1.57
CHRAC1	Chromatin accessibility complex 1	8q24.3	−1.76	−1.27
TRIM14	Tripartite motif containing 14	9q22.33	−1.73	−1.35
XK	X-linked Kx blood group (McLeod syndrome)	Xp21.1	−1.72	−1.21
TMEM237	Transmembrane protein 237	2q33.1	−1.72	−1.13
OLFML2A	Olfactomedin-like 2 A	9q33.3	−1.68	−1.39

Table 6 (continued)

Gene Symbol	Gene name	Location	Log_2 ratio *miR-320b* transfectant (IC/Normal)	Log_2 ratio *miR-320c* transfectant (IC/Normal)
SDC4	Syndecan 4	20q13.12	−1.68	−1.51
JAK3	Janus kinase 3	19p13.11	−1.68	−1.38
CTSV	Cathepsin V	9q22.33	−1.67	−2.24
BSPRY	B-box and SPRY domain containing	9q32	−1.65	−1.82
SPCS2	Signal peptidase complex subunit 2 homolog (S. cerevisiae)	11q13.4	−1.62	−2.35
CYP1B1	Cytochrome P450, family 1, subfamily B, polypeptide 1	2p22.2	−1.58	−1.13
LYPD6	LY6/PLAUR domain containing 6	2q23.2	−1.58	−1.04
ZWILCH	Zwilch kinetochore protein	15q22.31	−1.54	−2.16
RAB27B	RAB27B, member RAS oncogene family	18q21.2	−1.53	−1.93
DCAF11	DDB1 and CUL4 associated factor 11	14q11.2	−1.53	−1.57
RGS9BP	Regulator of G protein signaling 9 binding protein	19q13.11	−1.51	−2.27
TMEM64	Transmembrane protein 64	8q21.3	−1.49	−1.67
SYNGR2	Synaptogyrin 2	17q25.3	−1.45	−1.07
THBD	Thrombomodulin	20p11.21	−1.45	−1.29
EIF2AK3	Eukaryotic translation initiation factor 2-alpha kinase 3	2p11.2	−1.43	−1.23
ACTL6A	Actin-like 6 A	3q26.33	−1.41	−1.96
PIP5K1A	Phosphatidylinositol-4-phosphate 5-kinase, type I, alpha	1q21.3	−1.34	−1.37
SPIN4	Spindlin family, member 4	Xq11.1	−1.33	−1.81
POLE3	Polymerase (DNA directed), epsilon 3, accessory subunit	9q32	−1.31	−1.70
ARPP19	cAMP-regulated phosphoprotein, 19 kDa	15q21.2	−1.31	−1.81
RAD51	RAD51 recombinase	15q15.1	−1.31	−1.92
CXCL2	Chemokine (C-X-C motif) ligand 2	4q13.3	−1.31	−1.97
SRSF7	Serine/arginine-rich splicing factor 7	2p22.1	−1.31	−1.28
HOXC13	Homeobox C13	12q13.13	−1.30	−1.37
DUT	Deoxyuridine triphosphatase	15q21.1	−1.29	−1.48
HAS3	Hyaluronan synthase 3	16q22.1	−1.28	−1.38
STK4	Serine/threonine kinase 4	20q13.12	−1.27	−1.50
E2F1	E2F transcription factor 1	20q11.22	−1.26	−1.33
NFATC4	Nuclear factor of activated T-cells, cytoplasmic, calcineurin-dependent 4	14q12	−1.26	−1.37
SHCBP1	SHC SH2-domain binding protein 1	16q11.2	−1.24	−1.66
RCN2	Reticulocalbin 2, EF-hand calcium binding domain	15q24.3	−1.24	−1.51
MAML3	Mastermind-like 3 (Drosophila)	4q31.1	−1.23	−1.23
ZNF367	Zinc finger protein 367	9q22.32	−1.23	−1.00
PIGN	Phosphatidylinositol glycan anchor biosynthesis, class N	18q21.33	−1.21	−1.29
MTBP	Mdm2, transformed 3T3 cell double minute 2, p53 binding protein (mouse) binding protein, 104 kDa	8q24.12	−1.21	−1.11
RBP7	Retinol binding protein 7, cellular	1p36.22	−1.20	−1.64
RAB23	RAB23, member RAS oncogene family	6p11.2	−1.19	−1.02
FCGR2A	Fc fragment of IgG, low affinity IIa, receptor (CD32)	1q23.3	−1.19	−1.34
ITGB3	Integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61)	17q21.32	−1.18	−1.55
RIPPLY3	Ripply transcriptional repressor 3	21q22.13	−1.16	−1.67
MATN1	Matrilin 1, cartilage matrix protein	1p35.2	−1.15	−1.89
TERT	Telomerase reverse transcriptase	5p15.33	−1.11	−1.23
ARFIP1	ADP-ribosylation factor interacting protein 1	4q31.3	−1.11	−1.30
CEP85	Centrosomal protein 85 kDa	1p36.11	−1.11	−1.22

Table 6 (continued)

Gene Symbol	Gene name	Location	Log_2 ratio *miR-320b* transfectant (IC/Normal)	Log_2 ratio *miR-320c* transfectant (IC/Normal)
FAM89A	Family with sequence similarity 89, member A	1q42.2	−1.11	−1.13
LAPTM4A	Lysosomal protein transmembrane 4 alpha	2p24.1	−1.11	−1.39
ZMYM6NB	ZMYM6 neighbor	1p34.3	−1.11	−1.10
ESCO2	Establishment of sister chromatid cohesion *N*-acetyltransferase 2	8p21.1	−1.10	−1.04
BAK1	BCL2-antagonist/killer 1	6p21.31	−1.09	−1.15
E2F2	E2F transcription factor 2	1p36.12	−1.09	−1.28
RAD51AP1	RAD51 associated protein 1	12p13.32	−1.09	−1.56
WNT10B	Wingless-type MMTV integration site family, member 10B	12q13.12	−1.08	−1.61
BLOC1S5	Biogenesis of lysosomal organelles complex-1, subunit 5, muted	6p24.3	−1.08	−1.06
LAMP1	Lysosomal-associated membrane protein 1	13q34	−1.07	−1.27
CGN	Cingulin	1q21.3	−1.07	−1.16
C19orf25	Chromosome 19 open reading frame 25	19p13.3	−1.07	−1.06
CNOT6	CCR4-NOT transcription complex, subunit 6	5q35.3	−1.07	−1.63
SH2D4B	SH2 domain containing 4B	10q23.1	−1.06	−1.39
RAB21	RAB21, member RAS oncogene family	12q21.1	−1.05	−1.87
AK4	Adenylate kinase 4	1p31.3	−1.05	−1.49
RAB14	RAB14, member RAS oncogene family	9q33.2	−1.04	−1.37
HELLS	Helicase, lymphoid-specific	10q23.33	−1.03	−1.24
DSCC1	DNA replication and sister chromatid cohesion 1	8q24.12	−1.02	−1.66
ARPC5	Actin related protein 2/3 complex, subunit 5, 16 kDa	1q25.3	−1.02	−1.17
RIT1	Ras-like without CAAX 1	1q22	−1.02	−2.14
RIOK3	RIO kinase 3	18q11.2	−1.01	−1.41
NXPH4	Neurexophilin 4	12q13.3	−1.01	−1.26

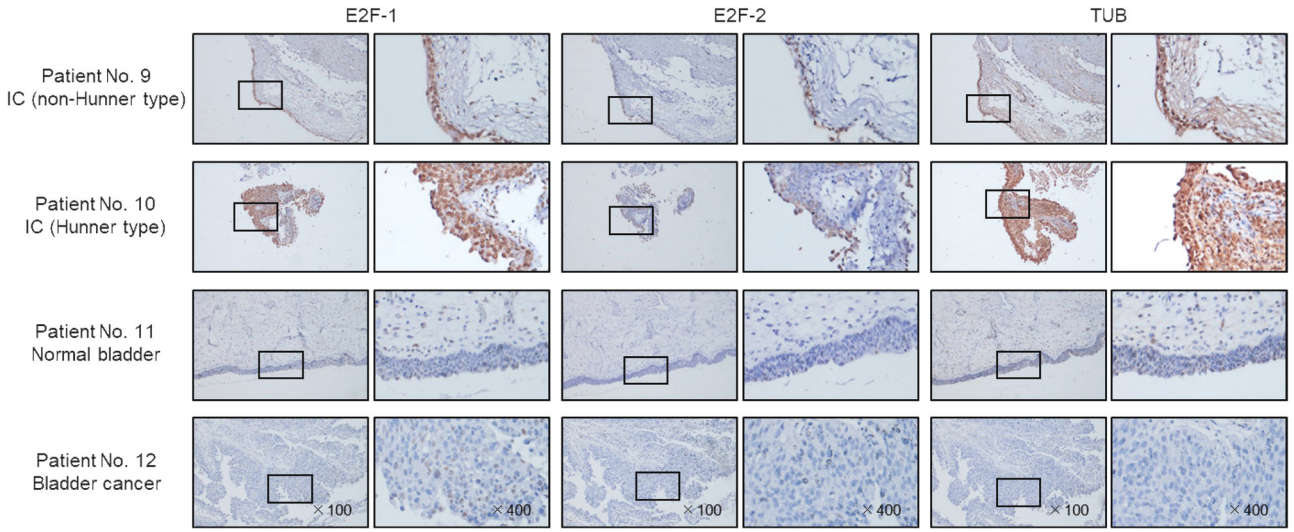

Fig. 2 E2F-1, E2F-2, and TUB expression in clinical bladder specimens. Immunochemical staining showed that E2F-1, E2F-2, and TUB proteins were strongly expressed in urothelial cells from IC specimens relative to normal tissues and BC tissues

model, together with an enhanced greater neuroprotective effect [41]. Based on these earlier findings, overexpression of *E2F-1* and *E2F-2* could promote inflammation associated with IC.

The tubby protein encoded by the *TUB* gene is a common upstream cell signalling protein in multicellular eukaryotes. The tubby protein acts as a signalling factor that potentially couples with G protein activity [42, 43].

Moreover, tubby proteins are involved in neuronal differentiation and development, whereas mutations in *tubby* genes in mammals are associated with delayed obesity, sensorineural hearing loss and retinal degeneration [43–45]. However, the functions of tubby proteins in humans are unclear and there is no report that they are involved in inflammatory diseases.

The expression changes in these three transcription factors and the networks induced by these changes may indicate that they play a significant role in IC onset and progression.

In conclusion, in this study we determined the miRNA expression signature in IC by RNA sequencing and successfully identified dysregulated expression of miRNAs. We found that all members of the *miR-320* family were downregulated in IC tissues and that *E2F-1*, *E2F-2*, and *TUB* were putative targets of regulation by these miRNAs. Immunohistochemistry showing increased levels of E2F-1, E2F-2, and TUB proteins in IC lesions support the sequencing results. In order to accurately understand the RNA network within the cell, it is ideal to use cells derived from the disease. From the latest genome technology, it is important that IC derived cell lines can be established and analyzed for its RNA networks. The miRNA signature of IC generated by RNA sequencing and in silico analyses could provide a basis to develop novel therapeutic targets for IC.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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