Differential distribution of U6 (*RNU6-1*) expression in human carcinoma tissues demonstrates the requirement for caution in the internal control gene selection for microRNA quantification

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Received March 15, 2015; Accepted August 24, 2015

DOI: 10.3892/ijmm.2015.2338

Abstract. Alterations in microRNA (miRNA) expression patterns have been associated with a number of human diseases. Accurate quantitation of miRNA levels is important for their use as biomarkers and in determining their functions. Although the issue of proper miRNA detection was solved with the introduction of standard reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays, numerous issues with the selection of appropriate internal control genes remain. U6 (*RNU6-1*) snRNA, the most commonly used internal control gene in miRNA RT-qPCR assays, was shown to be unstable in clinical samples, particularly cancer tissues. Identification of the distribution of U6 in different tissues is the premise of more accurate quantification of miRNAs. However, the distribution of U6 in human carcinoma tissues and corresponding normal tissues is unknown. In the present study, U6 levels were significantly higher in human breast carcinoma tissues compared with the corresponding normal tissues by RT-qPCR. In the carcinoma or corresponding adjacent normal tissues, the expression levels of U6 in epithelial cells were higher than those in the mesenchymal cells. Furthermore, the expression levels of U6 in the carcinoma tissues of the liver and intrahepatic bile ducts were higher than those in the adjacent normal tissues. These results suggest that the expression and distribution of U6 exhibits a high degree of variability among several types of human cells. Therefore, caution is required when selecting U6 as an internal control gene for evaluating expression profiles of miRNAs in patients with carcinoma, particularly carcinoma of the liver and intrahepatic bile ducts.

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Key words: microRNA, internal control gene, carcinoma, *in situ* hybridization, U6

Introduction

MicroRNAs (miRNAs) are 20-25 nucleotides in length, and are non-coding RNAs that incompletely bind to the 3' untranslated region (UTR) of multiple target mRNAs and thereby enhance their degradation and inhibit their translation. Increasing evidence indicates that miRNAs have critical roles in numerous human biological and pathological processes such as growth, apoptosis, development and tumorigenesis (1-5). miRNAs can regulate the expression of a variety of target genes and have been shown to function as tumor suppressors and oncogenes (6-8). In addition to their potential as novel molecules for cancer therapy (9), miRNAs also represent an emerging class of diagnostic and prognostic markers (10,11). Therefore, an accurate determination of miRNA expression levels is fundamental to the elucidation of their biological function.

Adaptation of existing technologies for profiling of miRNA expression includes reverse transcription-quantitative polymerase chain reaction (RT-qPCR), chip-based microarrays and next-generation sequencing. Among these technologies, RT-qPCR is widely used to quantify miRNA expression due to its sensitivity, specificity, speed, simplicity and the small quantities of template-RNA required. To correct for systematic variables, such as the quantity of starting template, RNA quality and enzymatic efficiency, RT-qPCR data are normalized against certain internal control genes that are ideally invariantly expressed across the test-sample set. The selection of a suitable internal control gene is an important first step in the accurate and reliable determination of miRNA expression levels. Although a consensus has not yet been reached on the optimal normalization strategy for miRNA in RT-qPCR studies, numerous RNA species, including rRNA (18S rRNA and 5S rRNA), snRNA (U6) and miRNAs (miR-191, miR-15a, miR-18a, let-7f and miR-16), have previously been used as internal control genes.

Numerous studies have proposed the use of U6 for normalization of tissue miRNAs, as U6 was shown to be consistently expressed in different tissues and cell types (12-15). However, it was recently suggested that U6 is unsuitable for normalization of tissue miRNA as the tissue levels of U6 exhibit high inter-individual variances and demonstrate instability in human lung, breast-tumor, liver and urothelial carcinoma, as well as in canine lymphoma (16-20). These published studies used RT-qPCR to compare the cycle-threshold (Ct) and applied numerous analytical tools, such as Normfinder, geNorm, ΔCt, stability index and Bestkeeper (21-24), to analyze the instability of U6 and identify the best internal control gene. These studies revealed the high variability of U6 expression. However, the reason for this variability remains to be elucidated. U6 snRNA is unique among the splicing snRNAs in that it is transcribed by RNA polymerase III (RNAP-III), and transcription by RNAP-III is strongly regulated, differing between diverse class III genes, among cell types. This finding led us to question whether there are U6 expression discrepancies in different cell types in human tissues and whether there are cellular composition changes between diseased and non-diseased tissues. In regards to these considerations, the present study analyzed the expression and maldistribution of U6 using miRNA *in situ* hybridization in human carcinoma tissues, and corresponding normal tissues, to explain the high variability of U6 obtained by RT-qPCR.

Materials and methods

Patients and pathology. All patients with cancer underwent curative resection of the primary tumor at the Second Affiliated Hospital of Harbin Medical University (Harbin, China) between April 2010 and 2014. No patients received neoadjuvant chemotherapy or radiotherapy prior to surgery. The following samples were included: 20 pairs of breast carcinoma (Table I), 5 of gastric carcinoma, 5 of colorectal carcinoma, 5 of esophageal squamous cell carcinoma, 5 of lung squamous cell carcinoma, 5 of hepatocellular carcinoma, 5 of intrahepatic cholangiocarcinoma and 5 of combined hepatocellular-cholangiocarcinoma (Table II). Resected carcinoma and corresponding normal tissues (>2 cm from carcinoma tissue) were immediately cut. Each tissue was divided into two sections. One section was frozen in liquid nitrogen and kept at -80˚C until RNA extraction. The other was cut into 5-mm slices, fixed in buffered 4% formaldehyde and embedded in paraffin. In addition, 4-μm histological sections were made and stained with hematoxylin and eosin. Histological type and grade were assessed by two pathologists (Yufei Jiao and Ge Lou) with considerable experience in clinical pathology, according to the World Health Organization criteria.

All the procedures were performed in accordance with the university's ethical standards and hospital criteria. All the participants provided informed consent.

miRNA isolation from tissue and RT-qPCR. RNA was extracted from tissues with TRIzol reagents (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Total RNA was reverse-transcribed to cDNA using a High-Capacity cDNA reverse transcription kit (Applied Biosystems, Beijing, China). RT-qPCR for U6 was performed using cDNA generated from 1 μg of total RNA using a SYBR-Green PCR Master mix (Applied Biosystems) according to the manufacturer's instructions. U6 amplification was performed using an RT-primer set: Forward, 5'-CGCTTC ACGAATTTGCGTGTCAT-3'; and a standard primer set: Forward, 5'-GCTTCGGCAGCACATATACTAAAAT-3' and reverse, 5'-CGCTTCACGAATTTGCGTGTCAT-3'. miR-16 was amplified using an RT-primer set: GTCGTATCCAGTGCA GGGTCCGAGGTATTCGCACTGGATACGACCGCCAA; and a standard primer set: Forward, 5'-CGCGCTAGCAGCACG TAAAT-3' and reverse, 5'-GTGCAGGGTCCGAGGT-3'. The reverse transcription reaction mixture (20 μl) was subjected to RT-qPCR analyses using a 7500 Real-Time PCR system (Applied Biosystems) according to the manufacturer's instructions. All the samples were performed in triplicate.

miRNA in situ hybridization (MISH)

Probe. Locked nucleic acid (LNA)-modified oligonucleotide probes for human, mature U6 and the scrambled negative control labeled with 5' end digoxigenin (DIG) were obtained from Exiqon (Vedbaek, Denmark). The sequences of the U6 probe and the scramble control probe were 5'-CACGAATTTGCGTGTCATCCTT-3' and 5'-GTGTAA CACGTCTATACGCCCA-3', respectively.

In situ hybridization. Sections (5-μm) from tissue blocks were deparaffinized, dehydrated and subsequently fixed in 10% neutral-buffered formalin for 10 min. Slides were subsequently immersed in acetylation solution for 10 min and incubated in proteinase K (20 μg/ml) for 5 min. After prehybridization at room temperature followed by incubation at 37˚C for 4-8 h, hybridization was performed at 37˚C overnight. On the following day the slides were washed in 5X standard saline citrate for 30 min at 37˚C, and subsequently, were washed twice for 30 min in 0.2X standard saline citrate. Following blocking in fetal bovine serum and hydrogen peroxide at room temperature for 2 h, the blocking buffer was replaced with blocking buffer containing anti-DIG-POD, Fab fragments from sheep (cat. no. 11 207 733 910; Roche Diagnostics GmbH, Mannheim, Germany). The slides were subsequently placed in a double-distilled H_2O box and incubated at 4˚C overnight.

Tyramide signal amplification (TSA) detection. Following *in situ* hybridization, excess antibody was removed in 0.1 M Tris-HCl (pH 7.5), 0.15 M NaCl and 0.05% Tween-20 (TNT) buffer three times for 15 min. The signal was amplified and visualized by tyramide signal amplification using the TSA-Plus Fluorescein System (Perkin Elmer, Waltham, MA, USA), according to the manufacturer's instructions. Amplification was performed in the dark for 7 min, with 100 μl/slide of TSA reagent diluted 1:50 with TSA diluents. Slides were washed in TNT buffer three times for 15 min. Slides were subsequently mounted in ProLong Gold with 4',6-diamidino-2-phenylindole and sealed with nail varnish.

Quantification of the U6 MISH signal. Images containing U6 fluorescence signals in the tissues were captured by an Olympus Bio Imaging Navigator (FSX100; Olympus, Tokyo, Japan). The MISH images were analyzed using ImageJ software (http://imagej.nih.gov/ij/).

Immunohistochemical staining (IHC). Formalin-fixed, paraffin-embedded tissue blocks of liver cancer were stained

Table I. Available clinical and pathological data of the breast carcinoma samples.

No. of patient	Patient age, years	Menopausal status	Size, mm	T	N	M	Grade	ER	PR	HER2/neu	Subtype
1	43	Pre	13	1	1	0	2	P	P	N	Luminal A
2	42	Pre	16	1	1	0	3	N	N	N	Basal
3	45	Pre	35	2	2	0	2	P	P	N	Luminal A
4	45	Pre	20	1	0	0	2	P	P	N	Luminal A
5	42	Pre	20	1	1	0	2	P	P	N	Luminal A
6	37	Pre	33	2	2	0	3	N	N	N	Basal
7	41	Pre	35	4	3	1	3	N	P	P	Luminal B
8	43	Pre	20	1	0	0	3	N	N	N	Basal
9	46	Pre	20	1	0	0	3	P	P	N	Luminal A
10	59	Post	20	1	0	0	3	N	N	N	Basal
11	49	Pre	30	2	1	0	2	P	P	N	Luminal A
12	35	Pre	20	1	1	0	1	P	P	P	Luminal B
13	50	Post	18	1	3	1	3	P	P	P	Luminal B
14	57	Post	16	1	1	0	3	N	N	P	HER-2
15	42	Pre	55	3	3	1	3	N	N	P	HER-2
16	40	Pre	20	1	1	0	2	P	P	N	Luminal A
17	61	Post	30	2	0	0	3	P	P	N	Luminal A
18	46	Pre	15	1	0	0	2	N	N	P	HER-2
19	71	Post	18	1	1	0	2	N	P	P	Luminal B
20	37	Pre	35	2	0	0	3	N	N	N	Basal

T, N and M refer to the primary tumor size, nodal status and distant metastases status according to the TNM breast cancer classification system. ER, estrogen receptor status; PR, progesterone receptor status; HER2/neu, c-erb-B2 human epidermal growth factor receptor 2. Subtype based on available hormone receptor status is provided.

Table II. Available clinical and pathological data of the carcinoma samples in the liver.

No. of patient	Patient age, years	Gender	Pathological type	Size, cm	Differentiation	Location	TNM stage	Viral infection	Liver cirrhosis
1	53	Female	Hepatocellular carcinoma	16	Moderate	Left lobe	IIIa	HBV	Y
2	44	Male	Hepatocellular carcinoma	20	Poor	Right lobe	IIIc	HBV+HCV	Y
3	55	Male	Hepatocellular carcinoma	4.8	Well	Left lobe	II	N	N
4	40	Male	Hepatocellular carcinoma	11	Well	Left lobe	IIIa	HBV	Y
5	33	Male	Hepatocellular carcinoma	10	Poor	Right lobe	IIIb	N	N
6	44	Female	Cholangiocarcinoma	18	Moderate-poor	Right lobe	IIIc	N	N
7	51	Female	Cholangiocarcinoma	8	Moderate	Right lobe	IIIb	N	N
8	44	Male	Cholangiocarcinoma	12	Moderate-poor	Right lobe	IIIb	HBV+HCV	Y
9	38	Male	Cholangiocarcinoma	9	Poor	Left lobe	IIIc	N	N
10	45	Male	Cholangiocarcinoma	8	Moderate	Left lobe	IIIa	HBV	Y
11	40	Male	Combined hepatocellular-cholangiocarcinoma	11	Moderate-poor	Right lobe	IIIc	HBV	Y
12	60	Female	Combined hepatocellular-cholangiocarcinoma	9	Moderate	Left lobe	IIIa	HBV	Y
13	39	Male	Combined hepatocellular-cholangiocarcinoma	18	Poor	Right lobe	IV	HCV	Y
14	35	Male	Combined hepatocellular-cholangiocarcinoma	12	Moderate-poor	Right lobe	IIIc	HBV+HCV	Y
15	47	Female	Combined hepatocellular-cholangiocarcinoma	10	Moderate	Left lobe	IIIa	HBV	Y

HBV, hepatitis B virus; HCV, hepatitis C virus.

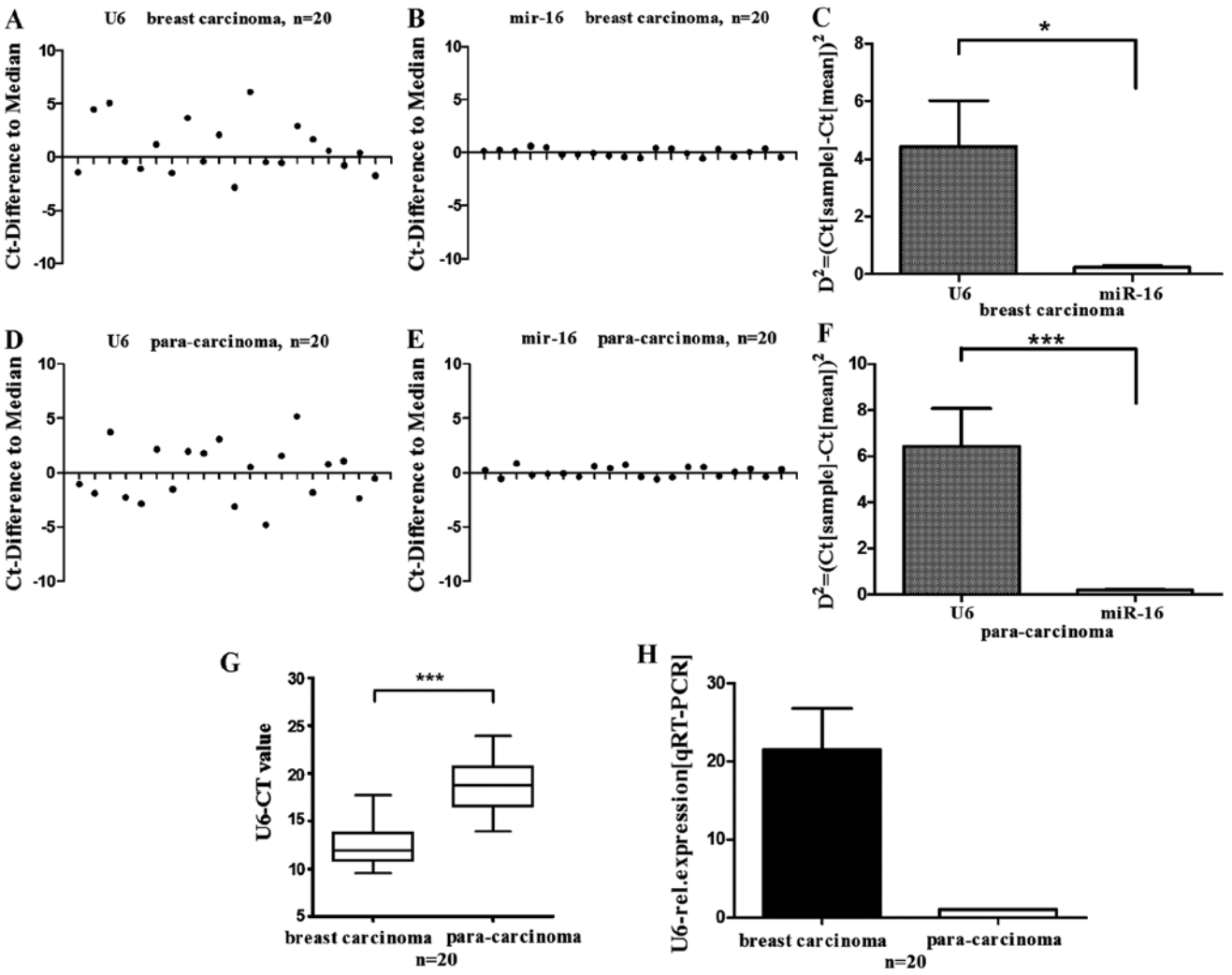

Figure 1. U6 levels exhibit high variability in human breast tissues. (A and D) U6 levels in tissue were analyzed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) in breast carcinoma tissues, and corresponding normal tissues, revealing an extremely high variability of U6 levels. (B and E) Levels of miR-16 were determined by RT-qPCR in the same samples and revealed an extremely low variability between the samples tested. (C and F) The variability of U6 and miR-16 values was statistically compared, revealing a significantly higher variability in U6 values compared with that of miR-16. (G) Raw U6 Ct values were compared between breast carcinoma and corresponding normal tissues, revealing significant differences between groups. (H) U6 tissue levels were compared between breast carcinoma and corresponding normal tissues, revealing significantly higher levels of U6 in the breast carcinoma group when data were normalized using miR-16. *P<0.05, **P<0.01 and ***P<0.001.

for hepatocyte and cytokeratin 19 (CK19) expression. Tissue sections (3-μm) were deparaffinized and hydrated following standard procedures. Following immersing in 3% hydrogen peroxide for 10 min to eliminate endogenous peroxidase, the sections were microwaved for antigen retrieval in 0.01 M sodium citrate for 15 min, followed by incubation with primary antibodies at 4˚C overnight. The sources of primary antibodies used for IHC were hepatocyte (MAB-0249) and CK19 (MAB-0056). The EliVision™ super detection kit (KIT-9921) (all from MaiXin-Bio, Fuzhou, China) was used as a secondary antibody. 3'3-Diaminobenzidine tetrahydrochloride was used as a chromogen. Hematoxylin was used to counterstain the sections.

Statistical analysis. Raw Ct values of U6 and miR-16 were shown as the difference to the median. To quantify the variability of U6 and miR-16 levels, 'mean of D^2-value' were used. Differences in the mean of these received D^2-values were analyzed with a t-test. The U6 Ct value differences discrepancies between two groups were compared using the Mann-Whitney U test. P<0.05 was considered to indicate a statistically significant difference.

Results

U6 levels exhibit high variability in human breast tissues. The variability of U6 levels in human breast tissues were examined and RT-qPCR analysis was performed on RNA extracts from tissue samples of breast carcinoma and corresponding normal tissues. Initial miRNA studies on breast tissues by Mattie *et al* (25) normalized miRNA expression to miR-16 and let-7, which were later shown to be stably expressed across malignant, benign and normal breast tissue by Davoren *et al* (17). Therefore, miR-16 was selected as a reference control gene. As shown in Fig. 1, in breast carcinoma tissues, U6 levels were significantly higher compared with corresponding normal tissues (Fig. 1H). Tissue levels of U6 exhibited high inter-individual variability in breast carcinoma

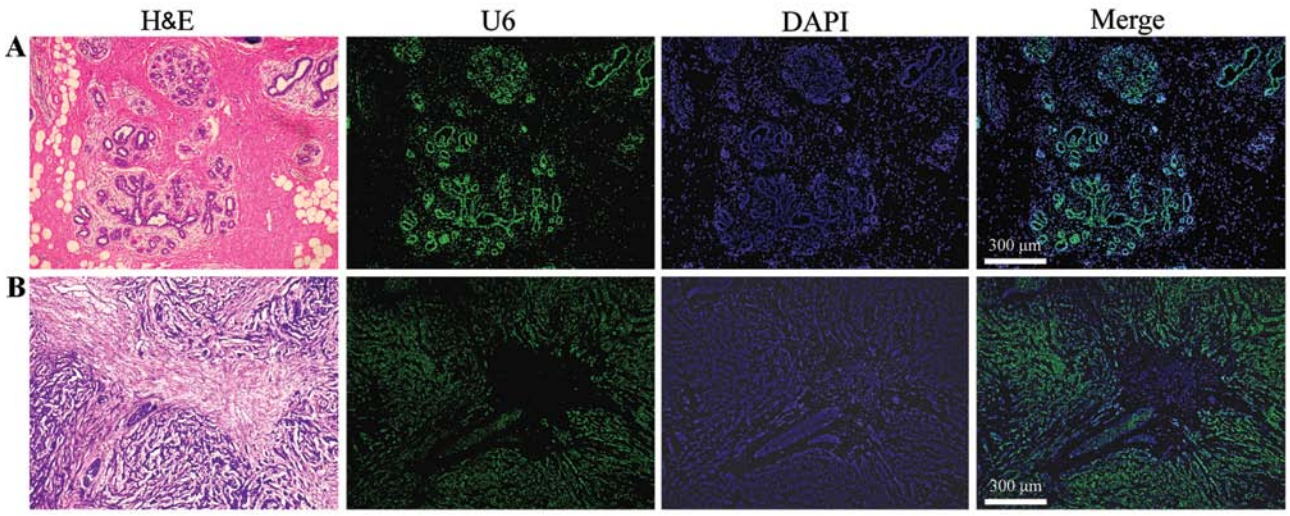

Figure 2. Detection of U6 expression in (B) human breast cancer tissues and (A) corresponding normal breast tissues by miRNA *in situ* hybridization. The breast tissue consists of an epithelial parenchyma and mesenchymal elements, which including varying quantities of fat, blood vessels, lymphatics and nerves. The epithelial component consists of ducts and acini, which together form the lobules that are the basic structural units of the mammary gland. The number of lobules varies in each female mammary gland. In the corresponding normal breast tissues, the fluorescence signals of the U6 probe (green) in normal mesenchymal cells were distinctly less and weaker than those observed in the normal epithelial cells. Breast cancer arises in the ductal and glandular structures of the breast. In human breast cancer tissues, the fluorescence signals of the U6 probe (green) in cancerous mesenchymal cells were clearly less and weaker than those in the cancerous epithelial cells. Original magnification, x42. H&E, hematoxylin and eosin stain; DAPI, 4',6-diamidino-2-phenylindole.

tissues and adjacent normal tissues, which was significantly higher compared with miR-16 (Fig. 1A-G). Consequently, if U6 is selected as an internal control gene for quantitative analysis of miRNA by RT-qPCR, the high level of U6 in human breast tissues may lead to a perceived variation of miRNA, and this misrepresentation of miRNA expression may obfuscate the understanding of miRNA function.

LNA-MISH provides specificity for the detection of U6 levels in human breast carcinoma and corresponding normal breast tissues. The RT-qPCR results were analyzed and interpreted by miRNA *in situ* hybridization. U6 *in situ* expression was detected in the aforementioned breast carcinoma tissues and corresponding normal tissues. Observations from Fig. 2 are summarized as follows. First, cancer is a type of malignant tumor characterized by the indefinite proliferation of epithelial cells, the epithelial:mesenchymal cell ratio in the cancerous tissues is much higher than that in the corresponding normal tissues. Second, mature U6 is present uniformly in the nucleus and in carcinoma tissues and corresponding normal tissues. The fluorescence signals of the U6 probe (green) in breast mesenchymal cells were distinctly less and weaker than in those of the epithelial cells. Therefore, the expression level of U6 in breast cancer epithelial cells was higher than that in the mesenchymal cells. The MISH experiments further confirmed the RT-qPCR results wherein U6 tissue levels in breast carcinoma were significantly higher compared to in the corresponding normal tissues.

Specificity of LNA-MISH for detecting U6 in other types of human cancer and corresponding normal tissues. In breast carcinoma tissues and corresponding normal tissues, the fluorescence signals of the U6 probe (green) in tumor mesenchymal cells were distinctly less and weaker than those observed in the epithelial cells. To confirm that the distribution of U6 is universal in various tumors, its expression was investigated in other types of carcinomas (gastric, colorectal, esophageal squamous cell and lung squamous cell carcinoma) with more or less of a mesenchymal element, and also revealed that the expression levels of U6 in cancer epithelial cells were higher than those in the mesenchymal cells (Fig. 3).

Specificity of LNA-MISH for detecting U6 in carcinoma tissues of the liver and intrahepatic bile ducts and adjacent normal tissues. The presence of U6 in carcinoma tissues of the liver, intrahepatic bile ducts and adjacent normal tissue is noteworthy. Expression levels of U6 in hepatoma cells and cholangiocarcinoma cells were higher compared to the adjacent normal hepatocytes, no matter which type of cancer was selected (Figs. 4-6). To identify and demonstrate the histological type of cancer, standard IHC was used to mark specific cell types. Antibodies of hepatocyte and CK19 (a marker of cholangiocytes) were used for the stain. The hepatocellular carcinoma was negative for the CK19 antibody; however, it demonstrated immunoreactivity to the hepatocyte antibody. The intrahepatic cholangiocarcinoma was negative for the hepatocyte antibody; however, it demonstrated immunoreactivity to the CK19 antibody. The combined hepatocellular-cholangiocarcinoma exhibited immunoreactivity to the hepatocyte and CK19 antibodies.

Discussion

The present study was designed to elucidate the critical issues associated with the use of U6 as an internal control gene in tissue miRNA quantization. More specifically, the study aimed to address why U6 is an unsuitable candidate for the normalization of tissue miRNA levels in patients with carcinoma. First, the expression levels of U6 were determined in human breast carcinoma and corresponding normal tissues by

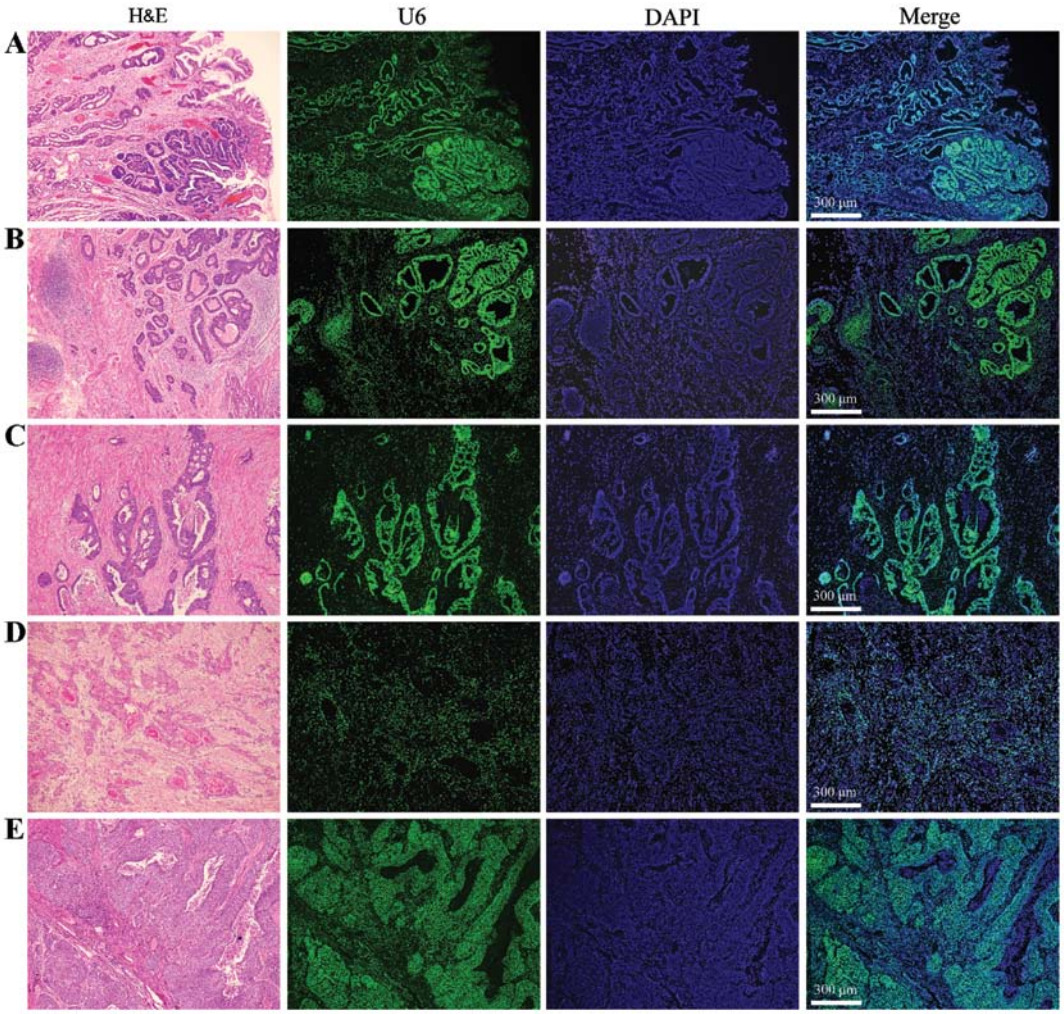

Figure 3. Detection of U6 expression in other types of human cancer and corresponding normal tissues by miRNA *in situ* hybridization. In the (A) gastric carcinoma and (B) adjacent normal tissues, the fluorescence signals of U6 probe (green color) in mesenchymal cells were clearly less and weaker than those observed in the epithelial cells. However, there was no significant difference in the fluorescence signals of U6 between normal and cancerous epithelial cells. In (C) colon, (D) esophageal squamous and (E) lung squamous cell carcinoma tissues, the fluorescence signals of the U6 probe (green) in the mesenchymal cells were evidently less and weaker than those observed in the cancerous epithelial cells. Original magnification, x42. H&E, hematoxylin and eosin stain; DAPI, 4',6-diamidino-2-phenylindole.

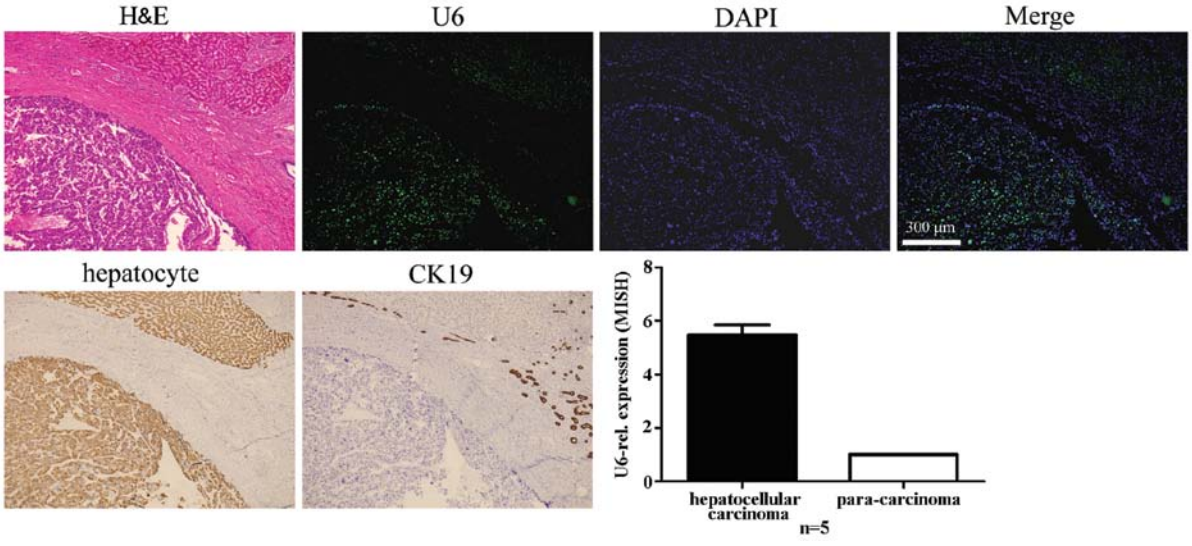

Figure 4. Detection of U6 expression in moderately differentiated hepatocellular carcinoma tissues (the lower left corner) and adjacent normal tissues (the top right corner) by miRNA *in situ* hybridization (MISH). The fluorescence signals of the U6 probe (green) in hepatoma cells were evidently more and stronger than those observed in the adjacent normal hepatocytes. The cancer cells are negative for the CK19 antibody, but exhibit immunoreactivity to the hepatocyte antibody. Original magnification, x42. H&E, hematoxylin and eosin stain; DAPI, 4',6-diamidino-2-phenylindole; CK19, cytokeratin 19.

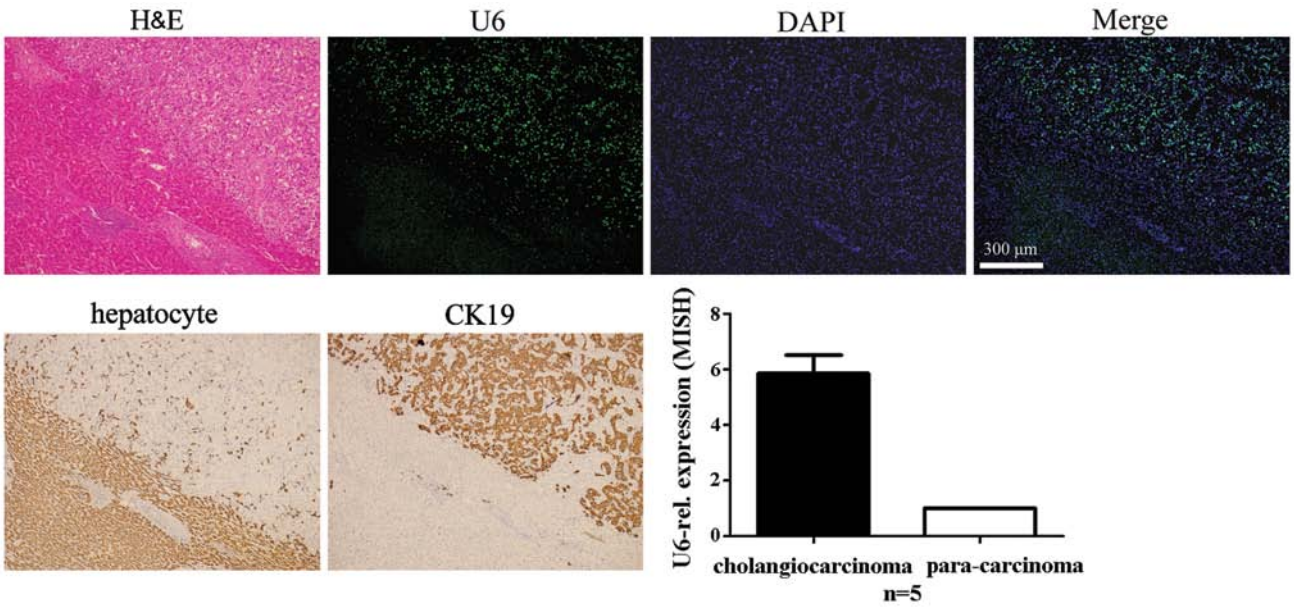

Figure 5. Detection of U6 expression in moderate to poorly differentiated intrahepatic cholangiocarcinoma tissues (the top right corner) and adjacent normal tissues (the lower left corner) by miRNA *in situ* hybridization (MISH). The fluorescence signals of the U6 probe (green) in cholangiocarcinoma cells were evidently more and stronger than those observed in the adjacent normal hepatocytes. The cancer cells are negative for hepatocyte antibody, but exhibit immunoreactivity to the CK19 antibody. Original magnification, x42. H&E, hematoxylin and eosin stain; DAPI, 4',6-diamidino-2-phenylindole; CK19, cytokeratin 19.

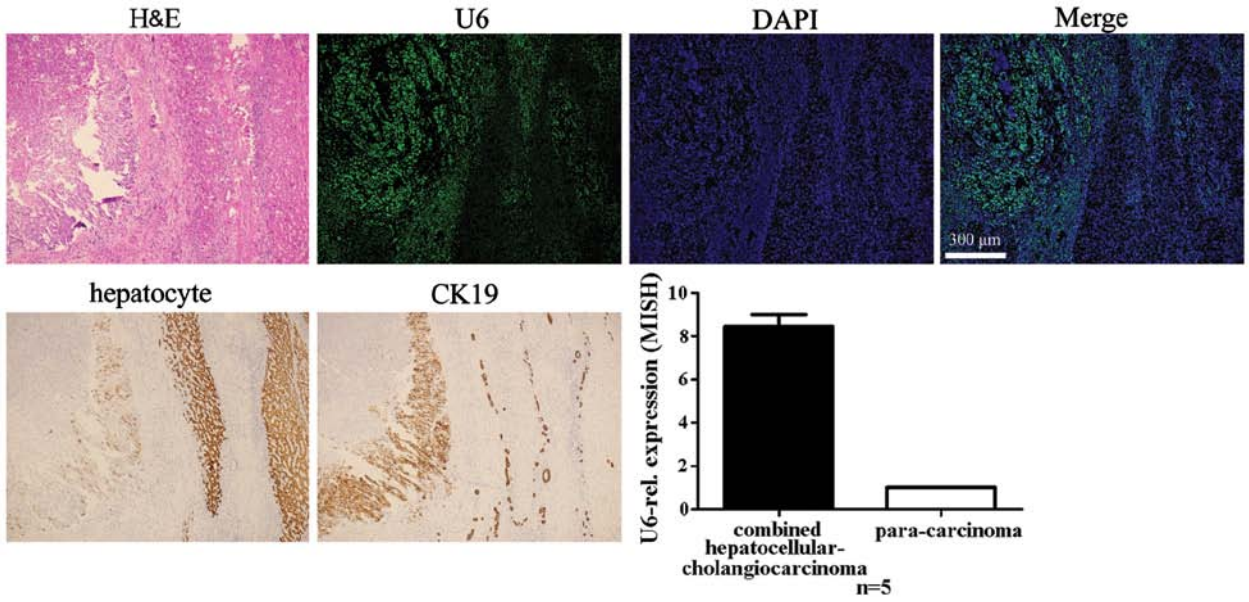

Figure 6. Detection of U6 expression in combined hepatocellular-cholangiocarcinoma tissues (the left portion) and adjacent normal tissues (the right portion) by miRNA *in situ* hybridization (MISH). The fluorescence signals of the U6 probe (green) in cancer cells were evidently more and stronger than those observed in the adjacent normal hepatocytes. The cancer cells exhibit immunoreactivity to the hepatocyte and CK19 antibodies. Original magnification, x42. H&E, hematoxylin and eosin stain; DAPI, 4',6-diamidino-2-phenylindole; CK19, cytokeratin 19.

RT-qPCR, and identified that U6 levels in breast carcinoma were significantly higher in comparison to those of corresponding normal tissues. Secondly, the study aimed to clarify the RT-qPCR ambiguities using miRNA *in situ* hybridization. All the neoplasms have a parenchyma and a stroma. The parenchyma comprises a neoplastic proliferation of cells. The stroma comprises the supporting connective tissue and blood supply that allows the neoplasms to grow. The stroma contains multiple cell types, including mixed inflammatory cells, endothelial cells, fibroblasts, smooth muscle cells and pericytes. The U6 *in situ* hybridization data shown here demonstrate that the expression level of U6 in cancer epithelial cells was higher than those in the mesenchymal cells in breast carcinoma and in other types of human carcinomas. The epithelial:mesenchymal cell ratio in the cancer tissues was much higher than that in the corresponding normal tissues. Therefore, U6 levels in carcinoma were significantly higher than those in the corresponding normal tissues.

In the aforementioned carcinoma tissues and adjacent normal tissues, the expression levels of U6 in epithelial cells

was higher than those in the mesenchymal cells; however, there was no significant difference in U6 fluorescence signals between normal and cancerous epithelial cells. Notably, the expression pattern of U6 in liver tissues is an exception. The expression level of U6 in hepatoma and cholangiocarcinoma cells was higher than that in the adjacent normal hepatocytes. Consequently, whether this abnormal transcription of U6 is associated with mechanisms of hepatocarcinogenesis and cholangiocarcinogenesis was questioned.

Preferably, a reliable internal control gene should exhibit invariant expression across all samples, regardless of disease status or other clinical variables. However, expression of U6 appears to vary for the following reasons: i) All tissues contain multiple cell types, each with their own unique miRNA and U6 expression patterns, and as noted in the study by Kent *et al* (26), miRNAs are expressed in cells and not expressed in tissues. Due to inherent cellular heterogeneity, the expression of U6 exhibited significant differences between epithelial cells and mesenchymal cells in human tissues. ii) In the transformation process from a normal to a carcinoma tissue, cellular composition (epithelial:mesenchymal cell ratio) changes substantially. iii) For each type of carcinoma, numerous subtypes are described, and the epithelial:mesenchymal cell ratio can vary greatly within these.

Peltier and Latham (16) supported the assertion that U6 is not suitable for use as an endogenous control for normalizing miRNA relative quantitation data in human lung, breast tumor, liver, urothelial carcinoma and canine lymphoma (17-20). Consequently, the use of U6 in this capacity could potentially lead to data-misinterpretation and erroneous conclusions. Therefore, we concluded that the root of the problem is the maldistribution of U6 snRNA among tissues, particularly the high expression observed in carcinoma tissues. The high variance in expression of U6 is a factor to be considered for quantification of miRNAs in all relevant studies.

U6 snRNA is one of 5 uridine-rich non-coding RNAs that forms the major spliceosome complex. As opposed to other U-snRNAs, U6 snRNA is transcribed by RNAP-III, and its maturation occurs exclusively in the nucleus. U6 snRNA has a central role in splicing, and thus its transcription, maturation, snRNP formation and recycling are essential for cellular homeostasis. The human U6 gene promoter and coding sequence contains a strong CpG island. Conservation and transcriptional activities of this gene are regulated by DNA methylation catalyzed by DNMT1 and DNMT3a (27). The human U6 gene promoter contains three regulatory elements: The distal enhancer-like sequence element, the proximal sequence element and the TATA box. Numerous proteins can bind to these regions to inhibit U6 gene transcription, such as p38 (28), BRCA1 (29), CK2 (30), RB (27) and Maf1 (31). Additionally, the majority of the signaling pathways activated in cancer, including Ras, Raf, PI3K, and AKT, enhance RNAP-III activity, while several tumor suppressors, including retinoblastoma, PTEN, p53 and BRCA1, decrease RNAP-III activity (32,33). The aforementioned factors may correlate with the inconsistency of U6 expression between epithelial and mesenchymal cells. Recent studies have identified a new U6 snRNA biogenesis factor, Usb1. Usb1 is an evolutionarily conserved exoribonuclease that is responsible for removing 3'-terminal uridines from U6 snRNA transcripts, which leads to the formation of a 2',3'-cyclic phosphate moiety. This maturation step is fundamental for U6 snRNP assembly and recycling (34,35). Due to the high expression of U6 in liver carcinomas, the association between Usb1 and liver carcinoma requires further research, which may provide novel insights into the processes of hepatocarcinogenesis and cholangiocarcinogenesis.

Acknowledgements

The present study was supported by the National Natural Science Foundation of China (grant no. 81270511); the Heilongjiang Postdoctoral Foundation (grant no. LBH-Z12172/LBH-TZ0415); the Research Fund for the Doctoral Program of Higher Education (grant no. 20122307110002); Yu Weihan Academician Fund for Distinguished Young Scholars of Harbin Medical University (2014) and the Science and Technology Project of Heilongjiang (grant no. 2013G1002). The authors would like to thank Mr. Dayong Wang (Department of Biochemistry and Molecular Biology, Harbin Medical University) and Mr. Kefei Wu (Department of Pathology, The Second Hospital of Harbin Medical University) for their excellent technical support and data analysis.

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