Quantitative analysis of *hTERT* mRNA levels in cells microdissected from cytological specimens

Hayato Fujita,[1] Kenoki Ohuchida,[1,2,6] Kazuhiro Mizumoto,[1,3,6] Takuya Egami,[1] Yoshihiro Miyasaka,[1,4] Hiroshi Yamaguchi,[4,5] Jun Yu,[1] Lin Cui,[1] Manabu Onimaru,[1] Shunichi Takahata,[1] Masazumi Tsuneyoshi[4] and Masao Tanaka[1]

[1]Department of Surgery and Oncology, and [2]Department of Advanced Medical Initiatives, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [3]Kyushu University Hospital Cancer Center, Fukuoka; [4]Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka; [5]Department of Pathology, Saitama Medical University International Medical Center, Saitama, Japan

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Clinicians frequently require cytopathological assessment of tumor samples for preoperative diagnosis, but in some specimens, diagnosis remains inconclusive after cytological examination. To date, several molecular markers, including human telomerase reverse transcriptase (hTERT), have been assessed for the ability to detect malignancy. However, analyses using whole cytological specimens are generally affected by contamination of untargeted cells. The present study investigated the feasibility of more sensitive examination by quantitative mRNA analysis of target cells microdissected from cytological specimens. Laser capture microdissection (LCM) was used to obtain target cells from cytological specimens. *hTERT* mRNA levels were then measured in target cells by quantitative real-time RT-PCR (qRT-PCR). The effect of RNA fragmentation on qRT-PCR was also assessed. Total RNA from cytological specimens was sometimes fragmented to a large degree. To avoid the effect of RNA fragmentation, gene specific priming and PCR primers generating short PCR products were used and no difference in delta Ct values between fragmented and non-fragmented RNA were found. *hTERT* mRNA levels were measured in cells microdissected from 33 cytological specimens. The levels of *hTERT* mRNA were significantly higher in malignant cases compared to those in non-malignant cases ($P = 0.0003$). The sensitivity was 96.2%, even when the specificities were 100%. High levels of *hTERT* mRNA were also found in three cases that were not diagnosed as malignant by cytological examination. Quantitative assessment of *hTERT* mRNA levels in cells microdissected from cytological specimens is a potential diagnostic tool to potentiate cytological examination in diagnosing malignancy. (*Cancer Sci* 2008; 99: 2244–2251)

Clinicians frequently need cytopathological assessment of various solid tumors for early diagnosis or to determine operative management. Fine-needle aspirations (FNA) of tumors in palpable organs, such as thyroid glands and breasts, have proven to be excellent indicators of malignancy.[1–3] Cytopathological assessment of pancreatic juice and urine is invaluable for early diagnosis or for screening of pancreatic and bladder neoplasms owing to the inaccessibility of these organs, despite improvements of diagnostic imaging.[4–6] Although cytopathological assessment of specimens is very useful when the verdict is clearly benign or malignant, there remains a substantial subset of cases for which diagnosis is inconclusive.[4–8] Other tools, such as molecular markers, are therefore needed to aid diagnosis in indeterminate cytological samples and to distinguish malignant from non-malignant cells.

Telomerase activity is a promising diagnostic marker for various tumors.[9] We, and other investigators, have reported that detection of telomerase activity in pancreatic juice or in urine is useful in the diagnosis of pancreatic cancer or bladder cancer, respectively.[10–13] In these studies, a modified PCR-based semiquantitative assay (conventional telomeric repeat amplification protocol [TRAP] assay) was used for analysis of telomerase activity. The sensitivity of these TRAP assays was 61.9–80%.[10–13] However, clinical introduction of this marker for cancer diagnosis is still problematic owing to difficulties in evaluating sample quality and quantitative measurement.

Human telomerase reverse transcriptase (hTERT) is one of the subunits of telomerase. We, and other investigators, have reported that quantification of *hTERT* mRNA is useful for cancer diagnosis in various organs, such as pancreas,[14] colon,[15,16] uterus,[17] thyroid,[18,19] breast,[16,20] stomach,[21] bile duct,[22] lung,[23] bladder[24] and prostate,[25] and that it eliminates the need for complicated procedures, such as the TRAP assay.[14,15,17,26] *hTERT* mRNA is observed at high levels in malignant tumors,[16] although *hTERT* mRNA can also be detected in some non-malignant cells such as germline cells, lymphocytes, hematopoietic progenitor cells and basal cells in the epidermis.[27–29] In general, cytological specimens contain various types of cells, such as blood, inflammatory and surrounding mesenchymal cells. The reliability of tests based on tissue or cell extracts is often crucially dependent on the relative abundance of the target cell population, and sampling errors or a large number of 'contaminating' cells can lead to false-negative results. This is especially true for the examination of early or precursor lesions, such as carcinoma *in situ* and epithelial dysplasias, which are of special interest, because they could be crucial stages of carcinogenesis. Therefore, we must exclude contamination of untargeted cells to enable more sensitive examination.

In the present study, we describe the use of laser capture microdissection (LCM), with direct microscopic visualization,[30] to obtain target cells from cytological specimens. Furthermore, using one-step quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR), with gene-specific priming, we measured levels of *hTERT* mRNA in microdissected target cells. *hTERT* mRNA levels were significantly increased in malignant cases, and could distinguish malignant from non-malignant cases in cytologically inconclusive cases. In addition, we assessed the implication of RNA degradation in clinical samples using a Bioanalyzer. Total RNA, extracted from cytological samples, was sometimes fragmented to a large degree. However, we could quantitatively measure *hTERT* mRNA levels, irrespective of RNA fragmentation, by using gene-specific reverse transcription (RT) and PCR primers that generate short PCR products. Diagnosis by cytopathological assessment and by *hTERT* expression in cells microdissected from cytological samples is a potent combination to distinguish malignant from non-malignant cells.

[6]To whom correspondence should be addressed.
E-mail: kenoki@med.kyushu-u.ac.jp or mizumoto@med.kyushu-u.ac.jp
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Table 1. Details of cytological specimens

Sample No.	Origin	Clinical diagnosis	Class	Cytological diagnosis	Histological diagnosis
Pancreas					
P-1	PJ	IPMN	II	Hyperplastic ductal cell clusters	No surgery
P-2	PJ	IPMN	III	Atypical ductal cell clusters	No surgery
P-3	PJ	Pancreatic tumor	III	Atypical ductal cell clusters	Unresectable
P-4	PJ	IPMN	III	IPMT with atypical cells	IDC, cystic type
P-5	PJ	Pancreatic cancer	IV	Adenocarcinoma suspected	IDC, mixed type
P-6	Ascites	Pancreatic cancer	V	Adenocarcinoma	No surgery
P-7	Ascites	Pancreatic cancer	V	Adenocarcinoma	No surgery
Breast gland					
B-1	FNA	Intraductal papilloma	II	Papillary lesion, intraductal papilloma	No surgery
B-2	FNA	Breast tumor	II	Papillary apocrine metaplasia	Mastopathy
B-3	FNA	Breast tumor	III	Papillary lesion	No surgery
B-4	FNA	Breast tumor	III	Atypical ductal cell clusters	No surgery
B-5	FNA	Breast cancer	III	Papillary lesion, intraductal papilloma	IDC
B-6	FNA	Breast cancer	V	Solid-tubular carcinoma	IDC
B-7	FNA	Breast cancer	V	Papillo-tubular carcinoma with scirrhous carcinoma	IDC
B-8	FNA	Breast cancer	V	Solid-tubular carcinoma	IDC
B-9	FNA	Breast cancer	V	Papillo-tubular carcinoma	IDC
B-10	Ascites	Breast cancer	V	Por	Metastatic breast carcinoma
B-11	PCE	Breast cancer	V	Adenocarcinoma	IDC
Others					
S-1	Ascites	Gastric cancer	V	Por with sig	Por
S-2	PE	Gastric cancer	V	Por with sig	No surgery
C-1	Bile	Bile duct cancer	III	Atypical columrar epithelium	Adenocarcinoma
C-2	Bile	Bile duct cancer	IV	Adenocarcinoma suspected	Well to por
L-1	BAL	Lung cancer	V	Mod	No surgery
L-2	PCE	Lung cancer	V	Adenocarcinoma, papillary	No surgery
L-3	Stump	Lung cancer	V	Well to mod	Mod
L-4	Stump	Lung cancer	V	Well to mod	Mod
L-5	Stump	Lung cancer	V	Por	Por
M-1	FNA	Mediastinal tumor	V	Germinoma suspected	Germ cell tumor suggestive of seminoma
G-1	Ascites	Ovarian tumor; MCT	IV	Mucinous carcinoma suspected	Mucinous cystic tumor of borderline malignancy
G-2	PE	Ovarian tumor	V	Adenocarcinoma	Serous adenocarcinoma
G-3	Ascites	Uterine body cancer	V	Por to mod	Adenocarcinoma
N-1	FNA	Neck tumor	V	Moderately to poorly differentiated SCC	Moderately to poorly differentiated SCC
N-2	FNA	Thyroiditis	V	Por	No surgery

BAL: bronchoalveolar lavage, FNA: fine-needle aspiration cytology, IDC: invasive ductal carcinoma, IPMN: intraductal papillary-mucinous neoplasm, MCT: mucinous cystic tumor, Mod: moderately differentiated adenocarcinoma, PCE: pericardial effusion, PE: pleural effusion, PJ: pancreatic juice, Por: poorly differentiated adenocarcinoma, SCC: squamous cell carcinoma, sig: signet-ring cell, Stump: stump cytology, Well: well differentiated adenocarcinoma.

Materials and Methods

Pancreatic cancer cell line. Pancreatic cancer cell line, SUIT-2 (provided by Dr H. Iguchi, National Shikoku Cancer Center, Matsuyama, Japan) was used. Cells were maintained as described previously.[31] Cell pellets were smeared on membrane slides (P.A.L.M. Microlaser Technologies, Bernried, Germany) for laser capture microdissection (LCM). These smears were dried and fixed in 100% methanol for 3 min. After fixation, smears were washed in diethylpyrocarbonate (DEPC)-treated water, and stained by four different methods, including Toluidine blue staining, Hemacolor rapid blood smear staining (simplified May–Giemsa staining, Merck KGaA, Darmstadt, Germany), standard May–Giemsa staining, and Papanicolaou's staining.[32]

Cytological specimens. Cytological specimens were obtained at the time of cytological examination and diagnosis from the pathological laboratory of Kyushu University Hospital (Fukuoka, Japan). In brief, cytological specimens were divided into three or more samples and smeared as soon as possible after retrieval. Smears were processed in three different ways. First, smears were mounted on standard glass slides for May–Giemsa staining, and then used for rapid cytological diagnosis. Second, smears were mounted on standard glass slides for Papanicolaou's staining and used for strict cytological diagnosis. The first and second smears were examined histologically by cytopathologists and diagnosis was confirmed according to Papanicolaou's classification.[32] Third, smears were mounted on membrane slides for LCM. These smears were dried and fixed in 100% methanol for 3 min. After fixation, smears were washed in DEPC-treated water and stained in 1% Toluidine blue staining solution or by Hemacolor staining. Thirty-three cytological specimens were obtained from various tumors from patients at the Kyushu University Hospital (Fukuoka, Japan), full details of which are described in Table 1. Nine breast FNA and three other FNA, including neck, thyroid gland and anterior mediastinal tumor, were obtained. Six ascites, two pleural effusions and two pericardial effusions were obtained by aspiration. Five pancreatic juice samples and two brushing cytology samples of bile duct were collected, as described previously,[10,11] from patients who underwent endoscopic retrograde cholangiopancreatography (ERCP) for suspected malignancy of the pancreas

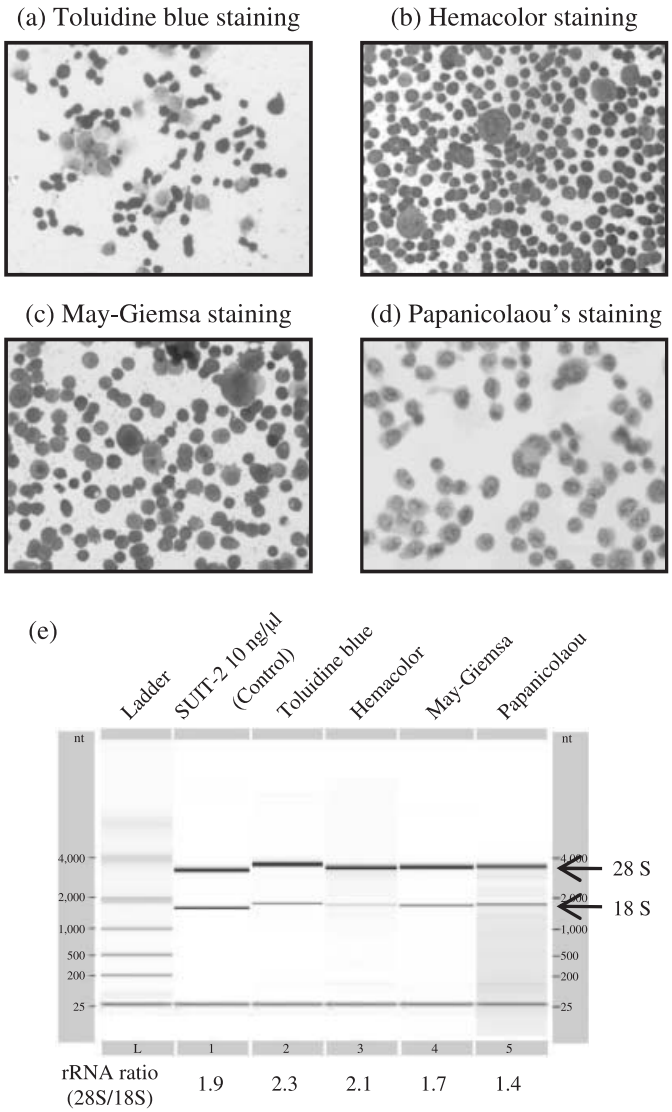

Fig. 1. Microphotographs of smears of SUIT-2 cells stained with Toluidine blue (a), Hemacolor (b), May–Giemsa (c), and Papanicolaou's staining (d). (e) Electrophoresis-like images of RNA extracted from microdissected SUIT-2 cells stained with the four different methods. 28S/18S rRNA ratios were maintained with three staining methods but not with Papanicolaou's staining.

or the bile duct. Three intraoperative stump cytology samples of lung tumor were obtained. One bronchoalveolar lavage (BAL) cytology sample was obtained. Written, informed consent was obtained from all patients, and the study was approved by our institution's surveillance committee and conducted according to the Helsinki Declaration.

RNA extraction. Total RNA was extracted from cultured cells with the High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany), according to the manufacturer's protocol, and with the standard acid guanidinium thiocyanate–phenol–chloroform (AGPC) protocol,[33] with or without glycogen (Funakoshi, Tokyo, Japan). Total RNA was extracted from cells isolated by microdissection using two methods: first, the AGPC protocol, and secondly, the PicoPure RNA isolation kit (Arcturus Bioscience, CA, US), according to the manufacturer's protocol.

Assessment of RNA concentration and RNA purity using the NanoDrop ND-1000 Spectrophotometer. Concentration of extracted total RNA was measured using the NanoDrop ND-1000 Spectrophotometer (NanoDrop technologies, DE, US), following the manufacturer's protocol. Purity of extracted RNA was evaluated by determining the ratio of sample absorbance at 260 and 280 nm (260/280 ratio).

RNA integrity assessment using the Agilent 2100 Bioanalyzer. Microchip electrophoresis was performed on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany). A RNA Pico Assay Kit was purchased from Agilent Technologies. As shown in Fig. 1(e), each total RNA extracted from microdissected cells was analyzed with RNA Pico Laboratory Chips (Agilent Technologies), according to the manufacturer's protocol.[34]

Quantitative analysis of *hTERT* mRNA by one-step real-time reverse transcription-polymerase chain reaction with gene-specific priming. We used one-step quantitative real-time RT-PCR (qRT-PCR), with gene-specific priming, to examine mRNA levels in various types of clinical sample that contained weakly or extensively fragmented RNA. A major advantage of this technology is its ability to reliably measure gene expression from fragmented RNA by synthesizing short amplicons of cDNA using gene-specific primers.[34–37] We designed specific primers (Table 2) and used BLASTN to confirm primer specificity. To confirm that the detected signal intensity was specific to the expected PCR product, we performed RT-PCR with each primer pair, with or without reverse transcription (RT). One-step qRT-PCR was performed with a QuantiTect SYBR Green RT-PCR Kit (Qiagen) incorporating a Chromo4 System (BIO-LAD, CA, US), as described previously.[14] In brief, the reaction mixture was first incubated at 50°C for 15 min to allow reverse transcription. PCR was initiated with one cycle of 95°C for 10 min, to activate modified *Taq* polymerase, followed by 45 cycles of 94°C for 15 s, 55°C for 20 s, and 72°C for 10 s and one cycle of 95°C for 0 s, 65°C for 15 s, and +0.1°C/s to 95°C for melting analysis to visualize non-specific PCR products. Each primer set used in the present study produced a single melting peak. Each sample was run in triplicate. The 10% deviation was calculated from the concentrations determined from the calibration curve. The level of *hTERT* mRNA was calculated from a standard curve constructed with total RNA from the SUIT-2 pancreatic cancer cell line. The quantitative range of threshold cycles observed was 20–35 cycles for *hTERT* primers,[26] 5–30 cycles for *β-actin* primers.[38] *hTERT* mRNA was normalized to that of *β-actin* mRNA. Threshold cycle values (Ct values) of six genes, including *hTERT*, *β-actin*, *18S rRNA*, *GAPDH*, *Ubiquitin C* and *β-2-microgloburin* (*B2M*) were analyzed to assess the influence of RNA fragmentation. Moreover, to confirm the reliability of analysis, we evaluated △Ct values ($\triangle Ct = Ct_{hTERT} - Ct_{reference\ gene}$).[34]

Microdissection-based quantitative analysis of *hTERT* mRNA. Neoplastic cells from 33 cytological specimens were selectively isolated with a laser microdissection and pressure catapulting system (P.A.L.M. Microlaser Technologies, Bernried, Germany), in accordance with the manufacturer's protocols. We microdissected 50–1000 target cells to perform reliable and reproducible measurements of *hTERT* mRNA levels. We microdissected most atypical cell clusters, which were determined by cytopathologists. In specimens without neoplastic cells (<Class III), we microdissected regenerative epithelial cell clusters to compare with specimens with neoplastic cells. We excluded specimens without epithelial cells. After microdissection, total RNA was extracted from the isolated cells and subjected to one-step real-time RT-PCR with gene-specific priming for quantitative measurement of *hTERT*, as described previously.[30]

Statistical analysis. Data were analyzed with the Kruskal–Wallis test for comparison of four groups and Mann–Whitney *U*-test for comparison of two groups because normal distribution was not obtained after logarithmic transformation. Statistical significance was defined as $P < 0.05$. Because we performed multiple comparisons of our real-time RT-PCR data, we conservatively used Bonferroni correction, and therefore, the adjusted

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Table 2. Primer sequences and product size

Primer	Forward Sequence 5′-3′	Reverse Sequence 5′-3′	Product size
hTERT	gcggaagacagtggtgaact	agctggagtagtcgctctgc	147
β-actin	aaatctggcaccacaccttc	ggggtgttgaaggtctcaaa	139
18S rRNA	gtaacccgttgaaccccatt	ccatccaatcggtagtagcg	151
GAPDH	caatgacccccttcattgacc	gatctcgctcctggaagatg	118
Ubiquitin C	atttgggtcgcggttcttg	tgccttgacattctcgatggt	133
B2M	tgctgtctccatgtttgatgtatct	tctctgctccccacctctaagt	87

significance level was $P < 0.017$. The optimal cut-off points for each marker for discriminating between malignant and non-malignant cells were sought by constructing receiver operating characteristic (ROC) curves, which were generated by calculating the sensitivities and specificities of a marker at several predetermined cut-off points.[39]

Results

Evaluation of staining methods with respect to RNA integrity. Although May–Giemsa staining and Papanicolaou's staining are usually used for cytological examination, the effect of these staining methods on RNA integrity was not clear. Therefore, we evaluated the effect of several staining methods on RNA integrity to select an appropriate staining method for RNA extraction from cytological specimens. As shown in Figure 1a–d, we stained smears of SUIT-2 cells with four staining methods, Toluidine blue staining, usually used for microdissection, Hemacolor staining (simplified May–Giemsa staining), standard May–Giemsa staining and Papanicolaou's staining. We microdissected 1000 SUIT-2 cells from smears stained with each method, and extracted total RNA according to the AGPC protocol. 28S/18S rRNA ratios of each total RNA were evaluated. The results are displayed as electrophoresis images (Fig. 1e). The first lane shows the control RNA with high integrity, extracted from cultured SUIT-2 cells without staining. 28S/18S rRNA ratios were satisfactorily maintained with 3 staining methods, but not with Papanicolaou's staining. Generally, Toluidine blue and Hemacolor staining, including preparation of smears and fixation, takes 10 or a few minutes, respectively, whereas Papanicolaou's staining takes about 1.5 h.[32] Therefore, in the following experiments, we used two staining methods, Hemacolor staining and Toluidine blue staining.

Microdissection from cytological specimens. To pick up only target cells and to exclude contamination of untargeted cells, we used LCM. Figure 2a shows representative microphotographs of microdissected intraductal papillary–mucinous neoplasm (IPMN) cells from a pancreatic juice specimen diagnosed as Class III by cytopathologists (P-2, Toluidine blue staining). There are two clusters of IPMN cells. The atypical grade of the upper cluster was Class III and that of the lower cluster was Class II. We microdissected each cluster and measured *hTERT* mRNA levels in each cluster separately. Figure 2b shows microphotographs of metaplastic cells microdissected from an FNA specimen of a patient with breast tumor (B-2, Hemacolor staining).

Evaluation of concentration, purity, and integrity of total RNA extracted from cytological specimens. Generally, cytological specimens contain small amounts of target cells. Therefore, a sensitive method is required to extract total RNA in sufficient concentration and quality to enable quantitative measurement of target mRNA. We used two methods to extract and evaluate total RNA from microdissected target cells, an AGPC protocol and the PicoPure RNA isolation kit. Concentration of total RNA extracted with the AGPC protocol ($n = 20$) was significantly higher compared to that extracted with the PicoPure RNA isolation kit ($n = 26$) ($P < 0.0001$, Fig. 2c). There were no significant differences in 260/280 ratios between the two methods ($P = 0.6$, Fig. 2d). These data suggest that the AGPC protocol was well-suited to extract total RNA from cells microdissected from cytological specimens. However, the RNA was sometimes fragmented to a large degree in both methods. As shown in Figure 2e, three of six total RNA samples extracted from cytological specimens maintained 28S/18S rRNA ratios (lanes 2, 3, and 4), but three other samples showed fragmentation of RNA.

Effect of RNA fragmentation on quantitative analysis of *hTERT* mRNA. Our data above suggest that total RNA extracted from cells microdissected from cytological specimens does not always maintain its integrity. Therefore, to avoid the effect of RNA fragmentation on qRT-PCR, we used one-step methods with gene-specific RT primers and PCR primers to produce short amplicons (Table 2). Total RNAs with high integrity extracted from cultured SUIT-2 cells were fragmented by incubation in a 37°C water bath from 0 to 120 h. Integrities of nine RNA samples were analyzed. RNA samples were gradually fragmented as incubation time passed (Fig. 3a). We then analyzed levels of *hTERT*, *β-actin*, *18S rRNA*, *GAPDH*, *Ubiquitin C* and *B2M* mRNA by qRT-PCR. We evaluated Ct values of each mRNA to investigate the effect of RNA fragmentation (Fig. 3b). Degradation of RNA results in a loss of intact amplicons in the RNA, thereby leading to an increase in Ct values.[34] Ct values of each mRNA slightly increased as RNA became fragmented. However, $\triangle$Ct values remained rather constant, even at advanced fragmentation of the RNA (Fig. 3c). These data suggest that mRNA is quantifiable by qRT-PCR using gene specific RT primers and PCR primers to produce short amplicons, even if the RNA samples are fragmented.

Quantitative analyses of *hTERT* expression in cells microdissected from cytological specimens. We measured *hTERT* expression in 33 samples microdissected from cytological specimens that were obtained from patients with various tumors, the details of which are described in Table 1. The samples were classified according to the final diagnosis based on histological diagnosis of resected tumors and/or by clinical observation (Fig. 4a). G-1 was excluded because this patient was subsequently diagnosed as mucinous cystic tumor of borderline malignancy after surgical resection. A significant difference in *hTERT* expression was observed between carcinoma ($n = 26$) and non-malignant disease ($n = 6$) ($P = 0.0003$). The median value of *hTERT* expression in non-malignant diseases was 0.5, whereas the median value of *hTERT* expression in carcinoma was 6.3, which was approximately 12-fold greater compared with that in non-malignant disease samples. The sensitivity of measurement of *hTERT* expression was 96.2% and the specificities were 100%, whereas the sensitivity of cytological examination was 84.6% with 100% specificity when cytological classification of Class IV or V was defined as positive for malignancy. According to ROC curve analysis, the sensitivity of measurement of *hTERT* expression was determined at several specificity levels. The areas under the ROC curve of measurement of *hTERT* expression and cytological examination were

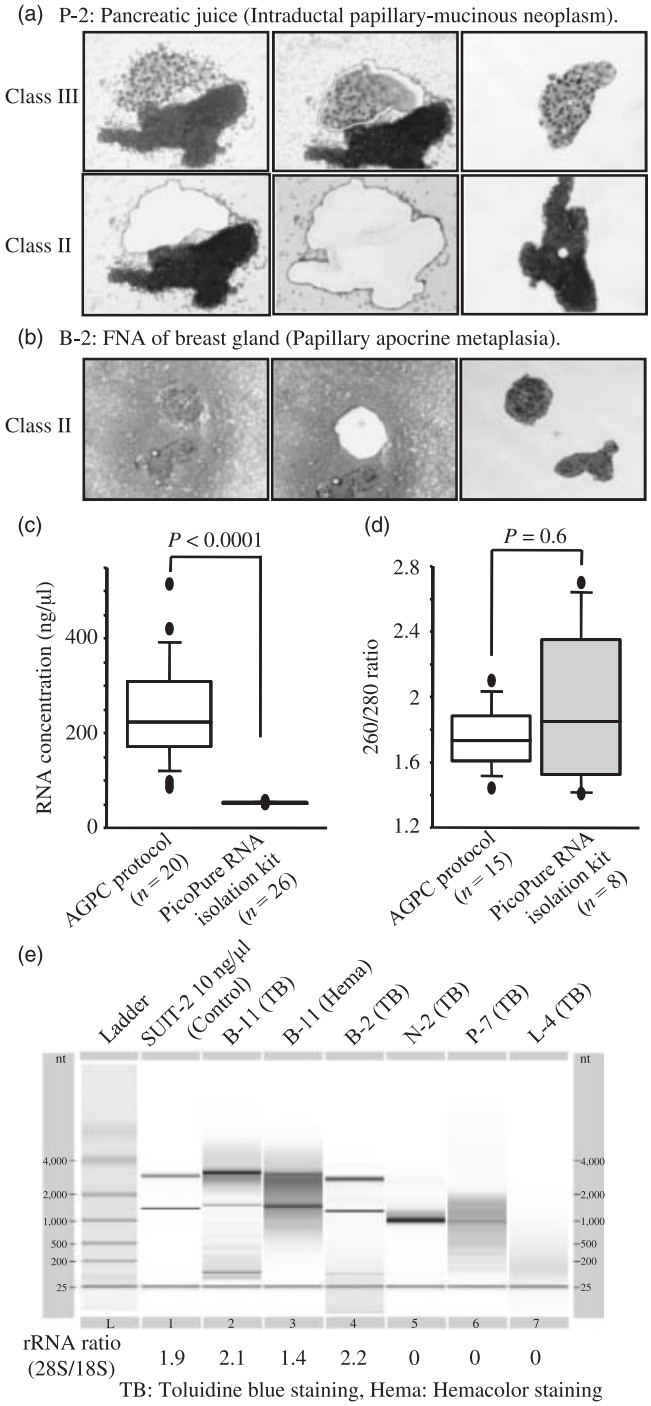

Fig. 2. (a) Representative microphotographs of microdissected intraductal papillary–mucinous neoplasm (IPMN) cells from a pancreatic juice specimen diagnosed as Class III by cytopathologists. (P-2, Toluidine blue staining). There are two clusters of IPMN cells. The upper cluster was formed with atypical Class III cells, and the lower cluster was formed with Class II cells. We could microdissect each cell cluster separately, and could measure respective *hTERT* mRNA levels. (b) Microphotographs of metaplastic cells microdissected from an FNA specimen of a patient with breast tumor (B-2, Hemacolor staining). (c) Concentration of total RNA extracted with the AGPC protocol (n = 20) was significantly higher compared to that extracted with the PicoPure RNA isolation kit (n = 26) (P < 0.0001). (d) There were no significant differences in 260/280 ratios between two methods (P = 0.6). (e), Electrophoresis-like images of six RNA extracted from cytological specimens (TB: Toluidine blue staining, Hema: Hemacolor staining). RNA from three samples (lane-5, 6 and 7) were fragmented, suggesting that total RNA extracted from microdissected cytological specimens do not always maintain their integrity.

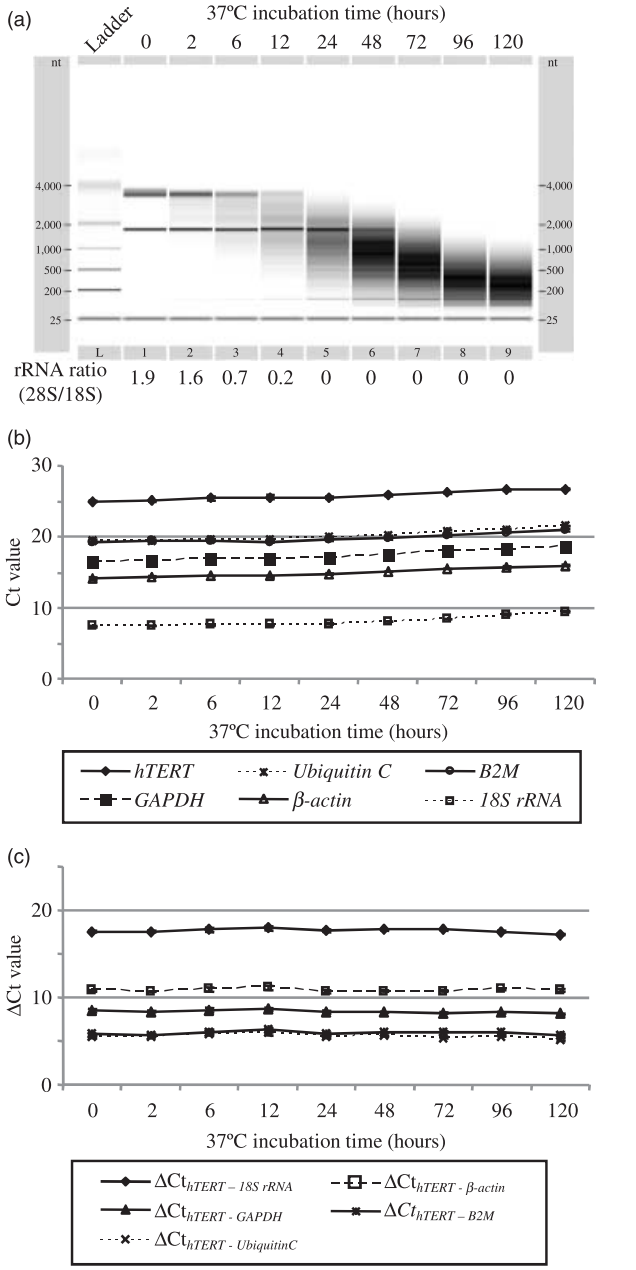

Fig. 3. (a) Electrophoresis-like images of experimentally fragmented RNA. Fragmentation of RNA samples gradually increased with incubation time. (b) The threshold cycle value (Ct value) of each mRNA slightly increased as they were fragmented. (c) △Ct values remained rather constant even at advanced fragmentation of the RNA.

0.98 and 0.96 for non-malignant cells *versus* malignant cells (95% confidence interval [95% CI], 0.86–0.99 and 0.83–1.00), respectively.

According to cytopathological diagnosis, confirmed by cytopathologists, three samples were Class II, seven samples were Class III, three samples were Class IV and 20 samples were Class V. The median values were 0.8 for Class II samples, 0.9 for Class III samples, 5.9 for Class IV samples and 7.5 for Class V samples. As shown in Figure 4b, a significant difference was found between Class III and Class V samples (P = 0.04) but not between Class III and Class IV samples (P = 0.2). The level of *hTERT* expression in Class III showed a wide range, possibly owing to three high *hTERT*-expressing samples, which were

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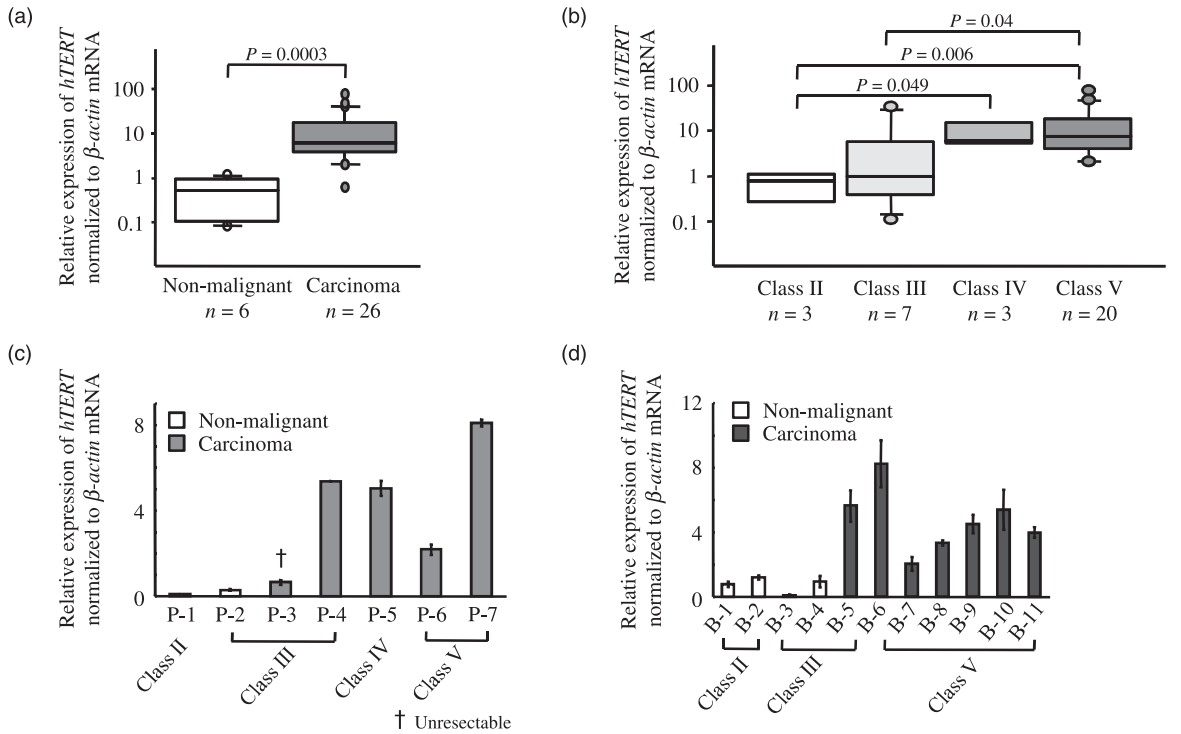

Fig. 4. (a) According to classification by the final diagnosis, there was a significant difference in *hTERT* expression in cells microdissected from cytological specimens between carcinoma ($n = 26$) and non-malignant cases ($n = 6$) ($P = 0.0003$). The median value of *hTERT* expression in non-malignant diseases was 0.5; whereas the median value of *hTERT* expression in carcinoma was 6.3. (b) Quantitative analysis of *hTERT* mRNA levels in cells microdissected from cytological specimens; Class II, $n = 3$, Class III, $n = 7$, Class IV, $n = 3$, and Class V, $n = 20$. The median values of *hTERT* in Class II, Class III, Class IV, and Class V specimens were 0.8, 0.9, 5.9, and 7.5, respectively. There were significant differences between Class V and Class III ($P = 0.04$) or Class II ($P = 0.006$) specimens and between Class IV and Class II specimens ($P = 0.049$). (c) Quantitative analysis of *hTERT* mRNA in cells microdissected from five pancreatic juice and two ascites samples with final diagnosis of pancreatic cancer (†: unresectable case). Although P-4 and P-5 samples were difficult to verify morphologically as Class V, they expressed higher levels of *hTERT* compared to those of two non-malignant samples (P-1, P-2). (d) Quantitative analysis of *hTERT* mRNA in cells microdissected from nine samples of breast FNA, one ascites and one pericardial effusion obtained from patients with breast carcinoma. One (B-5) of the Class III samples expressed higher levels of *hTERT* compared to those of four non-malignant samples (B-1, B-2, B-3, and B-4), and was subsequently diagnosed as invasive ductal carcinoma after surgical resection.

subsequently diagnosed as carcinoma by histological examination of surgically resected tumors (P-4, B-5, and C-1).

In pancreatic juice samples (Fig. 4c), although P-4 and P-5 samples seemed to be difficult to conclude morphologically as Class V, they expressed higher levels of *hTERT* compared with those of two non-malignant samples (P-1, P-2). Patient P-3 was clinically diagnosed as unresectable pancreatic cancer associated with severe stenosis of the main pancreatic duct. After sufficient follow-up periods, we confirmed the diagnosis. This is the only case with false-negative results of *hTERT* measurement. In this case, we were unable to brush the target lesion at the stenotic site to obtain a cytological specimen, possibly leading to failure of detection of *hTERT* expression.

In breast cancer-related specimens (Fig. 4d), one of the Class III samples (B-5) expressed a higher level of *hTERT* mRNA compared to those of four non-malignant samples (B-1, B-2, B-3, and B-4) and was diagnosed as invasive ductal carcinoma by pathologists after surgical resection.

Discussion

Although cytopathological assessment is very useful, the sensitivity of cytological diagnosis is insufficient (22.0–60.0%).[4–6,14] To distinguish malignancy from non-malignancy, various molecular approaches, such as K-*ras* mutations,[12,40,41] TRAP assay,[10–13] and RT-PCR,[14,24,42] have been applied to cytological samples. However, these approaches have not been widely used in the clinic because of the inability to evaluate sample quality. We, and other investigators, previously reported that the sensitivity of *hTERT* measurement in whole cytological samples was 43.5–86.2%, whereas the specificity was 61.5–100%.[14,24,42] We also suggested that the reliability of tests using whole cytological specimens was probably reduced owing to contamination with untargeted cells.[14] In the present study, we used microdissection to isolate target cells only and then used qRT-PCR with gene-specific priming and PCR primers producing short amplicons to perform reliable and sensitive measurement of *hTERT* mRNA. The sensitivity was 96.2%, even if the cut-off value was set to keep 100% specificity.

Notably, we found high levels of *hTERT* expression in cells microdissected from three samples that were cytologically diagnosed as Class III, but subsequently diagnosed as carcinoma by pathological examination of surgically resected tissues (P-4, B-5, and C-1). These observations suggest that our measurement of *hTERT* mRNA may potentiate cytological examination. The combination of cytological examination with the measurement of *hTERT* expression from microdissected cells may provide a significant advantage in discriminating malignant from non-malignant cells.

Several studies have revealed that the atypical category, based on cytological examination, contained a significant proportion of malignant cases in breast FNA,[2] and cervical[43] and urinary cytology.[44] As shown in Table 3, we also summarized the result of 261 pancreatic juice cytological examinations performed

Table 3. Pancreatic juice cytology (261 cases: 2003–07)

Cytology	Benign		Atypical	Suspicious	Malignant	Total
	Class I	Class II	Class III	Class IV	Class V	
Case number (%)	73 (28.0)	116 (44.4)	36 (13.8)	10 (3.8)	26 (10.0)	261 (100)

Table 4. Pancreatic juice cytology (110 cases confirmed histological diagnosis) (2003–07)

Cytology	Class I	Class II	Class III	Class IV	Class V	Total
Case number	18	47	23	8	14	110
Carcinoma† (%)	5 (27.8)	20 (42.6)	18 (78.3)	7 (87.5)	14 (100)	64 (58.2)

†Carcinoma includes pancreatic ductal adenocarcinoma (PDA), intraductal papillary-mucinous carcinoma (IPMC), endocrine carcinoma, bile duct carcinoma and ampullary carcinoma.

in the pathological laboratory of Kyushu University Hospital (Fukuoka, Japan) between 2003 and 2007. The ambiguous cytological diagnoses, such as Class III or Class IV, were 46 of 261 (17.6%). The follow-up study revealed that 18 of 23 patients (78.3%) in Class III and seven of eight patients (87.5%) in Class IV were carcinoma (Table 4). Generally, it is important to avoid surgical resection of benign pancreatic tumor because pancreatic surgery is too invasive for patients that do not have life-threatening malignancy. Therefore, it may be worth performing our present analysis to distinguish non-malignancy from malignancy in cases with ambiguous cytological diagnoses.

The fragmentation of RNA from clinical samples appears to be the most critical factor that reduces reliability of RT-PCR-based tests. In the present study, we performed experiments to improve the reliability of our measurement of *hTERT* mRNA in cells microdissected from cytological specimens. We found that introduction of gene-specific priming,[35,37] short amplicons,[34,36,37] and normalization to reference genes[34,37] enabled quantitative measurement of *hTERT* mRNA. Quantification of mRNA can be successfully performed with highly fragmented RNA, consisting of fragments smaller than 100 bp, by normalization to reference genes.[34,36,45–47] In the present study, using normalization to *β-actin*, we also successfully quantified *hTERT* mRNA levels in fragmented samples containing RNAs of less than 200 bp. As shown in Figure 2e, RNA fragments were larger than 200 bp in most RNA samples from microdissected clinical samples. Therefore, it may not be necessary to check the quality of all RNA samples, although it is easy and rapid to assess the integrity of RNA using an Agilent 2100 Bioanalyzer.

The amplification of expressed gene sequences by RT-PCR has been performed successfully from small numbers of cells isolated by LCM.[45–47] In our preliminary study, we microdissected 10–1000 SUIT-2 cells from smears, investigated the integrities of total RNA from each sample, and measured *hTERT* expression. Although total RNA with high integrity was extracted, even from 10 cells, and *hTERT* mRNA was measurable, Ct values of *hTERT* in samples from 10 or 30 cells were increased to over 35 (Supporting Information Fig. S1), in which range, the reliability and reproducibility of the test was impaired.[26] Therefore, in the present study, we microdissected more than 50 cells from cytological samples to perform reliable and reproducible tests.

Our data suggest that our microdissection method can be applied to various cytological specimens including FNA, pancreatic juice, ascites and pleural and pericardial effusions. The combination of cytological diagnosis with tumor-specific mRNA measurement, including *hTERT*, in microdissected target cells will enable more accurate diagnosis and more preferable management for patients with malignancy.

The present study consisted of a large number of Class V samples and included only a small number of Class III or IV samples. However, it is of special interest to distinguish malignant cells from non-malignant cells in cytologicaly inconclusive or suspicious samples, such as Class III or Class IV samples. Therefore, further studies involving a large number of Class III or Class IV samples are required before clinical introduction.

In conclusion, we compared molecular diagnosis, based on microdissection technology, to cytological examination and found high sensitivity and specificity of this method. We believe that quantitative assessment of *hTERT* mRNA in cells microdissected from cytological specimens is a potential diagnostic tool to determine malignancy.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. (a) Electrophoresis-like images of RNA extracted from microdissected 10, 30, 50, 100 and 500 SUIT-2 cells stained with Toluidine blue staining. 28S/18S rRNA ratios were satisfactorily maintained in each sample. (b) We could consistently quantify *hTERT* mRNA to the same level in the samples of different cell numbers. (c) The threshold cycle values (Ct values) of *hTERT* were increased to over 35 in RNAs extracted from 10 and 30 cells.

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