Telomerase Reverse Transcriptase Promoter Mutations in Human Hepatobiliary, Pancreatic and Gastrointestinal Cancer Cell Lines

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Abstract. *Background/Aim: The promoter region of the telomerase reverse transcriptase (TERT) gene is a regulatory element capable of affecting TERT expression, telomerase activity, and telomerase length. Mutations within the TERT promoter region are the most common mutations in many cancers. In this study, we characterized the TERT promoter mutation status in hepatobiliary, pancreatic, and gastrointestinal cancer cell lines. Materials and Methods: TERT promoter mutation status was assessed by digital PCR in 12 liver cancer, 5 cholangiocarcinoma (CCA), 12 pancreatic cancer, 17 gastrointestinal cancer, and 3 healthy control cell lines. Results: The C228T promoter mutation was detected in 9 liver cancer lines, and the C250T TERT mutation was detected in 1 oesophageal squamous cell carcinoma line. Conclusion: The C228T promoter mutation is specific to liver cancer cell lines among various gastrointestinal cancer cell lines. These data will contribute to future research on the tumorigenic mechanisms and clinical use of digital PCR to detect mutations.*

Telomerase is a ribonucleoprotein complex that maintains genomic stability and telomere length at the end of chromosomes and plays a key role in cellular immortality and tumourigenesis (1). Telomerase is estimated to be over-expressed in 85%-90%

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of all human malignancies (2). The catalytic subunit of telomerase is telomerase reverse transcriptase (TERT), which is transcriptionally activated in germline, stem, and cancer cells but silenced in somatic cells (3). Therefore, TERT is a gatekeeper for cellular immortalization while maintaining chromosomal integrity. Several studies have demonstrated the importance of transcriptional, post-transcriptional, and post-translational TERT regulation, which may play a role in cancer development (4-7). In particular, *TERT* promoter mutations are the most common mutations in many types of cancer (8).

Two particularly common *TERT* promoter mutations lead to increased telomerase expression and consist of cytosine to thymine transitions located −124 and −146 base pairs upstream of the *TERT* translational start codon (chromosome 5:1295228 and chromosome 5:1295250, termed C228T and C250T here, respectively) (9, 10). Together, these mutations have been identified in 20%-82% of hepatocellular carcinomas (HCC) (11) as well as in a significant percentage of other malignancies, including melanoma, glioma, urothelial cancer, and basal cell carcinoma (12, 13). Several clinical studies have revealed that the presence of *TERT* promoter mutations was closely correlated with poor prognosis in patients with solid tumours, such as lung and breast cancer (14).

The occurrence of *TERT* promoter mutations in liver carcinogenesis has been observed in precancerous lesions, with the prevalence of mutations gradually increasing in conjunction with the degree of dysplasia. These findings indicate that *TERT* promoter mutations are strongly associated with the gradual transformation of precancerous lesions from dysplastic nodules to malignant hepatocellular carcinoma (11). Moreover, the presence of a *TERT* C228T promoter mutation in the plasma of HCC patients was associated with a worse prognosis (15). *TERT* promoter mutations are observed in other cancers, such as cholangiocarcinoma (CCA) (11) and oesophageal squamous

cell carcinoma (16), but at a lower frequency. Despite the importance of *TERT* mutations in liver and other cancers, no studies have reported on the presence or absence of *TERT* promoter point mutations in various types of hepatobiliary, pancreatic, and gastrointestinal cancer cell lines using digital PCR (dPCR). In this study, we used dPCR to characterize C228T and C250T *TERT* promoter mutations in 46 cancer cell lines and three healthy control cell lines. Although the presence or absence of *TERT* promoter point mutations has been determined for some gastrointestinal cancers using real-time PCR and Sanger sequencing, there has been no comprehensive analysis of gastrointestinal cancers by dPCR. Therefore, we used dPCR as a first step to understand potential differences in mutation detection methods.

Materials and Methods

Cell lines. The histological characteristics of the investigated cell lines are presented in Table I. A panel of 46 established human cancer cell lines were cultured *in vitro*, including 12 liver cancer cell lines (Hep3B, HepG2, HLE, HLF, Huh-1, Huh-7, JHH-2, JHH-5, JHH-6, JHH-7, Li-7, and PLC/PRF/5), 5 CCA lines (HuCCT-1, Huh-28, RBE, TKKK, and TFK-1), 12 pancreatic cancer lines (KP-2, KP-3, KP-3L, PANC-1, PK-1, PK-8, PK-9, QGP-1, S2-CP8, S2-VP10, SUIT-2, and T3M4), 4 oesophageal squamous cell carcinoma lines (KYSE180, KYSE30, KYSE850, and KYSE960), 4 oesophageal adenocarcinoma lines (OACM5.1C, OE19, OE33, and SK-GT-4), 5 gastric cancer lines (GCIY, MKN1, MKN45, MKN7, and MKN74), and 5 colon cancer cell lines (CACO-2, CCK-81, COLO-320, and CW-2). Three primary cell lines derived from healthy human tissue served as controls: healthy hepatocytes (hNHeps), colon epithelial cells (HCoEpic), and fetal pancreatic cells (2C6). The HepG2 line was thought to be a hepatocellular carcinoma. However, there was a report that this cell line was derived from a hepatoblastoma. Therefore, we performed short tandem repeat profiling (BEX Company; Tokyo, Japan) to authenticate the HepG2 cell line (17). As a result, the HepG2 cells were confirmed to be identical to those reported in ExPASy (Expert Protein Analysis System, Swiss Institute of Bioinformatics portal) and were treated as hepatoblastoma cells. Ethical approval for experiments was not required because the cell lines are commonly available for purchase. All tested cell lines were obtained from the American Type Culture Collection (Rockville, MD, USA), Japanese Cancer Research Resources Bank (Tokyo, Japan), Institute of Physical and Chemical Research (Saitama, Japan), DS Pharma Biomedical (Osaka, Japan), Cosmo Bio Company (Tokyo, Japan), Institute of Development Aging and Cancer (Miyagi, Japan), Lonza Japan (Tokyo, Japan), and European Collection of Authenticated Cell Cultures (Public Health England, Salisbury, UK).

All cell lines underwent 1 or 2 passages after acquisition to increase cell numbers and were then stored at −80°C. The cells were thawed when ready for use, and DNA extraction was performed after 2 passages. Therefore, each cell line used in this study was unstimulated and had undergone less than 5 passages.

Cell culture. The cell lines were grown in their corresponding media and additives and were incubated with 5% CO_2 at 37°C. MKN1, MKN7, MKN45, MKN74, Li-7, HuCCT1, Huh28, RBE, TFK-1, OE19, OE33, OACM5.1C, SK-GT-4, KP-2, KP-3, KP-3L, PK-1,

Table I. *Characteristics of 49 cell lines evaluated for point mutations in the telomerase reverse transcriptase gene promoter.*

Cell line	Histology	Sex of donor
Extrahepatic bile duct		
TFK-1	Papillary adenocarcinoma	M
Intrahepatic bile duct		
HuCCT1	Moderately differentiated adenocarcinoma	M
Huh28	Cholangiocarcinoma	F
RBE	Cholangiocarcinoma	F
TKKK	Cholangiocarcinoma	M
Large intestine		
CACO-2	Adenocarcinoma	M
CCK-81	Adenocarcinoma	F
COLO-320	Adenocarcinoma	F
CW-2	Adenocarcinoma	F
HCoEpic	Control epithelial cells	Unspecified
Liver		
Hep3B	Carcinoma	M
HepG2	Hepatoblastoma	M
HLE	Undifferentiated carcinoma	M
HLF	Undifferentiated carcinoma	M
hNHeps	Control hepatocytes	Unspecified
Huh-1	Carcinoma	M
Huh-7	Well-differentiated carcinoma	M
JHH-2	Carcinoma	M
JHH-5	Carcinoma	M
JHH-6	Undifferentiated carcinoma	F
JHH-7	Carcinoma	M
Li-7	Carcinoma	M
PLC/PRF/5	Carcinoma	M
Oesophagus		
KYSE180	Well-differentiated squamous cell carcinoma	M
KYSE30	Well-differentiated squamous cell carcinoma	M
KYSE850	Moderately differentiated squamous cell carcinoma	M
KYSE960	Moderately differentiated squamous cell carcinoma	M
OACM5.1C	Adenocarcinoma	F
OE19	Adenocarcinoma	M
OE33	Adenocarcinoma	F
SK-GT-4	Adenocarcinoma	M
Pancreas		
2C6	Human line derived from embryonic pancreas	Unspecified
KP-2	Moderately differentiated tubular adenocarcinoma	F
KP-3	Adenosquamous carcinoma	M
KP-3L	Adenosquamous carcinoma	M
PANC-1	Ductal adenocarcinoma	M
PK-1	Ductal adenocarcinoma	Unspecified
PK-8	Carcinoma	Unspecified
PK-9	Ductal adenocarcinoma	M
QGP-1	Islet cell carcinoma	M
S2-CP8	Ductal adenocarcinoma	M
S2-VP10	Ductal adenocarcinoma	M
SUIT-2	Moderately differentiated tubular adenocarcinoma	M
T3M4	Ductal adenocarcinoma	M
Stomach		
GCIY	Adenocarcinoma	F
MKN1	Adenosquamous carcinoma	M
MKN45	Poorly differentiated adenocarcinoma	F
MKN7	Differentiated adenocarcinoma	M
MKN74	Moderately differentiated adenocarcinoma	M

F: Female; M: male.

PK-8, PK-9, PANC-1, QGP-1, COLO-320, and CW-2 cells were grown in Roswell Park Memorial Institute (RPMI 1640; FUJIFILM Corp.; Tokyo, Japan) supplemented with 10% foetal bovine serum (FBS; Corning, NY, USA) and 100 U/ml penicillin (Thermo Fisher Scientific; Waltham, MA, USA). SUIT-2 and CCK-81 cells were grown in minimal essential medium (MEM; FUJIFILM Corp.) supplemented with 10% FBS and 100 U/ml penicillin. Hep3B and HepG2 cells were grown in MEM supplemented with 10% FBS and 1% MEM non-essential amino acids solution (NEAA; Thermo Fisher Scientific). CACO-2 cells were grown in MEM supplemented with 1% NEAA, 20% FBS, and 100 U/ml penicillin. JHH-5, JHH-6, and JHH-7 cells were grown in Williams' Medium E (Sigma-Aldrich Corp., St. Louis, MO, USA) supplemented with 10% FBS and 100 U/ml penicillin. JHH-2 cells were grown in Williams' Medium E supplemented with 2% FBS and 100 U/ml penicillin. Huh-1, Huh-7, PLC/PRF/5, HLE, TKKK, S2-CP8 and S2-VP10 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% FBS and 100 U/ml penicillin. KYSE180, KYSE850, and KYSE960 cells were grown in a 1:1 mixture of RPMI 1640 and Ham's F12 medium (FUJIFILM Corp.) containing 2% FBS and 100 U/ml penicillin. HLF cells were grown in DMEM with 5% FBS and 100 U/ml penicillin. KYSE30 cells were grown in DMEM with 2% FBS and 100 U/ml penicillin. T3M4 cells were grown in Ham's F10 medium (FUJIFILM Corp.) supplemented with 10% FBS and 100 U/ml penicillin. The hNHeps cells were grown using a CS-C medium kit (DS Pharma Biomedical; Osaka, Japan) in accordance with the manufacturer's protocol. The 2C6 cells were grown in Ham's F10 medium supplemented with 5% FBS, 10% newborn calf serum (Thermo Fisher Scientific), 2.5% horse serum (Thermo Fisher Scientific), and 100 U/ml penicillin. The HCoEpiC cells were grown in Colonic Epithelial Cell Medium (SienCell Research Laboratories; Carlsbad, CA, USA) supplemented with Colonic Epithelial Cell Growth Supplement (SienCell Reasearch Laboratories).

DNA extraction. Genomic DNA was isolated using the PureLink Genomic DNA kit (Thermo Fisher Scientific) in accordance with the manufacturer's protocol. DNA concentration and purity were determined using a NanoDrop spectrophotometer (Thermo Fisher Scientific). DNA extraction of the various cell lines was performed after 2 passages.

Quantification of TERT promoter mutations by digital PCR. The dPCR analysis was performed with isolated genomic DNA using the QuantStudio™ 3D digital PCR system (Thermo Fisher Scientific) with custom TaqMan primers and probes labelled with 6-carboxyfluorescein (FAM) and 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC; Thermo Fisher Scientific) for detection of *TERT* C228T (−124 bp) and *TERT* C250T (−146 bp) promoter mutations. The Huh-7 cell line was used as a known positive DNA control, and Hep3B cells were the negative control in each dPCR run. Each dPCR reaction was performed using 7.5 μl of QuantStudio™ 3D digital PCR master mix, 0.375 μl of TaqMan primer/probe for each *TERT* mutation, 6.125 μl of water, and 10 ng of DNA in a final reaction volume of 14.5 μl; the mixture was loaded onto a QuantStudio™ 3D digital PCR chip (Thermo Fisher Scientific). PCR amplification was performed using the Applied Biosystems ProFlex PCR System under the following conditions: one cycle at 96˚C for 10 min followed by 39 cycles of 98˚C for 30 s and 60˚C for 2 min.
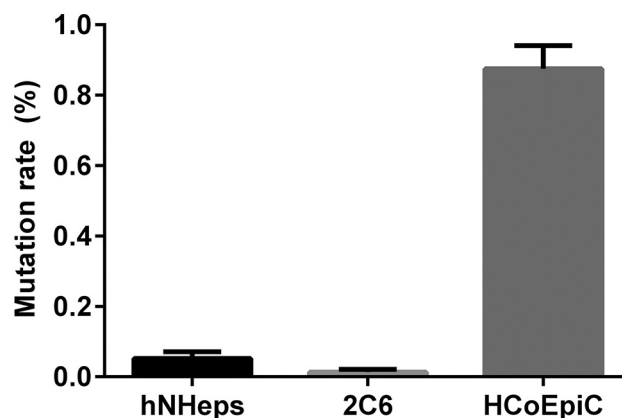

Figure 1. *Mutation rates are shown for the C228T TERT promoter point mutation in three types of healthy control cells. Among the three healthy control cell lines, HCoEpiC had the highest frequency of TERT promoter point mutations.*

Wild-type and mutant alleles were analysed using QuantStudio 3D AnalysisSuite software (Thermo Fisher Scientific). For each dPCR experiment, the mean number of copies per partition and the number of estimated copies per total reaction volume for the unknown samples were calculated. For each run, at least one negative template control, a wild-type positive control, and rare mutation positive controls were included. Each dPCR experiment was performed three times for each cell line on different days under the same PCR conditions.

Statistical analysis. Results are expressed as the mean±standard deviation (SD). All statistical analyses were performed using GraphPad Prism 6 software (GraphPad Software; La Jolla, CA, USA). Significant differences were determined between the reference cells (HCoEpiC) and each cell line by Student's *t*-test. *p*-Values less than 0.05 were considered significant.

Results

Prevalence of C228T TERT promoter mutations using dPCR. The presence of the C228T or C250T mutations within the *TERT* promoter region was analysed in cell lines derived from solid tumours and healthy human tissues as shown in Table I. DPCR was first performed on healthy control cell lines to detect the mutations. HCoEpic cells had the highest percentage of *TERT* promoter point mutations among the healthy human control cell lines, which included 2C6 and hNHeps cells (Figure 1); thus, this cell line was used as the control for comparisons with the cancer cell lines.

DPCR was used to compare the cancer cell lines with HCoEpic control cells to detect the C228T mutation in the *TERT* promoter (Figure 2, Figure 3 and Figure 4). The mutation was detected in 9 out of 12 liver cancer cell lines, including JHH-6, JHH-5, Li-7, HepG2, Huh-7, HLE, JHH-7, HLF, and JHH-2. No significant differences were observed
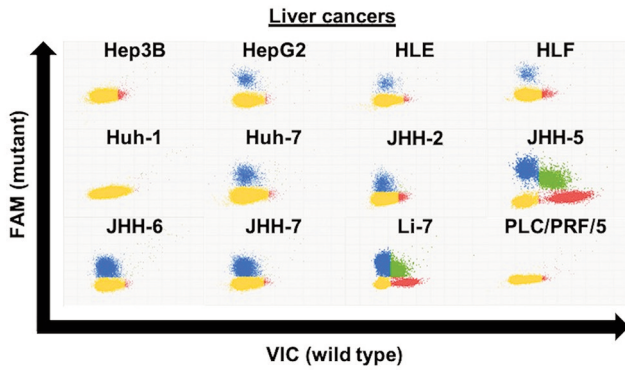
Figure 2. *The C228T TERT promoter mutation detected by dPCR in liver cancer cells. The results of dPCR for DNA extracted from liver cancer cell lines are plotted. Signals from FAM (C228T mutation; blue) and VIC (wild type; red) dyes are plotted on the x- and y-axis, respectively. Yellow clusters represent wells that were not amplified (negative calls), while green clusters indicate wells that contained both VIC and FAM signals. Among the 12 liver cancer cell lines, 9 showed a high frequency of TERT promoter point mutations. dPCR: Digital polymerase chain reaction; FAM: 6-carboxyfluorescein; HCC: hepatocellular carcinoma; VIC: 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein.*

Figure 3. *The figure compares nine liver cancer cell lines in which TERT promoter point mutations were frequently detected with HCoEpiC control cells. The mutation rates for all of the nine liver cancer cell lines were significantly different compared with the mutation rate for HCoEpiC cells. Values represent mean±SD; *p<0.05 vs. HCoEpiC control cells.*

between the control cells and the remaining three liver cancer cell lines (Hep3B, Huh-1, and PLC/PRF/5; Figure 3). Compared with those of the HCoEpic cells, the mutation rates were significantly higher in the following liver cancer cell lines: JHH-5 (51.2%-84.1%, *p*=0.011), JHH-6 (71.5%-74.5%, *p*<0.001), Li-7 (43.4%-70.0%, *p*=0.0013), Huh-7 (50.8%-51.7%, *p*<0.001), HLE (49.4%-51.4%, *p*=0.032), HepG2 (50.0%-51.1%, *p*<0.001), JHH-7 (38.4%-47.9%, *p*<0.001), HLF (33.9%-35.0%, *p*=0.01), and JHH-2 (24.5%-26.3%, *p*=0.018) (Figure 3). The mutant allele abundance of the remaining cell lines is shown in Figure 4; compared with that of control cells, the mutation rate was not significantly higher in cancer cell lines derived from the oesophagus, intrahepatic bile duct, large intestine, stomach, or pancreas.

Prevalence of C250T TERT promoter mutations using dPCR. As with the C228T mutation, the HCoEpiC cell line had the highest mutation rate for the C250T *TERT* mutation among the three healthy control lines tested and was used as the control for assessing the presence of this mutation in the cancer cells (Figure 5). Among oesophageal cancer cell lines, the C250T mutation was observed in KYSE850 cells (Figure 6), and the mutation rate in this line was significantly higher than that in the control cells (*p*=0.0012; Figure 7). With the exception of SK-GT-4, which had a *TERT* promoter point mutation rate of 0.1433%, the remaining oesophageal carcinoma cell lines (KYSE30, KYSE180, KYSE960, OE-19, OE-33, and OACM5.1C) had a *TERT* promoter point
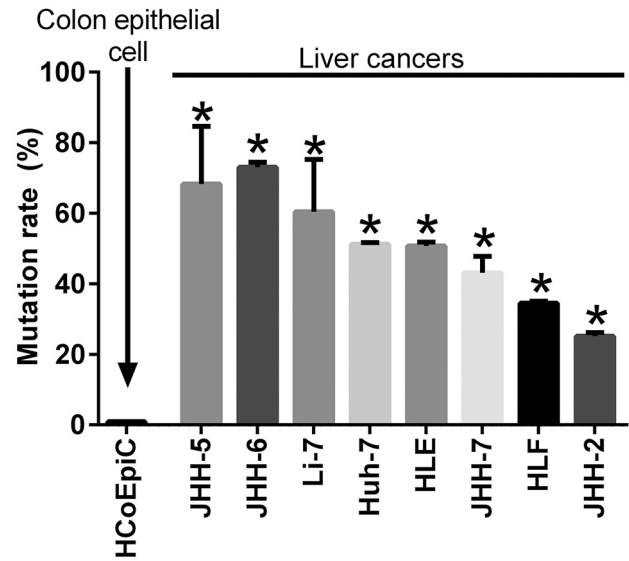
mutation rate of <0.1%, which was not significantly different from the control cells (Figure 8). The mutant allele abundance for the cell lines is shown in Figure 8. The mutation rates were not significantly different between the control cells and the hepatobiliary, pancreatic, gastric, or colonic cancer cell lines.

Discussion

TERT promoter mutations have been found in numerous cancers, but few studies have comprehensively examined large numbers of hepatobiliary and pancreatic cell lines to detect these mutations. Although large collections of cell lines, such as the NCI-60 panel (18), are available and have been characterized, the collection of hepatobiliary, pancreatic, and upper gastrointestinal cancer-derived cell lines is markedly smaller compared with that of other common tumour types, and these cells have been poorly characterized. Therefore, in this experiment, we evaluated cell lines from gastrointestinal malignancies that were not part of the NCI-60 panel collection and did not overlap with published literature.

In the present study, we used dPCR to analyse the presence of two *TERT* promoter mutations, C228T and C250T, in hepatobiliary, pancreatic, and gastrointestinal cancer cell lines. We detected the presence of C228T mutations in 9 of 12 liver cancer cell lines, but not in the cell
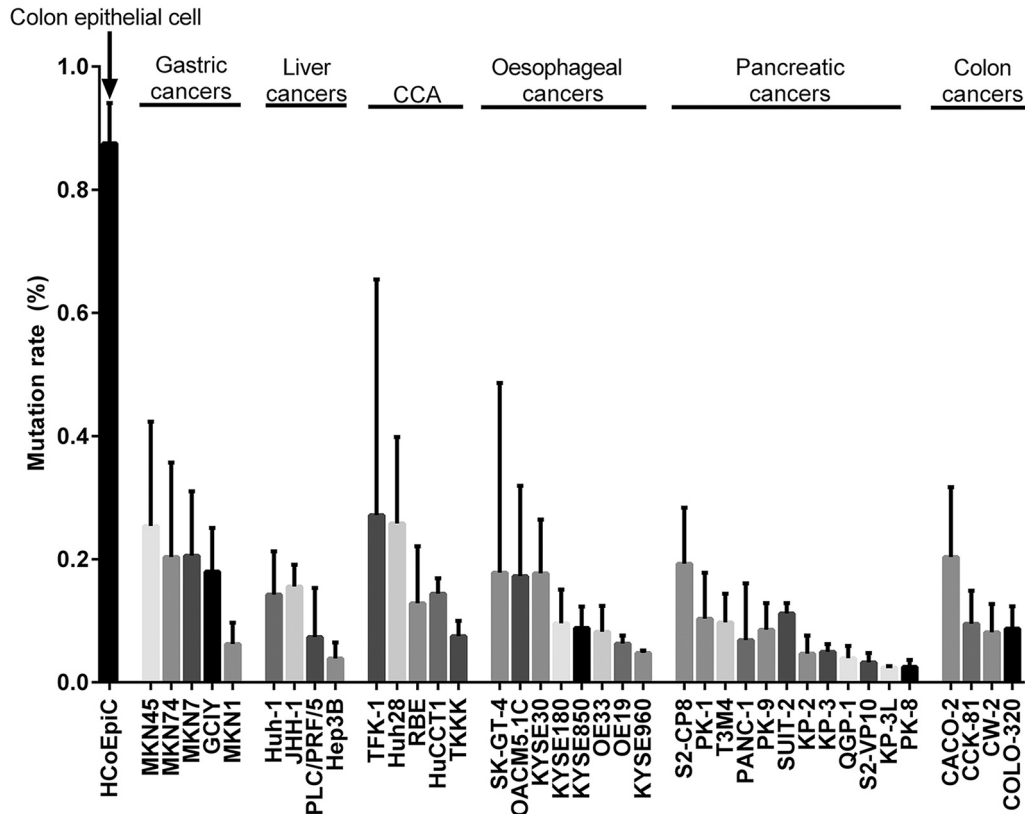
Figure 4. *The mutation rate of HCoEpiC control cells was compared with those of 37 cancer cell lines, excluding the liver cancer lines described in Figure 3. These cell lines demonstrated a low frequency of TERT promoter point mutations. There was no significant difference in mutation rates between any of the cancer cell lines and the rate for HCoEpiC cells.*

lines derived from pancreatic, intrahepatic bile duct, oesophageal, stomach, or colon cancers. The C250T mutation was only detected in 1 of 8 oesophageal cell lines (KYSE850) and was not detected in any of the remaining cell lines. Our results are consistent with those of another study that examined the presence of C228T/C250T mutations in a large panel of cell lines in which a high degree of variability in mutation status was observed depending on the cell line of origin (19). Furthermore, our results greatly expand on this study because of the inclusion of liver cell lines as well as the inclusion of numerous additional gastrointestinal, intrahepatic bile duct, oesophageal, and pancreatic lines.

Consistent with our results, the C228T mutation is frequently observed in patients with HCC (19). A previous study reported that *TERT* promoter point mutations were less frequent in HBV-positive HCC (20). In our study, there were 4 HBV-positive liver cancer cell lines, and 1 of these 4 lines showed a high frequency of *TERT* promoter point mutations, whereas the other 3 lines showed a low frequency of mutations. In our study, C228T *TERT* promoter point mutations were found in 75% of the liver cancer cell lines.
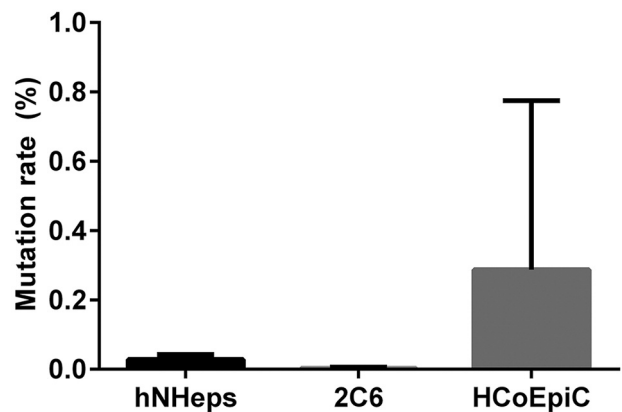

Figure 5. *Mutation rates are shown for the C250T TERT promoter point mutation in three types of healthy control cells. Among the three healthy control cell lines, HCoEpiC cells had the highest frequency of this TERT promoter point mutation.*

Another study reported 43.3% positivity for *TERT* promoter mutations in HCC, although this latter study evaluated human HCC clinical samples (21). DPCR is more accurate
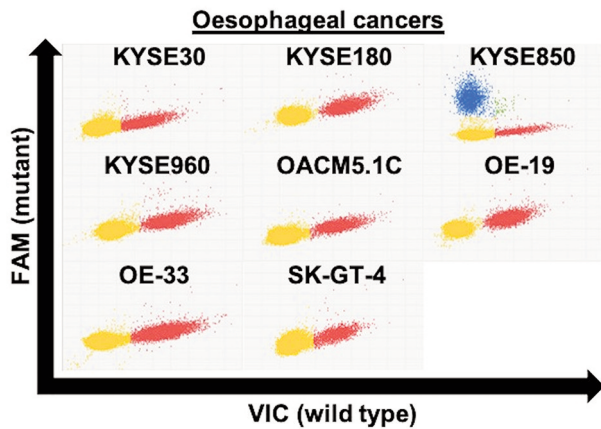
Figure 6. *The C250T TERT promoter mutation detected by dPCR in oesophageal carcinoma cell lines. The results of dPCR for DNA extracted from oesophageal carcinoma cell lines are plotted. Signals from FAM (C250T mutation; blue) and VIC (wild type; red) dyes are plotted on the x- and y-axis, respectively. Yellow clusters represent wells that were not amplified (negative calls). Among the eight oesophageal cancer cell lines, only KYSE850 showed a high frequency of TERT promoter point mutations. dPCR: Digital polymerase chain reaction; FAM: 6-carboxyfluorescein; VIC: 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein.*

Figure 7. *Mutant allele (C250T TERT) abundance expressed as the percent mutation rate in HcoEpiC control cells versus the KYSE850 oesophageal carcinoma line. The mutation rate was significantly higher in KYSE850 cells compared with that in HCoEpiC cells. Values represent mean±SD; *$p<0.05$ vs. HCoEpiC control cells.*

for quantifying relative abundance, while being less sensitive for absolute quantification because of the smaller reaction volumes compared with those of real-time PCR (22). Therefore, dPCR may be preferable to conventional real-time PCR for detecting *TERT* promoter point mutations.

Expression of TERT is critical for reactivation of telomerase, which plays an important role in the development and proliferation of cancer cells through telomere protection and avoidance of senescence (23). *TERT* promoter mutation rates vary significantly among human malignancies depending on their histological type and cellular origin (24). Our results are consistent with clinical data demonstrating the variability in *TERT* mutation rates among different types of cancers (25-28). For example, glioblastoma, malignant melanoma, and urothelial bladder cancers exhibit a high mutation rate, while thyroid cancer, hepatocellular carcinoma, and urinary carcinoma have an intermediate level; other tumours, such as those from the lung, breast, gastrointestinal tract, prostrate, and kidney, are characterized by a low mutation rate or lack of *TERT* mutations (25-28).

Although *TERT* promoter mutations have an important role in tumourigenesis and appear to have promise as biomarkers or prognostic factors (29-32), *TERT* promoter regulation is likely to be a multifactorial process involving genetic, epigenetic, transcriptional, and posttranscriptional factors (33). A comprehensive analysis of 31 cancer types found TERT expression in 73% of tumours; genetic
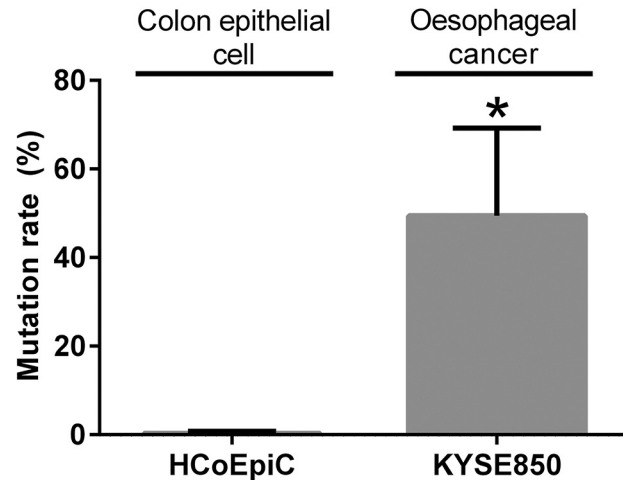
alterations as well as promoter methylation were predictive of telomerase activity (12). Furthermore, TERT expression was undetectable in an additional 22% or more of the tumours studied.

There are several limitations in our study. First, the concentration of DNA extracted from the HCoEpiC control cells was slightly lower than that of other cancer cell lines. This caused the total gene copy numbers to be lower, which resulted in a slightly higher relative percentage. For example, the C228T mutation rate in 2C6 cells, which had the lowest mutation frequency among the control cells, was 0.013% (0.007%-0.021%) compared with 0.875% (0.8280%-0.950%) in the HCoEpiC line. However, when compared with the real number of FAMs, 0.330 copies/μl (range=0.150-0.447 copies/μl) for HCoEpiC was less than 0.419 copies/μl (range=0.228-0.676 copies/μl) for 2C6. For this reason, the mutation rate of HCoEpiC was higher. Therefore, the values for mutation rates of HCoEpiC appear to be different in Figure 1 and Figure 5. Second, this study lacks experiments that analyse the association between *TERT* point mutations and function in each cell line. Further experiments are needed to evaluate the relationship between TERT mutation rates, telomerase length, and telomerase activity in these cells. Third, we did not evaluate *TERT* mutations in human clinical specimens. However, it will be desirable to conduct confirmatory studies to determine the consistency of the results of these basic experiments using human clinical samples in the future.

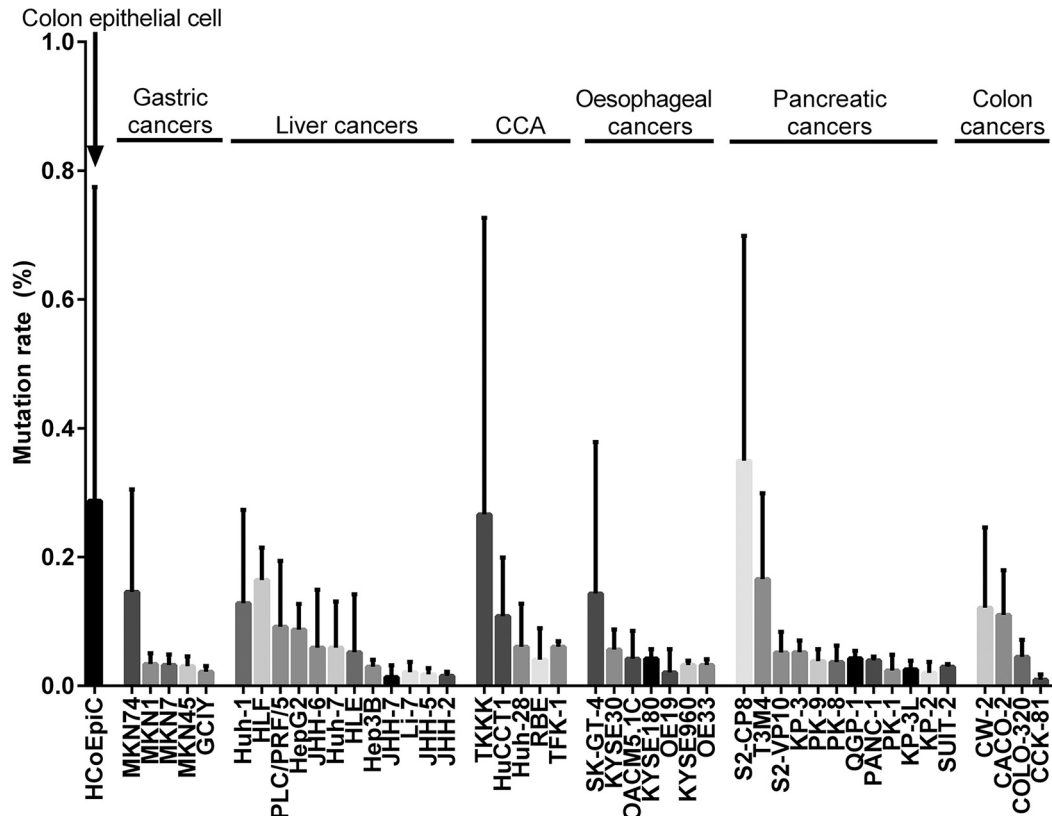In conclusion, we have comprehensively analysed the presence of *TERT* promoter point mutations in liver cancer

Figure 8. *The C250T TERT mutation rates are shown for HcoEpiC control cells versus all other cell lines except KYSE850. There was no significant difference in the mutation rate between the HCoEpiC control cells and any of the gastric cancer, liver cancer, CCA, oesophageal cancer (except KYSE850), pancreatic cancer, or colon cancer cell lines.*

cell lines, CCA, and gastrointestinal cancer cell lines using dPCR. By examining the presence or absence of the C228T and C250T *TERT* promoter point mutations in various cancer lines, we found that the C228T *TERT* promoter point mutation was specific for liver cancer lines. These data are expected to contribute to future research, including measurements of chemical biomarkers and elucidation of the oncogenic mechanisms.

Conflicts of Interest

The Authors declare that they have no competing interests in relation to this study.

Authors' Contributions

MH and TM designed the experiments. KF, TM, RN, SF, TK, DN, NF, HY, HKa, JT, HKo, KT, and HSM performed the experiments and analysed the data. TM performed final drafting and writing of the manuscript. YM contributed to the reanalysis of data for comparison of mutation rates of *TERT* point mutations. SF and MO contributed to drafting of the manuscript. All Authors have read and approved the final version of the manuscript.

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