ORIGINAL ARTICLE

Expression of human telomerase catalytic protein in gallbladder carcinogenesis

B Luzar, M Poljak, A Cör, U Klopčič, V Ferlan-Marolt

J Clin Pathol 2005;58:820–825. doi: 10.1136/jcp.2004.023143

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Correspondence to:
Assistant Professor B Luzar,
Institute of Pathology,
Medical Faculty, University
of Ljubljana, Korytkova 2,
1000 Ljubljana, Slovenia;
bostjan.luzar@mf.uni-lj.si

Accepted for publication
28 October 2004

Background: Telomerase catalytic subunit (hTERT) gene re-expression is a rate limiting step for the activity of telomerase, a key enzyme implicated in cellular immortalisation and transformation.

Aims: To determine the potential role of hTERT protein in gallbladder carcinogenesis.

Material/Methods: hTERT protein was analysed by means of immunohistochemistry in 89 gallbladder tissue samples: 16 normal epithelia, 14 reactive hyperplasias, 15 low grade dysplasias, 16 high grade dysplasias, and 28 adenocarcinomas. At least 200 nuclei were assessed for each slide and the mean number of positive signals for each nucleus was expressed as the hTERT index.

Results: The mean hTERT index increased progressively with the degree of gallbladder epithelial abnormalities: from 0.03 in normal epithelia, 0.04 in hyperplastic epithelia, 0.25 in low grade dysplasia, 0.82 in high grade dysplasia, to 0.93 in adenocarcinoma. Statistical analysis revealed that three different groups of gallbladder epithelial changes can be distinguished according to the number of hTERT signals for each nucleus: (1) normal and regenerative gallbladder epithelium, (2) low grade dysplasia, and (3) high grade dysplasia and adenocarcinoma ($p < 0.001$).

Conclusions: The occasional presence of hTERT protein in normal and regenerative gallbladder mucosa reflects their regenerative capacity. Nevertheless, significantly higher hTERT indices in low and high grade dysplastic epithelia and in gallbladder adenocarcinomas are probably a consequence of hTERT re-expression—an early event in the multistep process of gallbladder carcinogenesis.

Genetic changes and their exact sequence in the multistep process of gallbladder carcinogenesis are still not well characterised. Two main morphological pathways have been proposed for the development of gallbladder adenocarcinoma: the relatively common dysplasia–carcinoma sequence and a less frequent and rather controversial adenoma–carcinoma sequence.¹ The molecular abnormalities seen in dysplasia are similar to those in carcinoma, whereas gallbladder adenomas lack these molecular changes.^{2–4} In the dysplasia–carcinoma sequence, inactivation of the p53 gene, activation of the K-ras oncogene, and cyclin D1 overexpression have been characterised as early events in gallbladder carcinogenesis.^{5–6} In contrast, downregulation of p27^{Kip1} expression appears to be a late event in gallbladder carcinogenesis, associated with tumour progression and metastasis, and conferring a poor prognosis in these patients.⁷

“Whereas the RNA template and telomerase associated protein are expressed ubiquitously, telomerase catalytic protein expression is highly regulated and generally correlates with telomerase activity”

The proliferative capacity of normal somatic cells is limited by the gradual loss of tandem nucleotide repeats (TTAGGG)_n, termed telomeres, at the chromosomal ends, because of an end replication problem.^{8–10} One of the primary functions of telomeres is to mark the ends of linear chromosomes, as distinct from broken DNA, and to facilitate chromosome replication.¹¹ Telomeres are maintained by a specialised multisubunit ribonucleoprotein complex known as telomerase, which functions as a reverse transcriptase that can synthesise the telomeric ends at each cell division.¹² The basic components of telomerase have been identified as the RNA

template, telomerase associated protein, and reverse transcriptase (hTERT).^{13–15} Whereas the RNA template and telomerase associated protein are expressed ubiquitously, hTERT protein expression is highly regulated and generally correlates with telomerase activity.^{16–17}

The 40 kb single copy hTERT gene, mapping to chromosome 5p15.33, encodes a 127 kDa protein of 1132 amino acids contained in 16 exons.¹² Several splice variants of the hTERT gene transcript have been described.^{18–19} However, it is generally accepted that only the full length transcript of the hTERT gene can function as the catalytic subunit of telomerase, because it contains the whole core catalytic domain and the specialised C-terminus of this transcriptase.²⁰

Several studies have shown that, in adults, telomerase is only active in the germ line, certain stem cells, and activated lymphocytes, and is usually undetectable in differentiated cells, such as most human somatic cells.^{21–22} In contrast, telomerase reactivation has been found in approximately 85% of the most common human cancers, such as breast, lung, liver, pancreatic, colon, laryngeal/hypopharyngeal, oropharyngeal, and prostate cancer.²³ Providing the cell(s) with unlimited proliferative potential (immortality), telomerase is thought to promote the accumulation of additional genetic abnormalities, culminating in genomic instability of the cell.²⁴

To the best of our knowledge, the expression of hTERT protein during gallbladder carcinogenesis has not been analysed. The aim of our study was to analyse the expression of hTERT protein in normal gallbladder epithelium, regenerative epithelium, low and high grade dysplasia, and in gallbladder adenocarcinomas, to test the hypothesis that hTERT gene re-expression is an important, probably early, event in gallbladder carcinogenesis.

Abbreviations: hTERT, human telomerase reverse transcriptase; TRAP, telomeric repeat amplification protocol

Figure 1 (A) Normal gallbladder epithelium. Absence of human telomerase catalytic protein (hTERT) nuclear staining. (B) Low grade dysplasia; note the focal nuclear hTERT protein positivity. (C) Transition from low grade to high grade dysplasia; note several positive hTERT protein signals within high grade dysplasia (right) and the absence of nuclear reactivity in low grade dysplasia (left). (D) High grade dysplasia; almost all nuclei contain positive hTERT protein signals. (E) Gallbladder adenocarcinoma; several hTERT protein signals can be seen within the single nucleus in most nuclei.

MATERIAL AND METHODS

Tissue samples

A retrospective analysis was performed on 89 gallbladder tissue specimens, obtained from 89 patients treated with cholecystectomy in the period from 1998 to 2000 at the department of abdominal surgery, Clinical Centre, Ljubljana, Slovenia: 16 normal and 14 regenerative gallbladder epithelial samples originated from 30 patients undergoing cholecystectomy because of gallstones. No dysplastic and/or carcinomatous epithelial changes were seen in the gallbladders of these patients. In contrast, 15 samples of low grade dysplasia, 16 samples of high grade dysplasia, and 28 adenocarcinoma samples were obtained from 59 patients on whom cholecystectomy had been performed because of gallbladder adenocarcinoma. The patients with gallbladder cancer had not received chemotherapy or radiotherapy before surgery.

Criteria for light microscopical evaluation of gallbladder samples

Normal gallbladder epithelium is composed of a single layer of tall columnar cells with basally oriented nuclei.²⁵ Regenerative gallbladder epithelium is characterised by columnar cells with or without mucous, atrophic low cuboidal cells, and tall thin pencil-like cells, and is usually

found associated with acute inflammation and/or ulceration.²⁶ Dysplasia is defined histologically by varying degrees of pseudostratification, nuclear atypia, loss of polarity, and mitotic figures.²⁶ Depending on the severity of changes, dysplasia is usually graded as mild, moderate, or severe.²⁶ However, for practical purposes, only low and high grade dysplasia are usually distinguished. Carcinoma in situ is characterised by histological features of carcinoma without basement membrane penetration.²⁶ In our study, no distinction was made between high grade dysplasia and carcinoma in situ. According to the current World Health Organisation classification of "dysplasia and carcinoma in situ", the distinction between these two entities may be at least difficult, if not impossible, because of their sometimes overlapping histological features.²⁶ Adenocarcinoma of the gallbladder was classified according to the World Health Organisation classification of gallbladder tumours.²⁶

Immunohistochemical detection of hTERT protein

The presence of hTERT protein was investigated by immunohistochemistry. Tissue sections (4 µm thick) were dewaxed, rehydrated, and washed in phosphate buffered saline for 15 minutes. Antigen retrieval and staining with monoclonal antibody NCL-hTERT (Novocastra, Newcastle upon Tyne, UK), diluted 1/20, was performed in an automatic

Figure 2 Scatter plot of human telomerase catalytic protein (hTERT) indices in normal gallbladder epithelium (Norm), different grades of gallbladder epithelial abnormalities (regenerative epithelium (R) and high (HG) and low grade (LG) dysplasia (Dys)), and adenocarcinoma (Ca) of the gallbladder.

immunostainer (Discovery; Ventana, Tucson, Arizona, USA). After washing, sections were treated with biotinylated secondary antibody for 30 minutes, followed by incubation with peroxidase conjugated streptavidin for 30 minutes. Visualisation of the immunoreaction was carried out with 3,3' diaminobenzidine (Ventana detection kit) for five minutes. Finally, sections were counterstained with haematoxylin. Sections treated without primary antibodies served as negative controls. Normal human tonsil and testicular tissue were used as positive controls for hTERT protein.

Measurement of hTERT protein

Although diffuse finely granular cytoplasmic hTERT protein staining was noted in several cases (see below), only nuclear hTERT protein immunoreactivity was measured. A positive signal for hTERT protein was defined as a small or large dot(s) within the nucleoplasm and/or nucleoli of gallbladder epithelial cells. The area to be measured was randomly selected in the corresponding lesion, and at least 200 nuclei were assessed for each slide. The number of positive signals for each nucleus was expressed as the hTERT index for each corresponding epithelial lesion.

Statistical analysis

Statistical analysis was performed by a non-parametrical Mann-Whitney U test (non-parametrical Wilcoxon test) using SPSS 11.0 for Windows; p values lower than 0.05 were regarded as significant.

RESULTS

The presence of hTERT protein was analysed in a total of 89 samples of gallbladder tissue: 16 normal epithelia, 14 reactive hyperplasias, 15 low grade dysplasias, 16 high grade dysplasias, and 28 adenocarcinomas.

A positive signal for hTERT protein was identified as a small or large well defined dot(s) within the nucleoplasm and/or nucleoli of gallbladder epithelial cells. In addition to the gallbladder epithelium, positive signals for hTERT protein were also occasionally detected in the nuclei of lymphocytes, located in the gallbladder wall. In addition, finely granular cytoplasmic staining was detected in most cases of low and

Figure 3 Box and whiskers plot for the mean human telomerase catalytic protein (hTERT) indices in normal gallbladder epithelium (Norm), different grades of gallbladder epithelial abnormalities (regenerative epithelium (R) and high (HG) and low grade (LG) dysplasia (Dys)), and adenocarcinoma (Ca) of the gallbladder.

high grade dysplasia, and in adenocarcinomas, but only nuclear hTERT protein immunohistochemistry was further investigated.

We mostly failed to demonstrate hTERT protein nuclear immunoreactivity in normal and regenerative gallbladder epithelium (fig 1A). Only occasionally were isolated signals for hTERT protein found as small dots within the nuclei of epithelial cells.

Low grade dysplasia was characterised by focal clusters of hTERT protein positive cells (fig 1B). Several nuclei in high grade dysplasia and gallbladder adenocarcinoma contained one or more hTERT protein signal (fig 1C–E).

Figure 2 is a scatter plot of hTERT indices calculated in normal gallbladder epithelium, different grades of gallbladder epithelial abnormalities, and gallbladder adenocarcinoma. The mean number of hTERT signals for each nucleus increased progressively with the degree of gallbladder epithelial abnormalities: from 0.03 in normal epithelium, 0.04 in regenerative epithelium, 0.25 in low grade dysplasia, 0.82 in high grade dysplasia, to 0.93 in adenocarcinoma (fig 3).

Statistical analysis revealed that three different groups of gallbladder epithelial changes can be distinguished according to the number of hTERT signals for each nucleus, namely: (1) normal and regenerative gallbladder epithelium, (2) low grade dysplasia, and (3) high grade dysplasia and adenocarcinoma ($p < 0.001$). In addition, there were no significant differences in the number of hTERT signals for each nucleus among well, moderately, and poorly differentiated gallbladder adenocarcinomas ($p > 0.05$).

DISCUSSION

The role of telomerase in gallbladder carcinogenesis is at present largely unknown. Telomerase activity has so far only been analysed by the TRAP (telomeric repeat amplification protocol) assay in a few samples of gallbladder adenocarcinoma. Niiyama *et al* detected telomerase activity in eight of 11 gallbladder adenocarcinomas but not in benign biliary tract lesions.²⁷ Itoi *et al* detected telomerase activity in two of three gallbladder adenocarcinomas, whereas specimens of non-malignant biliary tissue had no detectable telomerase activity.²⁸ However, the TRAP assay has several potential limitations. Telomerase detection by the TRAP assay may be complicated by enzyme inhibitors, proteases, and RNases,

which may restrict the usefulness of the test and reduce its sensitivity.²⁹ In addition, recent studies have confirmed a close relation between hTERT mRNA expression and telomerase activity, suggesting that the assessment of hTERT gene expression could be used as an alternative to telomerase activity measurement.^{30–31}

Itoi *et al* have reported that hTERT mRNA was detected in the bile of three of four patients with gallbladder carcinoma.³² In contrast, telomerase activity as assessed by the TRAP assay was confirmed in only one of these patients.³² Recently, hTERT mRNA was detected preoperatively in bile from a patient with gallbladder cancer.³³ In addition, hTERT mRNA was found in the bile of a further three of eight patients with gallbladder cancer, and subsequently in the resected neoplastic tissue.³³ Interestingly, the bile samples of all four patients with benign gallbladder disease were negative for hTERT mRNA.³³

To the best of our knowledge, hTERT protein has not so far been studied in gallbladder carcinogenesis. In the present study, we analysed the expression of hTERT protein in normal gallbladder mucosa, regenerative gallbladder epithelium, low and high grade gallbladder epithelial dysplasia, and gallbladder adenocarcinoma, to gain insight into the potential role of the telomerase catalytic subunit, and indirectly also of the telomerase enzyme, in gallbladder carcinogenesis.

“Our results suggest that some cell clones might already be immortal in low grade epithelial dysplasia, because the hTERT protein positive nuclei were usually seen in clusters, probably representing the expansion of a single cell clone within the dysplastic gallbladder epithelium”

We selected monoclonal antibody NCL-hTERT from several commercially available anti-hTERT antibodies because it has been tested extensively, and has been reported to be sufficiently specific for use on formalin fixed, paraffin wax embedded tissue specimens.³⁴ It has also been shown that hTERT immunostaining with the NCL-hTERT antibody in fixed tissues agreed with telomerase activity and hTERT mRNA expression in the corresponding non-fixed samples,³⁴ suggesting that hTERT protein immunohistochemistry might be used as a surrogate marker for the estimation of telomerase activity in the tissue sample. Theoretically, nuclear localisation of the hTERT protein is required to promote elongation of telomere sequences, and hTERT protein immunoreactivity was generally found within the nucleoplasm and/or nucleoli as small or larger dots.³⁴ Wong *et al* have shown that catalytically active telomerase has a regulated intranuclear localisation that is dependent on the cell cycle stage, transformation, and DNA damage.³⁵ Whereas telomerase is released from sequestration at nucleolar sites into the nucleoplasm at the expected time of telomere replication in normal cell lines, there is almost a complete dissociation of telomerase from nucleoli at all stages of the cell cycle in tumour and transfected cells.³⁵ However, we and others have noted in a proportion of predominantly tumour cells, but also in samples of low and high grade gallbladder epithelial dysplasia, a fine granular cytoplasmic staining, either diffuse or confined to small areas, in addition to nuclear and/or nucleolar staining.^{34–36} Although the exact biological significance of cytoplasmic hTERT protein staining is at present unknown, cytoplasmic hTERT immunoreactivity might reflect a failure of nuclear translocation of a variant hTERT protein, as a result of alternative splicing of hTERT mRNA, or signify a yet unknown function of hTERT protein in the cytoplasm.^{34–36}

We mostly failed to demonstrate hTERT protein in normal and regenerative gallbladder epithelial cells. Cells with

hTERT protein signals did not differ morphologically from hTERT protein negative cells. Therefore, it appears that these cells correspond to committed descendants of stem cells undergoing terminal differentiation into columnar cells. Our results suggest that the occasional presence of hTERT protein in the nuclei of normal and regenerative gallbladder epithelial cells reflects the self renewal capacity of these cells. It has already been established that stem cells and their committed descendants, such as germline cells, possess telomerase activity that stabilises their telomeres and facilitates continuing proliferation for regenerative purposes.^{21–24} Telomerase activation may thus be an adaptive response to protect excessive telomere loss and may possibly help to extend the proliferative life span in highly regenerative cells.³⁷

The concept of dysplasia of the gallbladder as a precancerous condition was suggested by histological observations showing that dysplasia appeared to lie on a histological continuum with carcinoma, and that it was more common in gallbladders containing overt carcinoma than in non-cancerous gallbladders.^{5–38} According to Roa *et al*, the difference in the mean ages of patients with dysplasia and early gallbladder carcinoma (tumours with infiltration into the mucosa or muscular layer) is 12 years, and the difference between those with early carcinoma and advanced carcinoma is about two years.³⁸

We found a significantly higher number of hTERT signals for each nucleus in low grade dysplastic gallbladder epithelium than in normal and regenerative gallbladder epithelium. hTERT positive cells showed a dysplastic phenotype morphologically. Our results suggest that the significantly higher numbers of hTERT signals for each nucleus seen in low grade epithelial dysplasia than in normal and regenerative gallbladder mucosa probably reflects the re-expression of the hTERT gene as part of a multistage process of gallbladder carcinogenesis. Experimental studies have confirmed that re-expression of the hTERT gene in normal human somatic cells can reconstitute telomerase activity and extend the replicative life span of these cells beyond crisis, the last known proliferative blockade to cellular immortality.³⁹ Therefore, telomerase reactivation has been associated with the appearance of immortal cell populations, a crucial event in human carcinogenesis.⁴⁰ Furthermore, our results suggest that some cell clones might already be immortal in low grade epithelial dysplasia, because the hTERT protein positive nuclei were usually seen in clusters, probably representing the expansion of a single cell clone within the dysplastic gallbladder epithelium.

No direct data are available on the progression rate of high grade dysplasia/carcinoma in situ to invasive gallbladder adenocarcinoma. For comparison, high grade dysplasia in the stomach regressed in only about 5%, persisted in 14%, and progressed in most cases (81–85%).⁴¹ The time frame between a diagnosis of severe/high grade dysplasia and the identification of invasive gastric cancer was between less than one month and 39 months.⁴¹ Furthermore, data from the early 1960s show that carcinoma in situ of the larynx progressed to laryngeal squamous cell carcinoma in approximately 90% of cases in the subsequent six months.⁴²

Significantly higher numbers of hTERT protein signals for each nucleus were found in high grade dysplasia/intraepithelial carcinoma and adenocarcinoma than in normal gallbladder epithelium, regenerative epithelium, and low grade dysplasia. These data suggest that, as in low grade dysplasia, significantly higher hTERT indices in high grade dysplasia and adenocarcinoma are the consequence of hTERT re-expression. Namely, once telomerase reactivation has been established at a particular stage of gallbladder carcinogenesis (low grade dysplasia), it can be sustained in the subsequent

Take home messages

- Expression of the telomerase catalytic subunit (hTERT) increased progressively with the degree of gallbladder epithelial abnormality, probably as a consequence of hTERT re-expression—an early event in the multistep process of gallbladder carcinogenesis
- Three different groups of gallbladder epithelial changes could be distinguished according to the number of hTERT signals/nucleus: (1) normal and regenerative gallbladder epithelium, (2) low grade dysplasia, and (3) high grade dysplasia and adenocarcinoma
- The occasional presence of hTERT protein in normal and regenerative gallbladder mucosa probably reflects their regenerative capacity

stages, as we demonstrated here. Because telomerase reactivation conveys cellular immortality, cells can proliferate indefinitely, with clonal expansion and accumulation of additional genetic abnormalities, which drive the progression of epithelial changes towards gallbladder adenocarcinoma. Therefore, it is not surprising that similar numbers of hTERT signals in each nucleus are seen in high grade dysplasia/intraepithelial carcinoma and adenocarcinoma. Furthermore, these data suggest that genetic changes other than telomerase reactivation appear to be necessary for the progression of gallbladder epithelial abnormalities towards gallbladder adenocarcinoma. In agreement with our observations, down-regulation of p27^{Kip1} expression appears to be a late event in gallbladder carcinogenesis, associated with tumour progression and metastases.⁷

In conclusion, our study is the first to analyse the expression of hTERT protein in normal gallbladder epithelium, different grades of gallbladder epithelial abnormalities, and gallbladder adenocarcinoma. We have shown that the number of hTERT signals in each nucleus increases progressively with the degree of epithelial abnormalities. Statistical analysis revealed three groups of gallbladder epithelial changes, which differ significantly in the number of hTERT signals for each nucleus, namely: (1) normal and regenerative gallbladder mucosa, (2) low grade dysplasia, and (3) high grade dysplasia and adenocarcinoma of the gallbladder. Our results suggest that the occasional presence of hTERT protein in normal and regenerative gallbladder mucosa probably reflects its self renewal capacity—that is, regeneration. Nevertheless, significantly higher hTERT indices in dysplastic epithelium and gallbladder adenocarcinoma are probably the consequence of hTERT re-expression—an early event in the multistep process of gallbladder carcinogenesis.

Authors' affiliations

B Luzar, V Ferlan-Marolt, Institute of Pathology, Medical Faculty, University of Ljubljana, Korytkova 2, 1000 Ljubljana, Slovenia

M Poljak, Institute of Microbiology and Immunology, Medical Faculty, University of Ljubljana, Zaloška 4

A Cör, Institute of Histology and Embryology, Medical Faculty, University of Ljubljana, Korytkova 2

U Klopčič, Department of Cytopathology, Institute of Oncology, Zaloška 4, 1000 Ljubljana, Slovenia

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ECHO.....

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Molecular method simplifies diagnosis of infective endocarditis

Microbiologists in the UK are enthusiastic about the role of 16S rRNA sequencing in helping to identify endocarditis, after using it to diagnose a case of culture negative infective endocarditis.

Theirs was a classic case after previous recent antibiotic treatment, which sequencing confirmed as streptococcal endocarditis, but the method is as valuable for diagnosing endocarditis caused by fastidious bacteria, hazardous species, and rare pathogens.

The case was in a 62 year old previously fit man admitted after a month of vomiting, weight loss, and low grade fever, initially given antibiotics for suspected upper respiratory tract infection. However, tests disclosed acute renal failure with immune complex glomerulonephritis, and a transthoracic echocardiogram three days later showed a mobile mass on the right coronary cusp of the aortic valve. Empirical treatment for endocarditis based on probable streptococcal infection was started after three sets of blood cultures failed to grow pathogens.

After initial improvement fever returned on day 13, with a vasculitic rash on his legs and splinter haemorrhages on his metatarsals. Repeat echocardiography showed thickening of the entire left and right coronary cusps and severe aortic regurgitation, which led to valve replacement. The appearance was typical of subacute bacterial endocarditis histologically, with masses of Gram positive cocci. Cultures of the valve produced no growth, but 16S rRNA sequencing identified *Streptococcus sanguis*.

Traditional methods of diagnosis rely on culturing or serological testing for likely bacterial pathogens. But 10–15% of cases of infective endocarditis in the UK are culture negative, mostly because of previous antibiotic treatment.

▲ Shin GY, *et al*. *Heart* 2005;**91**:e47 (<http://www.heartjnl.com/cgi/content/full/91/6/e47>).