Pathology (April 2012) **44(3)**, pp. 209–215

ANATOMICAL PATHOLOGY

Telomerase expression as a predictive marker of radiotherapy response in rectal cancer: *in vitro* and *in vivo* study

Joo-Shik Shin*, Terence Foo†, Angela Hong‡¶, Mei Zhang‡, Trina Lum§, Michael J. Solomon||¶ and C. Soon Lee*§

*Department of Anatomical Pathology, Liverpool Hospital, and Discipline of Pathology and Cancer Biology Laboratory, School of Medicine, University of Western Sydney, Departments of †Clinical Biochemistry, ‡Radiation Oncology, §Anatomical Pathology, and ||Colorectal Surgery, Royal Prince Alfred Hospital, and ¶Clinical School of Medicine, University of Sydney, Sydney, New South Wales, Australia

Summary

Aims: To investigate telomerase as a predictive marker of radiotherapy response in rectal cancer.

Methods: Expression of the telomerase catalytic subunit hTERT was quantified with reverse transcription PCR in the radioresistant colorectal cancer cell line SW620 following exposure to 5Gy of radiation. Additionally, 52 rectal cancer cases were pre-operatively treated with either the short ($n=19$) or long ($n=33$) course radiotherapy (SCR, LCR, respectively) regimes, before and after which their hTERT expressions were semi-quantified with immunohistochemistry (IHC). This was correlated with the histological tumour regression in the resected bowel, dichotomised into good and poor responses.

Results: SW620 cells expressed gradually increasing levels of hTERT after radiation. hTERT IHC positivity of $\leq$75% tumour cells in pre-radiotherapied cancer was the optimal negative cut-off level [sensitivity 63.2%, specificity 45.8%, area under curve (AUC) 0.5362] in predicting good tumour response. As significantly more LCR cases showed good tumour response ($p<0.0001$), the SCR cases were excluded and AUC re-analysed, which still remained low (0.5357).

Conclusions: While our *in vitro* results suggest that hTERT up-regulation may contribute to radiation resistance of colorectal cancer cells, our *in vivo* results demonstrated poor ability of hTERT IHC in predicting histological tumour regression in rectal cancer.

Key words: Colorectal cancer, radiotherapy, rectal neoplasms, telomerase, tumour regression.

Received 8 June, revised 27 September, accepted 4 October 2011

INTRODUCTION

For curative surgical resection of low lying rectal cancers, a complete circumferential excision of the accompanying mesorectum with clear radial margins improves local disease control and ensures an adequate removal of the regional lymph nodes.[1] However, this can be especially challenging for locally advanced primary tumours, given the anatomical constraints of the pelvic floor that limit operative access. Radiotherapy may facilitate such surgery by pre-operative shrinkage of tumour volume, although the variable efficacy and the potential adverse effects of radiotherapy necessitate a practical means of predicting the extent of tumour response to this neoadjuvant treatment. Such an assessment would conceivably be based on molecular marker(s) that reflect tumour cell resistance or sensitivity to radiation induced death, which occurs mainly as a result of DNA damage in the form of double strand breaks.

Finding reliable such marker(s) has proved difficult. Some of the candidate molecules which have more recently demonstrated their potential to fulfill this role include those that are directly relevant to DNA damage response following irradiation, such as anti-apoptotic proteins bcl-2[2] and survivin,[3] as well as DNA double strand breaks recognising Ku80,[4] in addition to those that potentiate the malignant behaviour of the tumour cells, such as proliferation promoting epidermal growth factor receptor[5] and angiogenesis sustaining vascular endothelial growth factor.[2]

The telomerase enzyme, with its essential catalytic subunit reverse transcriptase (hTERT), is investigated here as a possible predictive marker in this context. Its substrates are the telomeres that comprise and protect the chromosomal ends. The repetitive sequence[6] of these DNA-protein complexes progressively shortens with successive mitoses, spawning the genomic instability that simultaneously limits the cellular replicative span whilst raising the possibility of new mutations that instead initiate oncogenesis. Considered necessary for the longevity and malignant progression of the thus transformed cell is telomerase expression or other means to negate the constitutional telomeric loss.

Of the proteins in complex with telomeric DNA are some of the established factors that are integral to the cellular DNA damage response after radiation injury, such as the Ku molecules that initially recognise and bind to DNA double strand breaks and the MRE11 complex that facilitates subsequent cell cycle arrest.[7] This has two implications. Firstly, proteins of the DNA damage response pathways may be necessary for the adequate functioning and maintenance of the telomeres. Secondly, critical shortening of the telomeric DNA as cells replicate and approach senescence may be accompanied by the loss of these proteins and thus demonstrate radiosensitivity. Supportive evidence can be found in two disorders of impaired DNA damage response, namely ataxia telangiectasia and Nijmegen breakage syndromes. Significantly increased rates of telomeric shortening per population doubling were seen in cell lines from these patients,[8] and exquisite sensitivity to ionising radiation is a clinical hallmark of these entities.[9] Likewise, an inverse relationship between

Print ISSN 0031-3025/Online ISSN 1465-3931
DOI: 10.1097/PAT.0b013e3283511cd5

telomere length and radiosensitivity has been observed in experimental models of both murine and human cancer cell lines.[10,11]

Compatible with the above findings is the hypothesis that the functions of normal telomeres and that of the DNA damage response are, at least in part, intrinsically related. Accordingly, as the level of telomerase expression in cancer cells should reflect the length and hence the state of their telomeres, it may in turn reciprocally predict their radiosensitivity.

The *in vitro* expression of hTERT was therefore quantitatively assessed at intervals following irradiation of SW620, a human colorectal cancer cell line which previously showed a relative resistance to radiotherapy.[12] In addition, the *in vivo* appraisal of hTERT expression was semi-quantitatively conducted in tissue microarrays (TMAs) of 52 rectal cancers both before and after radiotherapy, the values of which were then correlated with the histological extent of tumour regression. Further details are as detailed below. Appropriate approvals were obtained from the Sydney South West Area Health Service Ethics Review Committee.

MATERIALS AND METHODS

In vitro study

Cell lines and irradiation

Cells of human colorectal cancer SW620[13] and normal rectal epithelium Hs680.Rec were cultured, maintained in exponential growth as monolayers at 37°C in 5% CO_2/95% air, in RPMI 1640 medium (Thermo Trace, Australia) supplemented with 2 mM glutamine and 10% fetal calf serum. Routine testing for Mycoplasma was performed. Subsequently, these cells were treated with a single dose of 5 Gy using Varian Clinac 21Ex at 6 Gy/min. Radiation dose homogeneity was estimated at ±5% using existing clinical depth-dose data for the linear accelerator (Varian, Australia).

Total RNA extraction

At 2, 4, 6, 24 and 48 hours post-radiation, cells were trypsinised and washed with phosphate buffered saline (PBS) prior to collection as pellets through centrifugation at 1000 g for 5 min at 4°C. Total RNA was then extracted and purified using Qiagen RNeasy kit (Qiagen, Australia). The amount and purity of RNA obtained was determined spectrophotometrically.

Primers

Primers for hTERT and TATA box binding protein (TBP; endogenous reference) genes were purchased from Sigma-Proligo (Australia). The forward and reverse primer sequences were 5′ CGTACAGGTTTCACGCATGTG 3′ and 5′ ATGACGCGCAGGAAAAATG 3′, respectively, for hTERT[14] (PCR product size 176 bp); 5′ CACGAACCACGGCACTGATT 3′ and 5′ TTTTCTTGCTG CCAGTCTGGAC 3′, respectively, for TBP[15] (PCR product size 89 bp).

Real-time quantitative RT (reverse transcription)-PCR

RNA RT was carried out with Bio-Rad iScript cDNA synthesis kit (Bio-Rad, Australia) in a final volume of 10 μL containing a constant 0.2 μg of RNA. The samples were incubated at 25°C for 5 min then 42°C for 30 min, and reverse transcriptase was heat inactivated at 85°C for 5 min. For cDNA amplification, 4 μL aliquots of reverse-transcribed RNA were subjected to PCR with Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen, Australia) in a final volume of 25 μL. The reactions and analyses were performed in a Rotor-Gene 2000 real-time thermal cycler (Corbett Research, Australia). Initial activation of uracil-N-glycosylase (UDG) activity at 50°C for 2 min, then denaturation of UDG and activation of Taq DNA polymerase at 95°C for 2 min were common to reaction steps of both hTERT and TBP transcripts. These were then followed by, for hTERT, 40 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 30 s and acquisition of fluorescence signal at 82°C for 15 s; for TBP, 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C for 30 s, extension and acquisition of fluorescence signal at 72°C for 15 s. A melt analysis followed all PCR to confirm assay specificity.

Statistical analysis

Subsequent quantification of the relative expression of the hTERT gene was performed using the relative expression software tool (REST version 2) by Pffafl *et al*.[16] This Microsoft Excel based software consists of two steps: the initial calculation of the relative gene expression, followed by a statistical test for significance. The first step is based on the following formula, which corrects for the different PCR efficiencies (E) between the target (i.e., hTERT) and reference housekeeping (i.e., TBP) genes:

Ratio of relative expression of target gene in sample over control

$$= \frac{(E_{\text{target}})^{\Delta Ct\,(\text{MEAN control}-\text{MEAN sample})}}{(E_{\text{reference}})^{\Delta Ct(\text{MEAN control}-\text{MEAN sample})}}$$

For this study, adaptation of the above formula gives the following equation:

Ratio of relative expression of hTERT in irradiated SW620 over non-irradiated control

$$= \frac{(E_{\text{hTERT}})^{\Delta Ct(\text{MEAN non-irradiated SW620}-\text{MEAN irradiated SW620})}}{(E_{\text{TBP}})^{\Delta Ct\,(\text{MEAN non-irradiated SW620}-\text{MEAN irradiated SW620})}}$$

The ensuing test for statistical significance uses the mathematical model of a randomisation or permutation test, in which the Ct values for the matching target and reference genes are re-allocated together into either the sample or control groups during the procedure (so-called pair wise fixed re-allocation).

Based on the above principles, difference in the relative expression of hTERT (normalised to TBP) between irradiated and non-irradiated SW620 cells were assessed at each of the time points following exposure. For both hTERT and TBP, the respective means of PCR Ct values of non-irradiated control cells at 2, 24 and 48 h were used for calculation of delta Ct values at 4 and 6 h, as corresponding control cells at these time points were not collected. p values <0.05 were considered statistically significant.

In vivo study

Patients and radiotherapy

Fifty-two patients with primary rectal adenocarcinoma, who were treated at Royal Prince Alfred Hospital (Sydney, NSW, Australia) between 1998 and 2004 were randomly selected retrospectively and included in this study. They received pre-operative radiotherapy as either the short course (in 19 patients; 25 Gy in 5 fractions over 1 week) or the long course regime [in 33 patients; 50.4 Gy in 28 fractions over 5 weeks, with (for all but 1 patient) 5-fluorouracil in the first and last weeks], followed by surgical resection within 1 and 4–6 weeks, respectively.

Histological assessment of tumour regression

The extent of tumour regression following radiotherapy was graded in the resection specimens by reviewing the archived histological slides of the treated tumour. These were scored as per the tumour regression grade (TRG) scale by Mandard *et al*., described initially in the assessment of chemoradiotherapy response of oesophageal carcinomas[17] and later demonstrated as also applicable to irradiated rectal adenocarcinomas.[18] The sound inter-observer reliability of this grading system has been previously reported,[19] the five tier scale of which is as follows:

1. Complete regression with fibrosis only. No residual tumour seen.
2. Rare residual single cells or small aggregates of tumour scattered amongst fibrosis.
3. Residual large aggregates of tumour present, but fibrosis still predominates.
4. Abundant residual tumour outgrowing fibrosis.
5. Tumour without regression.

TMA

Formalin fixed, paraffin embedded tissues of each patient, including the primary tumour at initial diagnosis and the same tumour following radiotherapy in the resection specimen, were collected from the archives of the aforementioned institution. TMAs were subsequently built using the Beecher Manual tissue arrayer-1 (Beecher, USA), which consists of 1 mm diameter tissue cores embedded serially in a grid arrangement into multiple paraffin blocks (each produced from a standard 37 × 24 × 5 mm mould) (Fig. 1A). To orientate the ensuing TMA sections and to serve as positive control for hTERT