ORIGINAL ARTICLE

Telomerase activation and human papillomavirus infection in invasive uterine cervical carcinoma in a set of Malaysian patients

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Aim: Telomerase activity was studied in invasive uterine cervical carcinoma to assess whether it was activated during cervical malignant transformation and to look for a possible association with human papillomavirus (HPV) infection in a set of Malaysian patients.

Methods: Histologically confirmed invasive cervical carcinoma and benign cervices were assayed for telomerase activity using a commercial telomerase polymerase chain reaction (PCR) enzyme linked immunosorbent assay kit. The same cases were subjected to PCR detection of HPV using type specific (HPV types 6b, 11, 16, and 18) followed by L1 open reading frame (ORF) consensus primers.

Results: HPV was detected in 18 (13 HPV-16, one HPV-6b, four only L1 ORF) of 20 invasive cervical carcinoma and one (only L1 ORF) of 19 benign cervices. Raised telomerase activity ($A_{450\ nm} > 0.215$) was detected in 11 cervical carcinomas, with $A_{450\ nm}$ ranging between 0.238 and 21.790 (mean, 3.952) in positive squamous carcinomas, whereas $A_{450\ nm}$ was only 0.222 in the one positive adenosquamous carcinoma. Five of 11 cervical carcinomas in stage I, three of six in stage II, both in stage III, and the only case in stage IV showed telomerase activation. Increased telomerase activity was noted in five of the 12 lymph node negative, five of the seven lymph node status unknown cases, and the one case with presumed lymph node metastasis. Ten of 18 HPV positive and one of two HPV negative cervical carcinomas showed telomerase upregulation.

Conclusions: Telomerase is activated in invasive cervical carcinoma. Although larger studies are needed, there seems to be no clear association between telomerase upregulation and HPV status, although there is a suggestion of increased telomerase activity in squamous carcinomas and late stage disease.

Telomeres are specialised, non-coding regions at the ends of eukaryotic chromosomes comprising tandem TTAGGG repeats, with estimated lengths of 5–15 kb in humans.[1–8] Telomeres protect chromosomes from enzymatic breakdown of DNA,[9 10] and prevent aberrant recombinations and fusions, thus ensuring complete replication of the chromosome during cell division.[5 7] However, cellular DNA polymerase can normally only extend on an existing strand of RNA primer, synthesising DNA in a 5′ to 3′ direction. Therefore, a DNA segment equivalent in length to the RNA primer required for DNA replication is lost from the 5′ end of each DNA daughter strand with each replication, giving rise to the "end replication problem".[11 12] Hence, in most human somatic cells telomeres are lost with each replication.[5] At a critical telomere length (Hayflick limit), the cell undergoes replicative senescence and exits the cell cycle.[6 13] This progressive telomere shortening, likened to a "mitotic clock" by which cells count their divisions, is indicative of the residual cellular replicative capacity.[14 15]

However, normal germline and stem cells escape the inevitability of telomere shortening by expressing telomerase, a ribonucleoprotein enzyme that synthesises telomeric DNA on to chromosomal ends.[16 17] The induction of telomerase expression in normal human epithelial cells and fibroblasts[18] results in infinite replication and telomerase activity is evident in approximately 90% of human solid tumours,[19 20] suggesting that the expression of telomerase has a role in malignant transformation.

Subscribing to the current concept that the human papillomavirus (HPV) has an important aetiological role in cervical carcinogenesis,[21] and that most cervical carcinomas are associated with human papillomavirus (HPV) infection, we designed our study to determine whether (1) telomerase activity is indeed raised in uterine cervical carcinoma in a set of Malaysian patients and (2) there is an identifiable relation with HPV infection.

MATERIALS AND METHODS

Materials

Patients histologically diagnosed for the first time with cervical carcinoma at the University of Malaya Medical Centre in whom fresh, unfixed tumour tissue was available for study were considered for our study. Benign cervical tissue came from hysterectomies with or without salpingo-oophorectomies, performed for non-malignant lesions in the uterine corpus or ovary, in which adequate fresh, unfixed cervical tissue was available for study. Two pieces (one for HPV polymerase chain reaction (PCR) analysis and the other for telomerase assay) of the fresh benign or malignant cervical tissue were cut with a new scalpel blade from each sample and placed in separate metal foil containers, snap frozen in liquid nitrogen, and stored at −80°C. The diagnosis of each patient was reconfirmed by reviewing all the histological slides of that case. Before digestion and extraction of proteins and DNA from the fresh frozen tissue, one cryostat section stained with haematoxylin and eosin was made from each tissue to confirm the presence and adequacy of tumour

Abbreviations: $A_{450\ nm}$, absorbance at 450 nm; ELISA, enzyme linked immunosorbent assay; HPV, human papillomavirus; ORF, open reading frame; PCR, polymerase chain reaction

or benign cervical epithelium. In the event that the fresh frozen tissue was unsuitable for HPV PCR analysis, a paraffin wax block containing formalin fixed tumour or benign cervical epithelial tissue obtained from the same surgical specimen served as an alternative source of tissue for study.

Determination of HPV status by PCR analysis

HPV status was determined by PCR using type specific (HPV types 6b, 11, 16, and 18) and L1 open reading frame (ORF) consensus primers (MY09/MY11). DNA template was prepared by digestion of two 10 μm sections of the fresh frozen or formalin fixed, paraffin wax embedded tissue with 0.06 mg/ml proteinase K in 10 mM Tris, 0.1 mM EDTA (pH 8.0) at 48°C for five days.[22] The DNA was extracted with phenol/chloroform/isoamyl alcohol (25/24/1), with subsequent precipitation in 0.5 volume 7.5 M ammonium acetate and 2.5 volume ethanol. DNA template was only considered suitable for HPV PCR on amplification of a 268 bp human β globin segment using primers GH20/PCO4. For HPV PCR, 30 cycles of amplification were carried out, with DNA denaturation at 94°C for one minute, annealing at 55°C for two minutes, and extension at 72°C for three minutes, using the respective type specific or L1 ORF consensus primers, in an automated thermal cycler (DNA Thermal Cycler 480; Perkin Elmer, Norwalk, Connecticut, USA). Single bands of expected amplicon lengths identified on 2% agarose gel electrophoresis were considered positive and HPV type specific PCR positive cases were confirmed by dot blot in situ hybridisation using digoxigenin labelled oligoprobes. Cloned plasmids with full HPV genomes (HPV types 6b, 11, 16, and 18) served as positive controls for the relevant specific HPV types, whereas any of the cloned plasmids served as the positive control for L1 ORF consensus PCR, and were included in each amplification run. Heart muscle, acquired at postmortem examination from a patient who died of multiple injuries sustained in a motor vehicular accident, which was β globin segment amplifiable and known not to contain HPV DNA, was used as a negative control with each batch of HPV PCR analysis.

Telomerase activity assay

The assay was carried out using a telomerase PCR enzyme linked immunosorbent assay (ELISA) kit (Boehringer Mannheim) in accordance with the manufacturer's recommendations. Based on the telomeric repeat amplification protocol,[19] the method incorporated photometric enzyme immunoassay detection of the generated telomeric repeats. When present in the test samples, telomerase catalysed the addition of TTAGGG repeats[16 17] on to the synthetic biotin labelled primer using its intrinsic RNA as template. The extended biotin labelled telomeric products were further amplified using the above synthetic biotin labelled primer as one of the PCR primers in an automated thermal cycler (Perkin Elmer DNA thermal cycler 480), with 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 90 seconds. Controls were run with each batch and included a positive from the kit, made up of immortalised telomerase positive embryonic kidney cell line 293. The negative control consisted of 50 μg of extracted sample protein from a cervical carcinoma, previously shown to exhibit telomerase PCR ELISA assay absorbance $A_{450\ nm} > 1.5$ against a blank (reference wavelength of 690 nm), pretreated with 1 μg/μl DNase free RNase (Boehringer Mannheim, Mannheim, Germany) for 20 minutes at 37°C. Blanks, consisting of substitution of ultrapure distilled DNase, RNase free water for the sample, were also included in each run. Amplified biotin labelled telomeric products were denatured, hybridised with digoxigenin labelled TTAGGG specific probes, and immobilised in streptavidin coated microtitre plates.

Table 1 Demographic profile of cases of benign cervices (n=19), versus cervical carcinoma (n=20) assayed for telomerase activity

	Benign cervices	Cervical carcinoma
Age		
Range (years)	41–83	26–68
Mean (years)	49	46
Race		
Malay	5	1
Chinese	10	15
Indian	3	3
Others	1	1

Probe visualisation was via peroxidase conjugated antidigoxigenin–probe digoxigenin reaction and ensuing peroxidase action on 3,3′,5,5′-tetramethyly benzidine. Positivity was detected when the substrate turned blue and then yellow on addition of the stop reagent, the colour conversion maximising sensitivity of the readings. Sample absorbance was measured within 30 minutes using an ELISA reader at 450 nm ($A_{450\ nm}$) against a blank (reference wavelength of 690 nm).

Each batch of analysis was accepted as satisfactorily run only when the positive control had an $A_{450\ nm}$ of at least 1.5 and the negative an absorbance of not more than 0.25. All cases were initially tested with a sample protein concentration of 1 μg/μl. When telomerase activity was not raised, the case was re-tested at a protein concentration of 0.02 μg/μl. Whenever re-testing was conducted, the higher of the two sample absorbance values obtained was accepted if there was a difference between the two values.

RESULTS

Telomerase activity was assayed and HPV PCR detection was carried out in 20 invasive cervical carcinomas and 19 benign cervices. Table 1 shows the demographics of the cases of cervical carcinoma versus benign cervices entered into our study. The mean ages of both categories of patients were in the 4th decade. Ethnically, there was a preponderance of Chinese in the malignant category, with Chinese patients forming 75% of the cases, whereas they formed 53% of the benign cases. Histologically, the cervical carcinomas comprised 17 squamous carcinomas (10 non-keratinising, six keratinising, one lymphoepithelioma-like), two adenocarcinomas, and one adenosquamous carcinoma based on the histological classification system of the World Heath Organisation.[23] Eleven were in FIGO[24] stage I, six in stage II, two in stage III, and one in stage IV. Lymph nodes in the areas of drainage were sampled in 12 cases with none revealing metastatic disease. In seven, lymph nodes were not sampled. A supraclavicular lymph node was clinically palpated in the only patient with stage IV disease and assumed to be a tumour metastasis from the cervical carcinoma. Benign cervices came from hysterectomies, with or without salpingo-oophorectomies performed for benign ovarian cystic teratoma (two), ovarian serous cystadenoma (one), uterine leiomyoma (eight), uterovaginal prolapse (five), adenomyosis (one), benign endometrial polyp (one), and placenta praevia (one). Table 2 shows the HPV status and telomerase PCR ELISA assay activity ($A_{450\ nm}$) of the benign cervices (with reference to histological background) and cervical cancers (with reference to histological diagnosis, stage, and lymph node status). Statistical analysis was carried out using Fischer's exact test.

Table 2 HPV status and telomerase PCR ELISA assay absorbance read at 450 nm with a reference wavelength of 690 (A_{450}) versus histological background of benign cervices (n=19) and histological diagnosis, stage, and lymph node status of cervical carcinoma (n=20)

Benign cervices				Cervical carcinoma					
Case	Histological background	A_{450}	HPV	Case	Histological diagnosis	Stage	LN	A_{450}	HPV
1	Uterovaginal prolapse	–0.032	L1	1	Squamous, non-keratinising	II	–	1.469	L1
2	Leiomyoma	–0.028	–	2	Squamous, non-keratinising	I	–	3.781	16
3	Leiomyoma	–0.069	–	3	Squamous, non-keratinising	IV	+	1.935	16
4	Benign ovarian teratoma	0.032	–	4	Squamous, keratinising	I	NA	1.841	16
5	Leiomyoma	0.081	–	5	Squamous, keratinising	I	–	0.173	16
6	Leiomyoma	0.135	–	6	Squamous, keratinising	I	–	0.209	L1
7	Leiomyoma	0.011	–	7	Squamous, non-keratinising	II	NA	3.163	16
8	Benign ovarian teratoma	–0.017	–	8	Squamous, non-keratinising	I	–	1.632	6
9	Uterovaginal prolapse	–0.020	–	9	Adenocarcinoma	II	–	0.178	16
10	Adenomyosis	0.038	–	10	Adenocarcinoma	I	–	0.017	16
11	Placenta praevia	–0.073	–	11	Squamous, non-keratinising	III	NA	0.238	16
12	Leiomyoma	0.349	–	12	Squamous, non-keratinising	II	NA	–0.006	L1
13	Ovarian serous cystadenoma	0.096	–	13	Squamous, non-keratinising	I	–	0.067	–
14	Uterovaginal prolapse	–0.002	–	14	Squamous, non-keratinising	I	–	21.790	L1
15	Leiomyoma	0.015	–	15	Lymphoepithelioma-like	I	–	0.013	16
16	Leiomyoma	0.019	–	16	Squamous, non-keratinising	III	NA	3.045	16
17	Endometrial polyp	0.006	–	17	Squamous, keratinising	I	–	0.075	16
18	Uterovaginal prolapse	0.012	–	18	Adenosquamous	I	–	0.222	–
19	Uterovaginal prolapse	0.003	–	19	Squamous, keratinising	II	NA	–0.001	16
				20	Squamous, keratinising	II	NA	0.630	16

ELISA, enzyme linked immunosorbent assay; LN, lymph node status; NA, information not available.

HPV status

β Globin DNA was amplified in all fresh frozen samples of the benign cervices and 16 of the cervical carcinomas, which were subsequently subjected to HPV PCR. In the four cervical carcinomas with unsuitable fresh frozen tissue, HPV PCR was carried out in the β globin DNA amplifiable formalin fixed, paraffin wax embedded tissue. HPV was detected in one benign cervix and 18 cervical carcinomas. HPV-16 was identified in 13 cervical carcinomas and HPV-6b in one. Four cases negative on HPV type specific PCR showed HPV DNA on L1 ORF consensus PCR. The single HPV positive benign cervix was negative on type specific PCR, with HPV DNA detected only on L1 ORF consensus PCR.

Telomerase activity

Using the telomerase PCR ELISA kit, the telomerase activity ($A_{450\ nm}$) of the benign cervices ranged between –0.073 and 0.349, with a mean (2SD) of 0.029 (0.215). Therefore, 0.215 was accepted as the upper limit of the benign range.

In contrast, $A_{450\ nm}$ ranged between –0.006 and 21.790 in cervical carcinoma. $A_{450\ nm}$ was between –0.006 and 21.790 in the squamous carcinomas and between 0.017 and 0.222 in the adeno/adenosquamous carcinomas. Eleven cervical carcinomas, 10 squamous carcinomas, and one adenosquamous carcinoma had $A_{450\ nm}$ values above the cut off value. Of the 10 squamous carcinomas, eight non-keratinising and two keratinising carcinomas had $A_{450\ nm}$ values above the cut off point. Five of 11 stage I, three of six stage II, both stage III, and the only stage IV cancer showed $A_{450\ nm}$ above the cut off value. Five of 12 samples negative for lymph node metastasis and five of seven with unknown lymph node status had $A_{450\ nm}$ values above the cut off point. The only case with presumed lymph node metastasis was above the cut off value.

All the benign cervices and cervical carcinomas showed some inflammation. No apparent correlation was noted between the visual estimation of the amount of inflammatory cells and telomerase activity.

Correlation between HPV status and telomerase activity

Telomerase activity above the cut off point was found in 10 of 18 HPV positive and one of the two HPV negative patients. Of the 10 HPV positive invasive cervical carcinomas with telomerase activity above the cut off value, HPV-6b was identified in one, HPV-16 in seven, and only L1 ORF in two. In comparison, HPV-16 was identified in six and only L1 ORF in two of the eight HPV positive patients with telomerase below the cut off point.

DISCUSSION

Eleven of the 20 cervical carcinomas had absorbance values above the upper limit of the benign range. This is indicative of an increased number of telomeric repeats synthesised in cervical carcinomas compared with their benign counterparts, indirectly implying higher telomerase activity in the malignant category, and supporting telomerase induction in cervical carcinogenesis. Nonetheless, the proportion of invasive cervical carcinomas in our study estimated to show telomerase activation is lower than the 86–100% reported by other workers.[25–33] Although we attempted to eliminate PCR inhibitors by diluting the samples and used an ELISA method that was efficient in the assay of telomerase,[34 35] technical failure of ELISA and PCR can never be completely excluded. Nevertheless, our results do not differ from those reported by Mutirangura *et al*. Although telomerase activation was noted in 97% of invasive cervical carcinomas, it was also found in 46% of benign cervices in their study.[28] Hence, if a benign range of telomerase activity had been considered and an arbitrary cut off value applied (as in our study), a certain percentage of invasive cervical carcinoma may not have been considered "positive". It is also interesting to note that Mutirangura *et al* studied telomerase activity in a set of Thai patients, whereas the other studies quoted came from centres in Japan, Hong Kong, the Netherlands, and the USA. Thailand is an immediate neighbour of Malaysia and it is possible that geography influences the pathogenesis of cervical carcinoma in terms of telomerase activation.

Recently, some studies have suggested that telomerase induction is associated with high risk HPV infection,[29 36 37] whereas others were unable to demonstrate a clear association.[28 32 38 39] No correlation was apparent between telomerase activation in invasive cervical carcinoma and HPV status in our study, and the single benign cervix with HPV infection had telomerase activity towards the lower end of the normal range. Furthermore, high risk HPV-16 was

Take home messages

- Telomerase activity is increased in invasive cervical carcinoma
- There seems to be no clear association between telomerase upregulation and HPV status

distributed equally among the patients with and without telomerase activation. The role and point of effect of telomerase activity in cervical carcinogenesis needs further clarification in view of the fact that it is increased apparently irrespective of HPV status, and in some and not all invasive cervical carcinomas.

Like Zhang *et al*,[25] we found that telomerase activity was higher in all stages of cervical carcinoma, suggesting that it is an early event in the course of the disease. Approximately half of stage I and II tumours showed raised telomerase activity compared with all stage III and IV samples; however, because the number of cases was small firm conclusions cannot be made. Five of the 12 patients histologically confirmed to be without lymph node metastases, five of the seven in whom lymph node status was unknown, and the single patient with clinically assumed lymph node metastasis had telomerase activity above the cut off point. The higher proportion of those with unknown lymph node status exhibiting telomerase activity above the cut off point compared with node negative patients is interesting. Although purely speculative, it is possible that lymph node involvement was present in a proportion of the cases with unknown histological lymph node status, and could have contributed to the higher number of patients in this category exhibiting absorbance above the cut off point. More detailed investigations into the possibility of increased telomerase expression in tumours with lymph node metastasis may be worthwhile.

Although statistical analysis showed no significant difference between telomerase upregulation in keratinising and non-keratinising squamous carcinomas ($p > 0.05$), or between squamous carcinomas and adenocarcinomas ($p > 0.05$), $A_{450\ nm}$ values above the cut off point were noted in 10 of the 17 squamous (eight of 10 non-keratinising and two of six keratinising) and only one of the three glandular carcinomas. It is also interesting to note that the only case of adenosquamous carcinoma that had an absorbance above the cut off point (0.215) had a value of 0.222, only minimally above the cut off point. In contrast, the absorbance of the squamous carcinomas that were above the cut off value ranged from 0.238 to 21.790 (mean, 3.952), with seven of the 10 cases showing $A_{450\ nm} > 1.500$. Therefore, it is possible that telomerase activation is a more prominent process in squamous carcinomas of the cervix compared with glandular malignancies, but this requires further elucidation with studies on larger numbers of patients.

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