Amplification of the Telomerase RNA Component Gene in the Process of Human Esophageal Carcinogenesis

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Amplification of the human telomerase RNA component (hTERC) gene occurs early in cervical cancer development. Telomerase, the product of the hTERC gene, plays an important role in tumor cell apoptosis and genomic stability. Given the numerous similarities between esophageal and cervical cancers, we hypothesized that hTERC gene amplification may also be related with esophageal cancer development. We therefore examined 189 tissue sections from 63 cases of esophageal cancer and preneoplastic lesions. hTERC gene amplification in the lesions was detected by interphase fluorescence in situ hybridization. Of the 189 tissue sections, 149 were successfully evaluated (40 samples were excluded because of inappropriately preparation) and were classified as normal (n = 45), atypical hyperplasia I (n = 27), atypical hyperplasia II/III (n = 22), and squamous cell carcinomas (SCCs; the most common type of esophageal cancer) (n = 55). hTERC gene expression was not detected in normal esophageal tissue, whereas its expression was detected in atypical hyperplasias I (25.9%), atypical hyperplasia II/III (54.5%), and SCCs (90.9%) (p < 0.05). The average copy numbers of hTERC in atypical hyperplasias I and II/III, as well as SCCs were 2.19, 2.35, and 2.64, respectively. In particular, the numbers of abnormal nuclei in atypical hyperplasias II/III were significantly higher than those of in atypical hyperplasia I (p < 0.05). The hTERC gene amplification was not related with patient gender, histological stage, lymph nodes metastasis, and SCC differentiation grade (p > 0.05). All these findings suggest that hTERC gene amplification is associated with SCC development.

Keywords: esophageal cancer; fluorescence in situ hybridisation; gene amplification; human telomerase RNA component; telomerase

Tohoku J. Exp. Med., 2011, 224 (2), 99-104. © 2011 Tohoku University Medical Press

Esophageal cancer is one of the leading causes of cancer death, especially in developing countries. Squamous cell carcinoma (SCC) is the most common type of esophageal cancer in high-risk areas, but its etiology remains poorly understood. Early-stage esophageal cancer symptoms are also often not obvious. Recent studies demonstrated that invasive cervical carcinomas almost invariably conferred extra copies of chromosome arm 3q. As a result, the human telomerase RNA component (hTERC) gene, located in the chromosome 3q26 region, is amplified (Heselmeyer-Haddad et al. 2003, 2005). Telomerase, the product of the hTERC gene, directs the synthesis of a species-specific telomeric repeat sequence (5'-CUAACCCUA AC-3'). Telomerase also plays an important role in tumour cell apoptosis and genomic stability (Gordon and Santos 2010). Abnormal hTERC gene expression occurs early in cervical cancer formation (Umayahara et al. 2002; Hopman et al. 2006; Olaharski et al. 2006; Kloth et al. 2007). Additional copies of the long chromosome arm 3q also frequently appeared in esophageal cancers (Wang et al. 2006; Heselmeyer-Haddad et al. 2003). To date, the occurrence of abnormal hTERC gene expression in early-stage SCC formation remains unconfirmed.

In the present study, we examined hTERC gene copy numbers in esophageal cancer and preneoplastic lesions using interphase fluorescence in situ hybridization (FISH) with a chromosome enumeration double-colour DNA probe.

Patients and Methods

Tumor samples

Formalin-fixed paraffin-embedded tumor tissue specimens from 63 primary SCCs were obtained. They were diagnosed and surgically treated from January 2007 to December 2009 at the First Affiliated Hospital, Henan University of Science and Technology. All patients were identified by the Pathological Center of the Henan University of Science and Technology, Luoyang, 471003, P.R. China.

Received March 9, 2011; revision accepted for publication April 27, 2011. doi: 10.1620/tjem.224.99
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All tumor samples were collected with informed consent and approval from the ethics committee of the First Affiliated Hospital of Henan University of Science and Technology, China. The histopathological diagnosis was performed according to WHO criteria. The 63 tumors classified as SCCs were all without any squamous cell component. Based on histologic biopsies and pathologic diagnoses, 149 thin-layer slides from the 63 cases were divided into normal (n = 45), atypical hyperplasia I (n = 27), atypical hyperplasia II/III (n = 22), and squamous carcinomas of the esophagus (n = 55).

**FISH and signal enumeration**

A probe panel based on the hTERC gene sequence was designed to evaluate the gain of chromosome 3q. The probes and the FISH Kit were provided by the Beijing GP Medical Technology Co., Ltd. (China). The panel consisted of two probes: a centromer 3 with spectrum green (SG) and a bacterial artificial chromosome (BAC) contig. The BAC contig contained the hTERC gene sequence on chromosome region 3q26, which was labelled with spectrum red (SR) (Heselmeyer-Haddad et al. 2003). All probes were provided by the JinPuJia Company (China). Before in situ hybridization, the slides were removed by incubating them with xylene. They were rehydrated in an ethanol series (100%, 85%, and 70%) at room temperature for 2 min, and were boiled at 100°C for 40 min. Pepsin digestion, washing twice in 2 x saline sodium citrate (SSC) for 5 min, and dehydridation in an ethanol series (70%, 85%, and 100%) for 2 min followed. After the slides were denatured in 70% ammonium amide at 77°C for 5 min, they were gradient dehydrated in the above-mentioned ethanol series for 2 min at −20°C. After overnight hybridization at 42°C, the coverslips were gently removed. The slides were then washed in 2 x SSC for 5 min, rinsed with 0.3% NP40 for 2 min, and rewarshed with 0.1% NP40 for 30 s as well as 70% ethanol for 3 min. Finally, the slides were counterstained with 4, 6-diamidino-2-phenylindole (DAPI) and were embedded in an antifade solution. Microscopic images were acquired using an OLYMPUS B microscope equipped with custom optical filters for DAPI, SG, and SR, as well as a Plan Apo objective.

The signals were visually evaluated by screening the entire slide for the 3q probe (using the SR-specific optical filter). Cells with the normal 3q signal number were recorded as "diploid". Cells with abnormal 3q signal numbers were registered in patterns in the relocation charts for the whole probe panel. More than 2 hTERC signals and more than 2 centromeric region of chromosome 3 (CSP3) signals were used to define abnormal nuclei. The cutoff value was set at 17.3 per 100 random nuclei displaying increased hTERC signals and/or tumor ploidy. This cutoff value was based on 35 normal tissues (threshold = mean value ± 3 x standard deviation) (Solomon et al. 2002). A case was considered positive for the 3q assay when more than 17.3% of the cells exhibited more than 2 signals. Fisher’s exact test was used for a 2 x 2 contingency table analysis of the categorical data. The two categorical variables used were pathological assessment (progression and regression) as well as genomic aberration data. The two categorical variables used were pathological assessment (progression and regression) as well as genomic aberration data.

**Results**

**Hybridization results from the hTERC/CSP3 gene probe**

The probe panel was hybridized to 189 cases of thin-layer slides, including 63 cases of esophageal cancer and preneoplastic lesions. Among the 189 cases, 149 were histologically biopsied and pathologically diagnosed successfully. All 149 slides were independently screened and were classified as follows: normal (n = 45), atypical hyperplasia I (n = 27), atypical hyperplasia II/III (n = 22), and SCC (n = 55).

From the 45 normal tissues, 35 cases were selected for the random independent screening. The threshold mean value for the 35 normal tissues was 7.1, and the standard deviation was 3.4. A case was considered positive for the 3q assay when more than 17.3% of the cells exhibited a TERC signal number more than 2. This “gold standard” is based on a recent cervical cancer study (Solomon et al. 2002).

Diploid nuclei (nuclei with 2 signals for each probe; designated as pattern 2:2) dominated the normal tissues, whereas abnormal nuclei numbers (patterns 2:3 or 2:4) did not reach the threshold. Diploid nuclei (Pattern 2:2) also dominated the atypical hyperplasia I tissues, but abnormal nuclei numbers (pattern 2:3) reached the threshold. Pattern 2:4 (or > 4), nuclei with two signals for each probe, was seen occasionally. Patterns 2:2, 2:3, and 2:4 were also seen in atypical hyperplasia II tissues, but pattern 2:5 (or > 5) was seen occasionally. Nuclei with pattern 2:3 and 2:5 dominated the abnormal nuclei of atypical hyperplasia III tissues, and pattern 2:5 (or > 5) was seen occasionally (Fig. 1).

**Positive expression of the hTERC gene**

Positive expression rates of hTERC amplification were not detected (0%) in the 45 esophageal normal tissues, although four cases were near the threshold. Compared with the normal group, the positive hTERC gene expression rates of the atypical hyperplasias I and II /III, as well as esophageal cancer groups were significantly different (p < 0.05). The atypical hyperplasia I group was compared with atypical hyperplasia II/III and esophageal cancer groups. The atypical hyperplasia II/III group was compared with the esophageal cancer group. All the differences were statistically significant (p < 0.05). The positive predictive values of the atypical hyperplasia II/III group were 54.5%, 90.28%, 63.16%, and 86.67%. The hTERC amplification rate was consistent with the abnormal rates from the cytological and histological diagnoses. hTERC expression was negative in normal esophageal squamous epithelial cells. The positive hTERC expression rates were 38.2 % and 90.9% in the paracarcinoma epithelium and cancer tissues, respectively (p < 0.05) (Table 1).

**Characteristics of hTERC gene copy numbers**

The mean hTERC copy numbers in the atypical hyperplasia I, atypical hyperplasia II/III, and SCC groups were 2.19 ± 0.11, 2.35 ± 0.30, and 2.64 ± 0.27, respectively. The mean hTERC copy number in atypical hyperplasia was 2.46 ± 0.31 overall. Abnormal hTERC gene copy types followed patterns 2:3, 2:4, 2:5, 2:6, 2:7, 2:8, and 3:3 (representing nuclei with the signal numbers for each probe). Ten cases of atypical hyperplasia II, the main copy type of the
hTERC gene, followed patterns 2:2, 2:3, and 2:4. Only one case showed an abnormal hTERC gene copy type (patterns 2:5 and 2:6). Twenty cases were atypical hyperplasia III.

Ten cases showed abnormal copy types of the hTERC gene (patterns 2:5, 2:6, 2:7, 2:8), whereas the others showed normal copy types of the hTERC gene (patterns 2:2, 2:3, and 2:4) \( (p < 0.05) \). The diversification of amplified signals increased with unusual pathological alterations.

Positive hTERC gene amplification did not correlate with patient gender, histological stage, lymph nodes metastasis, and SCC differentiation grade \( (p > 0.05) \) (Table 2).

**Discussion**

As mentioned in the Introduction, the hTERC gene is located in the chromosome 3q26 region. Its product, telomerase, is involved in the maintenance of chromosome length and stability (Umayahara et al. 2002; Heselmeyer-Haddad et al. 2005; Kloth et al. 2007). hTERC gene amplification was reported to occur early in cervical carcinogenesis (Hopman et al. 2006). Chromosomal instabilities, such as increased aneuploidy and structural chromosome aberrations, play critical roles in intermediate- to late-stage cervical malignancies (Allen et al. 2000; Matthews et al. 2000; Yang et al. 2001; Hackett and Greider 2002; Zhang et al. 2002; Pasrija et al. 2007; Caraway et al. 2008; Goldman et al. 2008; Rankin et al. 2008; Alameda et al. 2009; Zhang et al. 2009; Atzmon et al. 2010; Li et al. 2010; Lacroix et al. 2011). Using two-colour FISH to detect atypical squamous cells of undetermined significance (ASCUS), as well as low-grade (LSL) and high-grade (HSL) squamous lesions in cervical smears, the chromosome 3q copy number was found to have an increased frequency and a diversified phenotype. As the disease progressed and became severe, aneuploidy incidences increased, especially the 3q copy number. The copy number was significantly higher than that in cervical cancer and in severe dysplasia or carcinoma in situ. The region where aneuploidy occurred was the pathogenetic cervical region (Umayahara et al. 2002; Heselmeyer-Haddad et al. 2003, 2005; Hopman et al. 2006; Olaharski et al. 2006; Kloth et al. 2007). The organization and the heredity relevance possibly revealed the similarities between esophageal and cervical cancers. However, hTERC gene expression in normal esophageal tissues, pre-cancerous lesions, and actual cancer cells is well reported. Amplifications of chromosomes 3q and 8q had the highest...
occurrence frequencies in primary esophageal cancer (Wang et al. 2006).

We hypothesized that hTERC gene amplification may also play a role in the development of esophageal cancer. To verify this, the copy numbers of the hTERC gene in esophageal cancer and preneoplastic lesions were examined. An interphase FISH with a chromosome enumeration double-colour DNA probe was used. Consistent with histologic biopsies and pathologic diagnoses, hTERC gene amplification was not detected in normal esophageal tissues, but was positively detected in atypical hyperplasias I and II/III, as well as SCCs ($p < 0.05$). The atypical hyperplasia I group was compared with the atypical hyperplasia II/III and esophageal cancer groups. The atypical hyperplasia II/III group was also compared with the esophageal cancer group. The significant differences ($p < 0.05$) demonstrated that different positive hTERC gene amplification rates existed between SCCs and all grades of esophageal dysplasia. In addition, the positive amplification rates gradually increased with disease deterioration. Associated with unusual pathological changes, the mean copy numbers of hTERC gradually increased. Abnormal hTERC gene copy types increased amplified signal diversification. These findings suggest that abnormal hTERC gene expression correlates with dysplasia severity, similar with cervical cancer (Olaharski et al. 2006). In the present study, the chromosome 3q copy number was abnormally amplified in grade II/III dysplasias and SCCs. We speculate that dysplasia may be related to chromosomal instability. Recent studies indicated that a double chromosome appears before the aneuploid in cervical cancer. A polyploidy appeared with lesion development, the chance of aneuploidy appeared, and the hTERC gene copy number increased (Allen et al. 2000; Matthews et al. 2000; Yang et al. 2001; Castle et al. 2002; Zhang et al. 2002; Böcking and Nguyen 2004; Hopman et al. 2006; Takeuchi et al. 2008; Costa et al. 2009; Hills and Lansdorp 2009; Zhang et al. 2009). All the above mentioned characteristics of dysplasia were similar with the SCCs. The positive amplification of the hTERC gene did not correlate with patient gender, histological stage, lymph nodes metastasis, and SCC differentiation grades ($p > 0.05$). hTERC gene expression has nothing to do at all with mid- and late-stages esophageal cancer. In particular, abnormal nuclei in atypical hyperplasia II/III tissues were significantly higher than those in atypical hyperplasia I ($p < 0.05$) tissues. Atypical hyperplasia II/III tissues had sensitivity and specificity. On the other hand, hTERC gene abnormalities and chromosome instabilities were closely related with the occurrence of esophageal cancer. The pathological changes in esophageal cancer may be attributed to abnormal chromosome 3q amplification.

In summary, abnormal hTERC amplification may occur early in the process of esophageal carcinogenesis. The pathological changes related to esophageal cancer may be attributed to abnormal chromosome 3q amplification. Therefore, hTERC gene detection may be helpful in diagnosing esophageal cancer and precancerous lesions, as well as in discovering novel therapeutic targets.

### Table 2. hTERC gene expression in cancer tissues and its significance.

<table>
<thead>
<tr>
<th>Clinical data</th>
<th>Number of cases (n)</th>
<th>Positive number</th>
<th>Positive number in percentage (%)</th>
<th>$\chi^2$</th>
<th>p value*</th>
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* A p value less than 0.05 is considered significant.
Acknowledgments

We thank Jin PuJia Company for technical assistance and IT support. Dr. Lan Zhang (Department of Pathology, Zheng Zhou University, Henan) provided specimens and Sun Ruotong (Department of Pathology, the First Affiliated Hospital of Henan University of Science and Technology) helped in reading the manuscript critically.

Conflict of Interest

All authors have no conflict of interest regarding this paper.

References


