Telomerase activity detection in cholesteatoma

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Abstract  Background: In cholesteatoma, there is lack of control over cell proliferation that is why ectodermal tissue grows to be an epidermoid cyst. So cholesteatoma may be considered as cell growth disorder but still the cause of this uncontrolled proliferation is unknown.

Objective: The aim of this study is to detect telomerase activity in cholesteatoma and meatal skin (as a control) and its relation to the clinical findings and severity of the disease.

Patients and methods: Samples of cholesteatoma and meatal skin were obtained from 31 patients during ear surgery. Telomerase activity was detected using polymerase chain reaction based on telomeric repeat amplification protocol assay then collected data were statistically analyzed.

Results: Telomerase activity was positive in 17/31 (54.8%) of cholesteatoma samples and was negative in all meatal skin samples. Significant high rates of intraoperative ossicular erosion and preoperative air bone gap more than 40 dB were associated with positive telomerase activity.

Conclusions: Telomerase activity may play a role in the hyperproliferative nature and uncontrolled aggressive growth pattern of cholesteatoma.

1. Introduction

The proliferative capacity of normal somatic cells is limited by gradual loss of tandem nucleotide repeat (TTAGGG), termed telomeres at chromosomal ends because of an end replication.1 Telomeres cap the ends of linear chromosomes and are essential for maintaining chromosome stability. In the majority of human cells, because of the end replication problem that affects DNA polymerase, telomere shortens 50–100 base pairs per cell division.2 When telomere reaches critical short length, they lose their function, and DNA damage checkpoints become activated, with either arresting in the cell cycle or undergoing apoptosis.3 However, cancer cells have a unique ability to turn on telomerase, an enzyme that elongates telomeres,
Telomerase is also capable of synthesizing telomere ends de novo.6

In the last years, studies on telomerase inhibition increased. In 2005, FDA approved GRN-163L use as telomerase inhibitor in patients with chronic lymphatic leukemia. From then, its use was tried for other neoplastic lesions.7

Cholesteatoma is characterized by the presence of keratinizing squamous epithelium in the middle ear cleft with hyperproliferative feature.8 Surgical management is the primary strategy used to treat and control the disease. Despite recent surgical advances,9 high rates of recurrences are still present.9,10 emphasizing the need for a better understanding of molecular mechanisms underlying cholesteatoma growth and recurrence.9

Not all ectodermal tissues which invade or remain as a vestige in the mesenchymal tissue grow and become a cholesteatoma and it is thought that in a normal human, such ectodermal tissues are naturally eliminated as a result of apoptosis.11 In cholesteatoma, there is lack of control over cell proliferation that is why ectodermal tissue grows to be an epidermoid cyst. For this reason, cholesteatoma may be considered as a cell growth disorder,12 but still the cause of this uncontrolled proliferation is unknown.13 So, cholesteatoma is considered a tumor-like growth in the middle ear.14

There is a controversy regarding the impact of telomerase activities on cellular proliferation in cholesteatoma.15

The aim of this work is to detect telomerase activity in cholesteatoma and metatal skin (as a control) and its relation with clinical findings and severity of the disease.

2. Materials and methods

This study was performed at the Otorhinolaryngology, Head and Neck Surgery department and Clinical Pathology department, Zagazig University Hospitals from February 2008 to December 2011 on 31 patients with cholesteatoma with pars flacida perforation. The patients were 18 males and 13 females aged between 28 and 66 years (mean age, 38 years).

All patients were subjected to full history taking, general and local examination including microscopic ear examination. Audiological and radiological evaluations were conducted to ensure the diagnosis and extent of the disease. Then routine preoperative laboratory tests were done.

Samples of cholesteatoma and samples of skin of the deep part of the bony external auditory canal (as a control) were obtained from all patients during ear surgery. These samples were stored in liquid nitrogen at −80°C until use for telomerase activity assay.

2.1. Measurement of telomerase activity

Telomerase activity was measured by Telo TAGGG telomerase PCR ELISAplus polymerase chain reaction-based telomeric repeat amplification protocol (TRAP) assay, first described by Kim et al.16 But, instead of radioactive nucleotide labeling, biotinylated primers were used during the extension reaction, thus enabling quantitative photometric detection by a streptavidin-DIG-peroxidase immunoreaction. Telo TAGGG telomerase PCR ELISAplus was supplied from Roche Molecular Biochemicals, Mannheim, Germany.

The TRAP assay is a two-step process in which the telomerase-mediated elongation products are subsequently amplified by PCR to allow highly sensitive detection of telomerase activity. Some samples contain inhibitors of Taq DNA polymerase, thus giving false negative results when analyzed for telomerase activity. To overcome this problem, the internal standard provided with the Telo TAGGG telomerase PCR ELISAplus, is a 216 bp homologous standard that allows a clear detection of Taq DNA polymerase inhibitors. In total, 10–20 μm-thick frozen sections of each specimen were cut. Thin slices of frozen tissue specimens were prepared on sterile petri dishes with surgical disposable knife blades to obtain thin flakes, immediately lysed in 200 μl of ice-cold lysis buffer, incubated on ice for 30 min and centrifuged at 16,000g for 20 min at 4°C. Five microliters of each sample was heat-inactivated by incubation at 85°C for 10 min prior to TRAP assay to inactivate telomerase protein to produce negative controls. For amplification; 25 μl of reaction mixture (biotinylated telomerase substrate P1-TS, optimized anchor-primer P2, nucleotides and Taq DNA polymerase) and 5 μl of the internal standard (216 bp DNA) were added per tube (a) 1–3 μl sample volume (corresponding to 0.5 μg/μl protein, which was measured by Bradford method) and 1–3 μl negative controls; (b) 1 μl control template; and (c) lysing reagent as a blank for control template. The total volume was completed up to 50 μl with nuclease-free water. The PCR conditions were 1 cycle for primer elongation (25°C, 20 min), 1 cycle for telomerase inactivation (94°C, 5 min), followed by amplification in the form of 30 PCR cycles (94°C for 30 s, 50°C for 30 s, 72°C for 90 s and 72°C for 10 min). PCR cycling was carried out in Perkin Elmer cycler 9700. For each 2.5 μl of the amplification product, 10 μl of denaturing reagent was added into two separate tubes.

Hybridization buffer T (DIG-labeled detection probe complementary to telomeric repeat sequences P3-T) was added to one tube and Hybridization buffer IS (DIG-labeled detection probe complementary to the internal standard P3-IS) was added to the second tube. One hundred microliters of each mixture was transferred into each well. After incubation and washing, 100 μl of Anti-DIG-HRP working solution (Polyclonal antibody from sheep, conjugated to horseradish peroxidase) was added per well and incubated on ice for 30 min and centrifuged at 16,000g for 20 min at 4°C. Five microliters of each sample was heat-inactivated by incubation at 85°C for 10 min prior to TRAP assay to inactivate telomerase protein to produce negative controls. For amplification; 25 μl of reaction mixture (biotinylated telomerase substrate P1-TS, optimized anchor-primer P2, nucleotides and Taq DNA polymerase) and 5 μl of the internal standard (216 bp DNA) were added per tube (a) 1–3 μl sample volume (corresponding to 0.5 μg/μl protein, which was measured by Bradford method) and 1–3 μl negative controls; (b) 1 μl control template; and (c) lysing reagent as a blank for control template. The total volume was completed up to 50 μl with nuclease-free water. The PCR conditions were 1 cycle for primer elongation (25°C, 20 min), 1 cycle for telomerase inactivation (94°C, 5 min), followed by amplification in the form of 30 PCR cycles (94°C for 30 s, 50°C for 30 s, 72°C for 90 s and 72°C for 10 min). PCR cycling was carried out in Perkin Elmer cycler 9700. For each 2.5 μl of the amplification product, 10 μl of denaturing reagent was added into two separate tubes.

2.2. Statistical analysis

Clinical features such as age and sex of the patients, hearing loss, and intraoperative ossicular bone erosion were reported and prepared for statistical analysis.

All data were subjected to computerized statistical analysis by using SPSS program (Statistical package for the Social Science, version 10, Chicago, IL).
Data were expressed as mean (±) ± standard deviation (SD) for quantitative variables. Student’s “t” test was used to assess significance.

3. Results

Telomerase activity was found to be positive in 17 patients (54.8%) and negative in 14 patients (45.2%) of cholesteatoma samples, while telomerase activity was found to be negative in all meatal skin samples.

Extracts from all telomerase negative samples gave a positive signal for the internal telomerase assay standard, excluding Taqman polymerase inhibition. The relation of telomerase activity to the patient’s age and sex, which has been found to be non significant, is summarized in Table 1.

All the seventeen patients with positive Telomerase activity were found to have ossicular erosion intraoperatively while such finding was found in only 9 patients (64.2%) with negative telomerase activity and this difference was found to be statistically significant. At the same time, 15 patients (88.2%) with positive Telomerase activity were found to have air bone gap more than 40 dB, however it was found in only 7 (50%) patients with negative telomerase activity.

This difference was also statistically significant. This relation of telomerase activity with preoperative audiolometric results and intraoperative ossicular erosion is summarized in Table 2

4. Discussion

The activation of telomerase enzyme has been found to be a necessary step for the immortal growth of human cancer cell. Telomerase is activated in 85–90% of human cancer.

The natural history of entrapped epithelial tissue (cholesteatoma pearl) in the middle ear cleft is unknown, some may disappear spontaneously, some remain static and others progressively enlarge to become cholesteatoma. The factors that determine the fate of this entrapped epithelial tissue in the middle ear cleft have not been detected.

Cholesteatoma is considered as a tumor-like growth in the middle ear because it shows persistent hyperproliferation. Although various theories have been developed regarding the cause of the disease, the basic mechanisms of cholesteatoma formation have still not been fully characterized. Although generally understood to be a benign process, cholesteatoma exhibits several clinical aspects of tumors as destruction of adjacent tissue, bone erosion, and frequent recurrences after surgery.

Some previous studies found that telomerase activity was closely related to cellular proliferation in chronic hyper-proliferating tissue such as cholesteatoma.

In our study, telomerase activity was positive in 54.8% of cholesteatoma samples and none of meatal skin was positive, suggesting that telomerase activity may have a role to play in continuous cell proliferation and growth (expansion). This result was not surprising as several molecular markers of tumor formation had been found in cholesteatoma e.g. upregulation of matrix metalloproteinases, c-myc, and activation of angiogenesis.

Only few studies have been performed on telomerase activity in cholesteatoma. Kojima et al. reported that telomerase was detected in acquired cholesteatoma at a low frequency (one out of five), suggesting that telomerase activity is specific for malignant tumors but not for cholesteatoma. Nevertheless, the number of cholesteatoma samples in their study was very small (only five cases). Also Kuczkowski et al. detected telomerase activity as low as 2 (8.7%) of 23 samples of cholesteatoma, and Watabe-Rudolph et al. found telomerase activity positive in 1 of 29 cholesteatoma samples (3.4%) but they demonstrated that the telomere lengths were similar in cholesteatoma and squamous cell carcinoma (SCC). They proposed that the absence of telomerase activity limits the proliferative capacity of cholesteatoma by induction of apoptosis. With respect to this explanation Kuczkowski et al. assumed that the absence of telomerase may lead to generation of dysfunctional telomerates what in turn may impair the proliferative capacity of cholesteatoma.

On the other hand, Lee et al. found that telomerase activity was positive in 21/40 cholesteatomas (52.9%) and absent in the control group, but the telomerase activity in SCC in the same study was lower (66.7%) than the high rate reported by other studies. Goh et al. also found a higher expression of telomerase activity in 77.3% of cholesteatoma cases.

Age and sex of the patients did not significantly affect the detection of telomerase activity in cholesteatoma samples of our study. A finding that was also reported by Goh et al. and Lee et al.
Telomerase activity was significantly higher in cholesteatoma samples of patients with more preoperative hearing loss and patients with intraoperative ossicular erosion and this agrees with the conclusion of study of Lee et al. who reported that cellular proliferation was significantly higher in cases where the telomerase activity was positive. While a previous study found that these relations were insignificant.

Our results support the suggestion that the expression of telomerase may be related to the proliferative nature of cholesteatoma. So telomerase activity may be one of the factors responsible for the pathogenesis of cholesteatoma. The significant difference between cases with positive and negative telomerase activity in relation to ossicular erosion in our study indicates that telomerase activity is not only related to cellular proliferation but may also be related to the pathogenesis of bone erosion associated with cholesteatoma.

As FDA approved GRN-163L use as a telomerase inhibitor in patients with chronic lymphatic leukemia, it is needed to investigate if telomerase inhibitor can be used to limit the progress of cholesteatoma and decrease its recurrence rates.

5. Conclusion

Telomerase activity was detected in 54.8% of cholesteatoma samples and not in adjacent skin. Expression of telomerase was significantly higher in patients with more preoperative hearing loss and intraoperative ossicular erosion suggesting that the telomerase activation may be closely linked to the hyperproliferative nature of cholesteatoma as well as bone erosion associated with it.

References