

Telomerase Reactivation Is an Early Event in Laryngeal Carcinogenesis

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The exact role and timing of reactivation of telomerase, a key enzyme implicated in cellular immortalization and transformation in the multistep process of laryngeal carcinogenesis, is still unknown. We attempted to (1) determine that quantitative differences exist in the levels of telomerase catalytic subunit (*hTERT*) mRNA expression among different grades of laryngeal epithelial abnormalities classified according to the Ljubljana classification; (2) determine that telomerase reactivation is an important, most probably early event in laryngeal carcinogenesis; and (3) analyze whether the relative quantity of *hTERT* mRNA can be used as a molecular biomarker in the early detection of precancerous lesions. The relative quantity of *hTERT* mRNA, expressed as an hTERT index, was analyzed in 140 frozen laryngeal tissue specimens representing different morphological stages of laryngeal carcinogenesis by using a commercially available LightCycler Telo TAGGG *hTERT* Quantification kit. The presence and relative quantity of *hTERT* mRNA in laryngeal epithelium increases progressively with the degree of epithelial abnormalities. *hTERT* mRNA was detectable in 1/15 normal laryngeal epithelia (7%, mean hTERT index 0.02), 3/15 simple hyperplasias (20%, mean hTERT index 0.09), 10/27 abnormal hyperplasias (37%, mean hTERT index 0.18), 9/12 atypical hyperplasias (75%, mean hTERT index 0.74), 8/9 intraepithelial carcinomas (89%, mean hTERT index 1.82), and 53/62 invasive laryngeal squamous cell carcinomas (85%, mean hTERT index 2.51). Statistical analysis revealed two groups of laryngeal epithelial changes with significant differences in the levels of *hTERT* mRNA expression ($P < .0033$): (1) normal and reactive hyperplastic laryngeal epithelium (simple and abnor-

mal hyperplasia) and (2) atypical hyperplasia (precancerous lesion), intraepithelial and invasive laryngeal squamous cell carcinoma. The results of the present study suggest that telomerase reactivation is an early event in laryngeal carcinogenesis, detectable already at the stage of precancerous laryngeal epithelial changes. Nevertheless, other genetic abnormalities appear to be necessary for progression of these epithelial abnormalities toward invasive laryngeal squamous cell carcinoma.

KEY WORDS: Carcinogenesis, Catalytic subunit, *hTERT* mRNA, Larynx, Ljubljana classification, Telomerase.

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Genetic abnormalities in laryngeal carcinogenesis are morphologically expressed in different grades of laryngeal epithelial abnormalities (1–3). Epithelial abnormalities are cumulatively called epithelial hyperplastic laryngeal lesions and, according to the Ljubljana classification, range from benign or reactive to potentially malignant lesions and intraepithelial carcinoma (2, 3). The majority of current histological classifications of epithelial hyperplastic laryngeal lesions, including the WHO classification of the upper respiratory tract, follow criteria similar to those commonly used for cervical epithelial lesions: epithelial abnormalities progress from squamous cell hyperplasia, different grades of dysplasia and intraepithelial carcinoma, to invasive squamous cell carcinoma (1). However, because of the different etiopathogenesis of laryngeal and cervical epithelial changes (cigarette smoking and alcohol abuse, especially in combination in the larynx *versus* human papillomaviruses in the cervix), as well as therapeutic and prognostic implications, the Working Group on Epithelial Hyperplastic Laryngeal Lesions of the European Society of Pathology has recently recommended the use of the Ljubljana classification as an alternative to the WHO classification (2, 3). It has been shown not only that the Ljubljana classification is more precise for daily

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diagnostic work than other grading systems but that different degrees of epithelial abnormalities also signify their different biological behavior, thereby influencing the most appropriate treatment possibilities (3). The main differences between the Ljubljana and WHO classifications are (1) the distinction between benign (simple and abnormal hyperplasia) and potentially malignant lesions (atypical hyperplasia) and (2) separation of atypical hyperplasia from intraepithelial carcinoma because of their different risk for progression to invasive squamous cell carcinoma and their different treatment options (3).

The proliferative capacity of normal somatic cells is limited by the gradual loss of tandem nucleotide repeats (TTAGGG), termed *telomeres*, at the chromosomal ends because of an end-replication problem (4–6). On the other hand, malignant cells have the ability to proliferate indefinitely, because they are able to compensate for telomeric loss (7). The telomerase enzyme is a specialized multisubunit complex, with telomerase catalytic subunit (hTERT = human telomerase reverse transcriptase) functioning as a reverse transcriptase that can synthesize the telomeric ends at each cell division (8). Telomerase has been found to be reactivated in 90% of malignant neoplasms, including laryngeal squamous cell carcinomas (7, 8). Telomerase reactivation has therefore been regarded as a critical step in cellular immortalization, a crucial event in the multistage process of human carcinogenesis (8).

Recent studies have confirmed a close relationship between *hTERT* mRNA expression and telomerase activity, suggesting that quantification of *hTERT* gene expression can be used as an alternative to telomerase activity measurement (9, 10). At present, no data are available on the quantities of *hTERT* mRNA in different morphological stages of laryngeal carcinogenesis. We have recently shown that *hTERT* mRNA may occasionally be detected in a normal laryngeal epithelium, but the frequency and relative quantity of *hTERT* mRNA is significantly higher in laryngeal squamous cell carcinomas (11). To the best of our knowledge, only six studies to date have been published using a telomeric repeat amplification protocol (TRAP) for the measurement of telomerase activity in the larynx and/or hypopharynx (12–17). The main drawback of these studies is the lack of uniformly applied terminology for different grades of epithelial abnormalities, as well as imprecise methodology used for detection of telomerase activity. Namely, TRAP assay has been identified as having several limitations, because an active telomerase enzyme complex is obligatory for detection of telomerase activity (18). Nevertheless, telomerase activity has been detected in 0–20% of normal epithelia (12, 14, 17), 100% of hyperplastic epithelia (17), and 0–100%

of dysplastic epithelia, depending on the degree of epithelial dysplasia (12, 16, 17), and in 68–100% of invasive squamous cell carcinomas (12–17). It can be deduced from these studies that telomerase reactivation represents an important, most probably obligatory event in laryngeal carcinogenesis. However, the exact position in the multistep process of laryngeal carcinogenesis at which telomerase reactivation occurs still remains to be identified.

The current study was designed to (1) test the hypothesis that quantitative differences exist in the levels of *hTERT* mRNA expression among different grades of laryngeal epithelial abnormalities, (2) test the hypothesis that telomerase reactivation is an important, most probably early event in laryngeal carcinogenesis, and (3) analyze whether the relative quantity of *hTERT* mRNA can be used as a molecular biomarker in the early detection of precancerous lesions.

METHODS

A retrospective analysis was performed on 140 frozen laryngeal tissue specimens obtained from 57 laryngeal cancer patients treated with laryngectomy or hemilaryngectomy in the period from 1998–2001 at the Department of Otorhinolaryngology and Cervicofacial Surgery in Ljubljana, Slovenia. Not later than 30 minutes after the surgical procedure, tumor samples from a resected specimen were snap-frozen in liquid nitrogen and stored subsequently at -80°C for later analysis. Before the surgery, patients did not receive any chemotherapy or radiation therapy.

Dissection of Frozen Laryngeal Tissue Specimens

Frozen laryngeal tissue specimens were obtained by dissecting cancerous from noncancerous tissue in the same frozen material. Briefly, one 4- μm -thick section was stained with hematoxylin and eosin (H&E). Combining the use of a low-power field lens ($\times 2.5$) of a Nikon Eclipse 6000 microscope, a thin surgical blade, and the H&E-stained slide as a reference, the frozen tissue block was divided into two parts, one containing cancerous tissue and the other, noncancerous tissue. Afterward, one 4- μm tissue section (reference section) from each tissue block was cut, stained with H&E, and verified under the microscope for adequate dissection. Thereafter, serial sections of 10- μm thickness to obtain approximately 2.5 mm^3 of tissue were performed. The last section was again stained with H&E, and only if the last section corresponded histologically to the reference one, did we proceed with a subsequent total RNA isolation.

Total RNA Isolation

Total RNA was isolated from laryngeal tissue specimens by using a High Pure RNA Tissue Kit (Roche Diagnostics, Mannheim, Germany), strictly following the manufacturer's recommendations. Eluted total RNA was stored at -80°C for later analysis.

Quantification of hTERT mRNA

A commercially available LightCycler *TeloTAGGG* hTERT Quantification kit (Roche Diagnostics, Mannheim, Germany) was used for real-time amplification and quantification of *hTERT* mRNA (19) according to manufacturer's instructions. Briefly, the *hTERT* mRNA was reverse transcribed and a 198-bp fragment of generated cDNA was amplified with specific primers in a one-step RT-PCR reaction. The amplicon was detected by fluorescence using a specific pair of hybridization probes, consisting of two different oligonucleotides that hybridize to an internal sequence of the amplified fragment during the annealing phase of the amplification cycle. In a separate one-step RT-PCR, mRNA encoding for porphobilinogen deaminase (*PBGD*) was processed for use as a housekeeping gene. The reaction product served as both a control for RT-PCR performance and as a reference for relative quantification.

For each tested sample, $2\ \mu\text{L}$ of the eluted total RNA was used. Quantification was performed by real-time monitoring for identification of the exact time point at which the logarithmic linear phase could be distinguished from the background. External standards containing 10^6 , 10^5 , 10^4 , 10^3 , and 10^2 copies of *hTERT* mRNA per $2\ \mu\text{L}$ were used in each run. The cycle numbers of the logarithmic linear phase were plotted against the logarithm of concentration of *hTERT* mRNA. By comparing the crossing line intercept of an unknown sample with the standard curve, a quantitative estimate of the starting copy number of *hTERT* mRNA (as well as *PBGD* mRNA) was calculated. The normalized *hTERT* mRNA value (hTERT index), a measure for *hTERT* mRNA quantity, was calculated by dividing the amount of hTERT transcript by the amount of endogenous housekeeping gene *PBGD* mRNA of the same sample, multiplied by 100.

Light Microscopical Evaluation of Frozen Tissue Sections

The changes in the surface laryngeal epithelium were classified independently of the knowledge of *hTERT* mRNA relative quantity according to the Ljubljana classification of epithelial hyperplastic lesions into four groups (2, 3; schematic presentation

of Ljubljana classification is presented at the bottom of Fig. 1):

1. Simple hyperplasia: the epithelium is thickened as a result of an increased prickle cell layer. The cells of the basal and parabasal region, which comprise one to three layers, remain unchanged;

2. Abnormal hyperplasia: basal and parabasal cells in the lower part of the epithelium are augmented. Rare regular mitoses may be seen, always located in or near the basal layer;

3. Atypical hyperplasia (precancerous lesion) or "risky epithelium": stratification is still preserved. Nuclei of most epithelial cells show the changes of so-called atypia, such as nuclear enlargement with irregular contours, hyperchromasia, increased nuclear/cytoplasmic ratio, and increased number of nucleoli. Mitoses are increased and are usually found in the lower two thirds of the epithelium. Dyskeratotic cells are frequent within the entire epithelium. Civatte bodies (apoptotic cells) may be present;

4. Intraepithelial carcinoma (carcinoma *in situ*): the lesion is characterized by three distinct morphologic characteristics: (1) loss of stratification of the epithelium, (2) markedly increased mitotic figures throughout the whole epithelium, often more

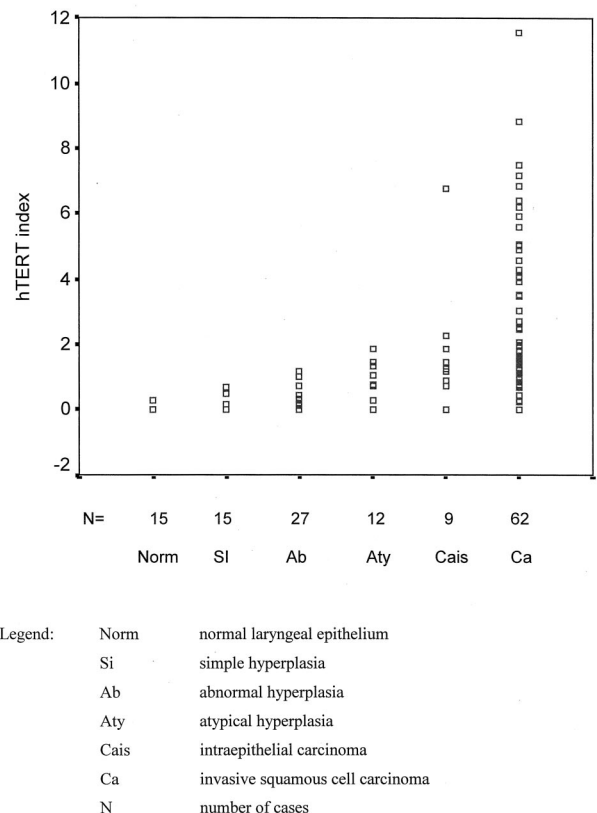


FIGURE 1. Mean hTERT indices in different grades of epithelial hyperplastic laryngeal lesions and invasive squamous cell carcinoma of the larynx. Norm, normal laryngeal epithelium; Si, simple hyperplasia; Ab, abnormal hyperplasia; Aty, atypical hyperplasia; Cais, intraepithelial carcinoma; Ca, invasive squamous cell carcinoma; N, number of cases.

than five per high-power field, and (3) pronounced cellular alterations resembling those seen in invasive carcinoma.

Statistical Analysis

Statistical analysis was performed by a nonparametric Mann-Whitney *U* test with Bonferroni correction (nonparametric Wilcoxon test) using SPSS 10.1 for Windows; *P* values < .0033 were regarded as statistically significant.

RESULTS

The presence of *hTERT* mRNA was analyzed in 78 laryngeal epithelial samples adjacent to the invasive laryngeal squamous cell carcinomas (15 normal laryngeal epithelia, 15 simple hyperplasias, 27 abnormal hyperplasias, 12 atypical hyperplasias, and 9 intraepithelial carcinomas) and 62 invasive laryngeal squamous cell carcinomas. In contrast to other types of epithelial abnormalities, intraepithelial carcinoma was found exclusively in close contact with invasive laryngeal squamous cell carcinoma. Of >200 frozen laryngeal cancer tissue blocks, 9 cases containing a sufficient amount of intraepithelial carcinoma were obtained. This was because, despite meticulous tissue dissection, deeper 10- μ m sections (taken to obtain sufficient amount of frozen tissue for total RNA isolation) of an initial intraepithelial carcinoma usually revealed an underlying invasive laryngeal squamous cell carcinoma.

The presence of *hTERT* mRNA was detected in 1/15 normal laryngeal epithelia (7%), 3/15 simple hyperplasias (20%), 10/27 abnormal hyperplasias (37%), 9/12 atypical hyperplasias (75%), 8/9 intraepithelial carcinomas (89%), and 53/62 invasive laryngeal squamous cell carcinomas (85%; Table 1). A scatterplot of all *hTERT* indices calculated in the present study is shown in Figure 2.

The mean *hTERT* index was 0.02 in normal laryngeal epithelium (0–0.27, SD \pm 0.07), 0.09 in simple hyperplasia (0–0.67, SD \pm 0.20), 0.18 in abnormal hyperplasia (0–1.16, SD \pm 0.32), 0.74 in atypical hyperplasia (0–1.84, SD \pm 0.60), 1.82 in intraepithelial carcinoma (1.16–6.76, SD \pm 1.96), and 2.51 in laryngeal squamous cell carcinoma (0–11.50; SD \pm

2.52; Fig. 1). Figure 3 shows 95% confidence interval for the mean *hTERT* indices in normal laryngeal epithelium, different grades of epithelial abnormalities, and laryngeal squamous cell carcinoma.

Statistical analysis revealed two groups of laryngeal epithelial abnormalities with significant differences in the levels of *hTERT* mRNA expression (Fig. 3): (1) normal and reactive hyperplastic laryngeal epithelium (simple and abnormal hyperplasia) and (2) atypical hyperplasia, intraepithelial, and laryngeal squamous cell carcinoma. Namely, significantly higher *hTERT* indices were present in atypical hyperplasias and in intraepithelial carcinomas than in normal laryngeal epithelia and simple or abnormal hyperplasias (*P* = .003, *P* < .001, respectively). In addition, *hTERT* indices in atypical hyperplasias were generally lower than in intraepithelial carcinomas, but the differences were not statistically significant (*P* = .074). Significantly higher relative quantities of *hTERT* mRNA were found in laryngeal squamous cell carcinomas than in normal laryngeal epithelia and in simple or abnormal hyperplasias (*P* < .001). However, no significant differences existed among the relative quantities of *hTERT* mRNA in atypical hyperplasias or intraepithelial carcinomas and laryngeal squamous cell carcinomas (*P* = .01, *P* = .515, respectively).

DISCUSSION

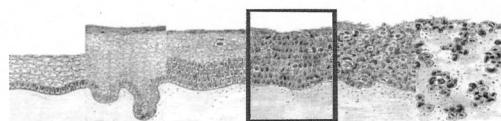
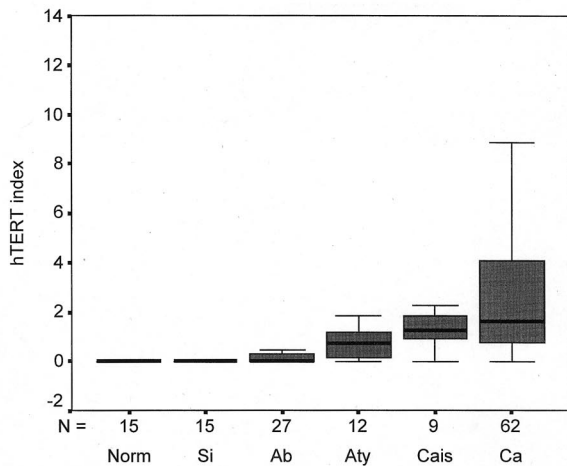
Squamous cell carcinoma of the larynx arises from a clonal population of cells that accumulate genetic alterations in a multistep process of laryngeal carcinogenesis (20). Genetic abnormalities and the sequence of genetic events underlying the progression of normal laryngeal mucosa to squamous cell carcinoma are still not well understood. From 6 to 10 independent genetic events involving oncogenes and tumor suppressor genes within a single cell have been estimated to be necessary for invasive cancer development (21). It is believed that the loss of the chromosomal region 9p21 with inactivation of the *p16* gene, found in 80% of head and neck cancers, occurs early in carcinogenesis of these tumors, and may already be detected at the level of

TABLE 1. *hTERT* mRNA and *hTERT* Indices in Normal Laryngeal Epithelium, Different Grades of Epithelial Abnormalities and Invasive Squamous Cell Carcinoma of the Larynx

Laryngeal Tissue	Number of Cases	<i>hTERT</i> mRNA (Number)	+ Cases (%)	<i>hTERT</i> Index		
				Mean	Range	Standard Deviation
Normal epithelium	15	1	7	0.02	0–0.27	0.07
Simple hyperplasia	15	3	20	0.09	0–0.67	0.20
Abnormal hyperplasia	27	10	37	0.18	0–1.16	0.32
Atypical hyperplasia	12	9	75	0.74	0–1.84	0.60
Carcinoma <i>in situ</i>	9	8	89	1.82	1.16–6.76	1.96
Invasive carcinoma	62	53	85	2.51	0–11.55	2.52

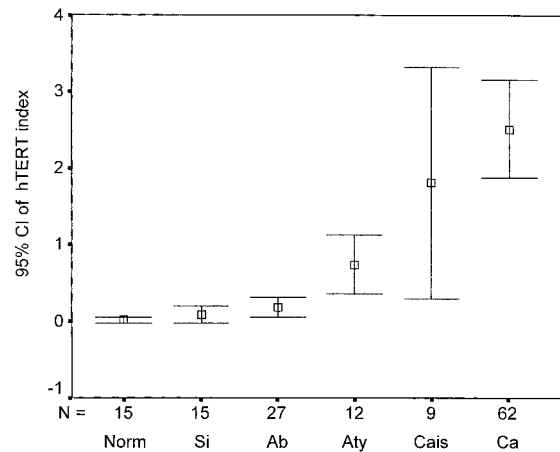
epithelial hyperplasia (21, 22). Loss of p53 function, present in 50% of head and neck cancers, most probably occurs later and influences the development of preinvasive and invasive lesions (21, 22). In addition, amplification of the oncogene cyclin D1, found in about one third of head and neck squamous cell carcinomas, is usually associated with invasive cancer and is thought to represent a late event in laryngeal carcinogenesis (21, 22).

Reactivation of telomerase, a ribonucleoprotein multi-subunit enzyme, has been implicated in the development of various human malignancies, including laryngeal squamous cell carcinomas (7). Providing the cells with unlimited proliferative potential, that is, immortality, telomerase has been thought to promote accumulation of additional genetic abnormalities, culminating eventually in invasive tumors (23). In the carcinogenesis of squamous cell carcinoma in the oral cavity, telomerase reactivation most probably represents an early event and is already detectable in a moderately dysplastic oral squamous epithelium (22). In the larynx, from 68–100% of laryngeal squamous cell carcinomas show telomerase reactivation (11–17), although the exact role and timing of telomerase



Legend: Norm normal laryngeal epithelium
Si simple hyperplasia
Ab abnormal hyperplasia
Aty atypical hyperplasia
Cais intraepithelial carcinoma
Ca invasive squamous cell carcinoma
N number of cases

FIGURE 2. Scatterplot of hTERT indices in normal laryngeal epithelium, different grades of epithelial abnormalities, and invasive laryngeal squamous cell carcinoma.



Legend: Norm normal laryngeal epithelium
Si simple hyperplasia
Ab abnormal hyperplasia
Aty atypical hyperplasia
Cais intraepithelial carcinoma
Ca invasive squamous cell carcinoma
N number of cases

FIGURE 3. Confidence interval (95% CI) for the hTERT indices in normal laryngeal epithelium, different grades of epithelial abnormalities, and invasive laryngeal squamous cell carcinoma. Norm, normal laryngeal epithelium; Si, simple hyperplasia; Ab, abnormal hyperplasia; Aty, atypical hyperplasia; Cais, intraepithelial carcinoma; Ca, invasive squamous cell carcinoma; N, number of cases.

reactivation in laryngeal carcinogenesis are still unknown.

To the best of our knowledge, the present study is the first to evaluate systematically the relative quantity of *hTERT* mRNA, a measure for the level of telomerase activity, in normal laryngeal epithelium and different grades of laryngeal epithelial abnormalities.

Normal Laryngeal Epithelium and Simple and Abnormal Hyperplasia

Simple and abnormal hyperplasia of the laryngeal epithelium represent a benign epithelial proliferation (2, 3). A >10-year follow-up of patients with simple and abnormal epithelial hyperplasia of the larynx showed that only 1.0% of patients with simple hyperplasia and 0.9% of patients with abnormal hyperplasia developed laryngeal squamous cell carcinoma in the examined period (2, 3).

In the present study, we detected the presence of *hTERT* mRNA in 7% of normal epithelia and in 20% of simple hyperplasias and 37% of abnormal hyperplasias. The mean hTERT indices were 0.02 in normal epithelium and 0.09 in simple hyperplasias and 0.18 in abnormal hyperplasia. There were no statistically significant differences in the relative quantities of *hTERT* mRNA among these groups of epithelial changes.

So far, no data are available about the relative quantity of *hTERT* mRNA in benign (e.g., reactive) epithelial hyperplastic lesions of the larynx. Our results are comparable with those of three studies analyzing the quantity of *hTERT* mRNA in oral mucosa (24–26). Thus, Sumida *et al.* (25) detected *hTERT* mRNA in one third of normal epithelia and one half of hyperplastic oral epithelia, whereas Lee *et al.* (26) and Kim *et al.* (24) did not find *hTERT* mRNA in any normal epithelium or cases of low grade epithelial dysplasia of the oral cavity.

The presence of *hTERT* mRNA in a normal and/or reactively hyperplastic laryngeal epithelium is not surprising and probably originates from the stem cells known to reside in the basal layer of the squamous epithelium (27). It has already been established that stem cells, like germline cells, possess telomerase activity that stabilizes their telomeres and facilitates continuing proliferation for self-renewal purposes (23, 28). Nevertheless, it has also been proven that activated lymphocytes may have telomerase activity for their clonal expansion (29). Lymphocytes, a normal constituent of the subepithelial stroma, are part of the mucosal defense mechanisms and can also occasionally be found within the laryngeal epithelium (30). In several cases of normal laryngeal epithelia and in simple and abnormal hyperplasias, we even found pronounced aggregates of lymphocytes in the subepithelial stroma, but *hTERT* mRNA was not detectable in these samples. It therefore seems unlikely that low relative quantities of *hTERT* mRNA in normal laryngeal epithelia, as well as in simple and abnormal hyperplasias, would have originated from lymphocytes.

In summary, although we have not directly proven that low relative quantities of *hTERT* mRNA in a minority of normal and reactively hyperplastic laryngeal epithelia (simple and abnormal hyperplasia) are derived from stem cells, this is the most likely explanation. We believe that *hTERT* mRNA detected in these epithelia most probably reflect the proliferative capacity of the laryngeal squamous epithelium and is not the consequence of telomerase reactivation.

Atypical Hyperplasia, or “Risky Epithelium”

In contrast to benign hyperplastic laryngeal epithelial lesions, a marked increase of malignant transformation of $\leq 10\%$ was observed in patients with atypical hyperplasia during a >10 -year follow-up period (2, 3). Atypical hyperplasia has therefore a central position in the Ljubljana classification and has been regarded by the Working Group on Epithelial Hyperplastic Laryngeal Lesions of the European Society of

Pathology as a potentially malignant lesion, also called *risky epithelium* (2).

In the present study, we detected *hTERT* mRNA in 75% of atypical hyperplasias, with a mean *hTERT* index of 0.74. Interestingly, *hTERT* indices were significantly higher in atypical hyperplasia than in normal and benign hyperplastic laryngeal epithelia ($P < .001$). The results of our study are comparable with those of Kim *et al.* (24), who demonstrated *hTERT* mRNA in 87.5% of cases with moderate and severe dysplasia in the oral cavity. In agreement with these observations, Shibuya *et al.* (31) found a progressive increase of *hTERT* mRNA with the degree of epithelial abnormalities during bronchial carcinogenesis; *hTERT* mRNA was present in 35% of dysplastic lesions and 100% of invasive squamous cell carcinomas.

Significantly higher relative quantities of *hTERT* mRNA in atypical hyperplasias than in the normal laryngeal epithelia and benign epithelial hyperplastic laryngeal lesions detected in the present study cannot be explained by a lateral migration of neoplastic cells from the invasive part of the tumor into the surrounding epithelium or subepithelial stroma. This can be excluded on the basis of meticulous laryngeal tissue dissection, where no effort was spared to ensure that such cells were not overlooked. Morphologically, no cells different from the cells seen in atypical hyperplasia were observed either within the epithelium or in the subepithelial stroma. Furthermore, infiltrating lymphocytes, already discussed under benign laryngeal epithelial hyperplastic lesions, do not seem to have any significant influence on the relative quantity of *hTERT* mRNA in atypical hyperplasia, either.

We believe that by excluding lateral migration of tumor cells and infiltrating lymphocytes as a possible source of *hTERT* mRNA in atypical hyperplasia, significantly higher relative quantities of *hTERT* mRNA in this type of epithelial abnormality most likely represent telomerase reactivation. Recent experimental studies have shown that reexpression of *hTERT* in normal human somatic cells can reconstitute telomerase activity and extend their replicative life span beyond crisis, the last-known proliferative blockade to cellular immortality (32). Telomerase reactivation has therefore been associated with the appearance of immortal cell populations, a crucial event in human carcinogenesis (33).

In summary, high relative quantities of *hTERT* mRNA in the great majority of atypical hyperplasias are probably the consequence of telomerase reactivation being an early event in the multistep process of laryngeal carcinogenesis and are most likely derived from immortal cell populations.

Intraepithelial (Carcinoma *In Situ*) and Invasive Squamous Cell Carcinoma

Intraepithelial carcinoma has all the morphological features of squamous cell carcinoma, except that the basement membrane is still preserved and there is no penetration of tumor cells into the subepithelial stroma (2). The data from the early sixties show that carcinoma *in situ* of the larynx progressed to laryngeal squamous cell carcinoma in approximately 90% of cases in the subsequent six months (34). No recent data are available on the progression rate of laryngeal intraepithelial carcinomas, because these patients require immediate adequate treatment. For comparison, high-grade dysplasia in the stomach regressed in only about 5%, persisted in 14%, and progressed in the great majority of cases (81–85%) (35). The timeframe between a diagnosis of severe or high-grade dysplasia and the identification of invasive gastric cancer was between <1 month and 39 months (35).

We detected *hTERT* mRNA in nearly 90% of laryngeal intraepithelial carcinomas and 85% of laryngeal squamous cell carcinomas, with a mean *hTERT* index of 1.82 and 2.51, respectively. Although the relative quantity of *hTERT* mRNA in intraepithelial carcinomas was generally higher than in atypical hyperplasias and lower than in laryngeal squamous cell carcinoma, the differences failed to reach statistical significance. This observation is not surprising. Namely, once telomerase reactivation has been established at a particular stage of laryngeal carcinogenesis, it can be sustained in the subsequent stages, as has been proven in the present study. Because telomerase reactivation conveys cellular immortality, cells can proliferate indefinitely and accumulate additional genetic abnormalities, which drive progression of epithelial changes toward laryngeal squamous cell carcinoma. Similar levels of *hTERT* mRNA in intraepithelial and laryngeal squamous cell carcinomas can also be justified morphologically. Intraepithelial carcinoma has all the morphological features of malignancy, except invasion. In agreement with our observations, overexpression of cyclin D1, a late event in laryngeal carcinogenesis, has been linked to progression of intraepithelial carcinoma into laryngeal squamous cell carcinoma (21, 22). Nevertheless, at a particular stage of laryngeal carcinogenesis, telomerase may no longer be necessary and alternative mechanisms for stabilization of telomeres can become operative, at least in a subset of carcinomas, a phenomenon already described in human tumors (36). Similarly, 15% of laryngeal squamous cell carcinomas in the present study had undetectable levels of *hTERT* mRNA (11).

There are some potential limitations of the present study. First, although several studies have

confirmed that quantification of *hTERT* mRNA can be used as an estimate for telomerase activity, one has to be aware that relative quantity of *hTERT* mRNA is rather a surrogate marker and not a definite proof of telomerase activity. The second potential drawback of our study is the fact that patients with only preneoplastic changes were not studied. Rather, the different histological grades (*e.g.*, epithelial abnormalities) were selected from lesions that already contained areas of frank carcinoma in their vicinity. At present, it is still unclear whether different areas within a lesion that overall demonstrates a capacity for invasion have the same molecular constituency and biologic capacity for invasion as do lesions that do not show any component of invasion.

In conclusion, to the best of our knowledge, the present study is the first to analyze systematically the relative quantity of *hTERT* mRNA in different stages of laryngeal carcinogenesis. We have clearly shown that the presence and relative quantity of *hTERT* mRNA in laryngeal epithelium increases progressively with the degree of epithelial abnormalities. Low levels of *hTERT* mRNA expression detected in normal and reactively hyperplastic laryngeal epithelia most probably reflect the regenerative capacity of the squamous epithelium and are believed to be derived from stem cells in the basal epithelial layer. In contrast, significantly higher *hTERT* mRNA relative quantities in the majority of atypical hyperplasias and intraepithelial and laryngeal squamous cell carcinomas are likely to be the consequence of telomerase reactivation and most probably reflect the appearance of immortal cell populations. The present study suggests that telomerase reactivation is an early event in laryngeal carcinogenesis, detectable already at the stage of precancerous laryngeal epithelial changes, but that other genetic abnormalities appear to be necessary for progression of these epithelial abnormalities toward invasive laryngeal squamous cell carcinoma.

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