Increased telomerase activity and hTERT expression in human salivary gland carcinomas

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Abstract. Approximately 85% of human malignant tumors express increased levels of telomerase. The marked association of telomerase activity with malignant tissue provides strong evidence that telomerase activity is a significant marker for the diagnosis of cancer. In this study, telomerase activity was examined in 12 benign salivary gland tumors (8 pleomorphic adenomas and 4 adenolymphomas), 24 malignant tumors (15 mucoepidermoid carcinomas, 6 adenoid cystic carcinomas and 3 acinic cell carcinomas) and 6 non-neoplastic salivary glands. The mRNA expression of the human telomerase reverse transcriptase (hTERT) and additional telomerase-associated proteins (hTEP1, p23, Hsp90 and dyskerin) was also examined. Of the 24 malignant tumors, 15 revealed strong telomerase activity. The non-neoplastic salivary glands appeared to have a negative telomerase expression. Furthermore, telomerase activity was significantly higher in high-grade mucoepidermoid carcinomas compared to low-grade ones (Student's t-test, $p<0.05$). A significant correlation was found between telomerase activity and mRNA expression of hTERT in 15 cases, including non-neoplastic salivary glands and tumors (Spearman's rank correlation test, $p<0.05$). Furthermore, a significant correlation was found between telomerase activity and mRNA expression of EGFR (Spearman's rank correlation test, $p<0.001$). The results suggest that not only hTERT, but also EGFR play a significant role in the activation of telomerase. In conclusion, the results suggest that telomerase activity and hTERT/EGFR mRNA expression are useful markers for the detection of malignant cells in salivary gland carcinomas. Moreover, our results indicated that telomerase activity determines the degree of malignancy of mucoepidermoid carcinoma.

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Introduction

Telomeres are comprised of tracts of guanine-rich nucleotide repeats that serve as binding sites for telomere binding proteins (1,2). The main action of telomeres is to cap the chromosome ends and prevent chromosomal instability (2). Telomerase is a ribonucleoprotein complex that adds telomere repeats at the ends of chromosomes and is required to maintain telomeric length in order to escape replicative senescence (2-4). Telomerase activity has been detected in germ cells and most cancer cells, whereas most normal somatic cells reveal no clearly detectable telomerase activity (5-8). Approximately 85% of human malignant tumors express increased levels of telomerase (8). On the other hand, most of the remaining 15% maintain their telomere lengths in the absence of telomerase by one or more mechanisms referred to as alternative lengthening of telomeres. Furthermore, several studies demonstrated that telomerase expression is correlated

with clinical outcome in human cancers (8-10). The marked association of telomerase activity with malignant tissue provides strong evidence that telomerase activity may serve as a significant marker for diagnosing cancer.

Human telomerase is a ribonucleoprotein that consists of the human telomerase RNA component (hTR), the human telomerase reverse transcriptase (hTERT) and additional telomerase-associated proteins (hTEP1, p23, Hsp90 and dyskerin) (11-13). Chang *et al* reported that only the hTERT subunit revealed differential expression in normal and cancer tissues and concluded that hTERT is a subunit of telomerase activity that can be regulated (14). A significant correlation between the expression level of hTERT mRNA and telomerase activity was previously reported in oral squamous cell carcinomas (15). Hsp90 interacts with hTERT and enhances hTERT expression and telomerase activity in oral cancer cells (16). These results indicate that hTERT plays a pivotal role in the activation of telomerase in human cancers.

For cancer diagnosis, the current gold standard is histopathology, which in most cases provides an accurate assessment of tissue biopsies in suspected cases. In salivary gland tumors, however, traditional histopathology or fine-needle aspiration cytology occasionally runs up against indeterminate cases (17). Therefore, more sensitive and specific markers are essential for identifying the presence of malignant salivary gland tumor cells. In addition, whether or not telomerase activity correlates with the prognosis of patients with salivary gland tumors is of great interest. In this study, telomerase activity and the expression of telomerase components was investigated in salivary gland tumors to examine whether the components could be used as diagnostic markers for tumor malignancy. In addition, the correlation between telomerase activity and the expression of telomerase-associated components, including hTERT, hTEP1, p23, Hsp90 and dyskerin, was examined.

The activation of epidermal growth factor receptor (EGFR) signaling causes a variety of downstream biological processes associated with tumor growth, invasion and angiogenesis (18). Previously, we reported that the expression level of EGFR mRNA was increased in oral squamous cell carcinomas compared to those in normal gingivae (19). An increased expression of EGFR was also reported in salivary gland carcinomas (20). However, the critical role of EGFR in malignant salivary gland tumors remains to be determined. To ascertain whether there is a correlation between EGFR and telomerase activity, the mRNA expression of EGFR (HER1) was elucidated in this study.

Materials and methods

Tissue samples. We examined 12 benign salivary gland tumors (8 pleomorphic adenomas and 4 adenolymphomas), 24 malignant tumors (15 mucoepidermoid carcinomas, 6 adenoid cystic carcinomas and 3 acinic cell carcinomas) and 6 non-neoplastic submandibular glands. Specimens of non-neoplastic salivary glands and salivary gland tumors were obtained with informed consent, and approval from the institutional review board at the Hiroshima University, Japan, and related facilities. Non-neoplastic salivary glands were obtained from patients who had received operative treatment for head and neck cancer. We confirmed that non-neoplastic tissues did not contain tumor cells. Each sample was divided into two sections. One section was snap-frozen in liquid nitrogen immediately after excision and maintained at -80˚C until use. The other section was examined histologically. Salivary gland tumors were diagnosed according to the World Health Organization classification (17).

Telomerase assay. Telomeric repeat amplification protocol (TRAP) assay was performed as described by Wright *et al*, with a minor modification (21). For telomerase extraction, frozen tissue samples (~20 mg) were rinsed with ice-cold lysis buffer [10 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 1 mM EGTA, 0.5% 3-[(3-cholamidopropyl)-dimethlamino]1-propanesulfonate (CHAPS), 10% glycerol, 5 mM β-mercaptoethanol, 4 mM DTT, 0.5 units RNase inhibitor and 0.1 mM AEBSF] and then homogenized with 100 μl ice-cold lysis buffer. After 30 min of incubation on ice, the lysate was centrifuged at 16,000 x g for 20 min at 4˚C. The supernatant was snap-frozen in liquid nitrogen and stored at -80˚C until use. The protein concentration in each extract was measured by the Coomassie brilliant blue assay. An aliquot (0.1 μg protein) of the extract was incubated for 30 min at 20˚C in 50 μl reaction mixture containing 20 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 68 mM KCl, 0.05% Tween-20, 1 mM EGTA, 50 μM dNTPs, 344 nM of TS primer (5'-AATCCGTCGAGCAGAGTT-3'), 2 units of Taq DNA polymerase and 10 attograms internal standard (150 bp). After the mixture was heated at 90˚C for 3 min to inactivate the telomerase, 344 nM CX primer (5'-CCCTTACCCTTACCCTTACCCTAA-3') was added and the mixture was subjected to 33 cycles of PCR with the following cycle conditions: 94˚C for 45 sec, 50˚C for 45 sec, 72˚C for 60 sec and finally 72˚C for 2 min. PCR products from each sample (25 μl) were analysed by electrophoresis on 12% polyacrylamide non-denaturing gels. Following the analysis, the gel was gently agitated in 1X TBE (Tris-borate-EDTA) with SYBR-Green I nucleic acid gel stain (FMC Bioproducts, Rockland, ME, USA) for 30 min. The TRAP signals were visualized with a fluorescence image analyzer (FUJI FLA-2000, Tokyo, Japan) and quantified by densitometric scanning using a Scientific Imaging System (Kodak Digital Science 1D, Rochester, NY, USA), normalized to the internal telomerase assay standard (ITAS) signals. To evaluate the activity of telomerase in each case, a sample showing ≥4.0 sequential bands was defined as telomerase-positive (22). We calculated the ratio of the density of the total number of ladder bands to the ITAS signal. When the ratio was <3.0, telomerase activity was classified as weak. When the ratio was ≥3.0, the activity was classified as strong.

RNA extraction and real-time quantitative PCR analysis. Total RNAs were extracted with an RNeasy mini kit (Qiagen). Total RNA (1 μg) was subjected to a RT reaction using the High Efficient Reverse Transcription kit (Toyobo, Osaka, Japan). The following PCR reactions (20 μl) were performed: the thermal cycle for hTERT consisted of 33 cycles of amplification at 94˚C for 2 min, at 55˚C for 2 min, at 72˚C for 3 min and finally at 72˚C for 5 min. For hTEP1, the thermal cycle consisted of 30 cycles at 94˚C for 1 min, at 50˚C for 1 min, at 72˚C for 1 min and finally at 72˚C for 5 min. For hTR and β-actin it was 27 cycles

Table I. Primer sequences used for RT-PCR analysis.

Gene	Sequence
hTR	S (5'-GAAAAACGTAGGCGCCGTGCTTTTGC-3') AS (5'-GTTTGCTCTAGAATGAACGGTGGAAGG-3')
hTERT	S (5'-CCTCTGTGCTGGGCCTGGACGATA-3') AS (5'-ACGGCTGGAGGTCTGTCAAGGTAG-3')
hTEP1	S (5'-TCAAGCCAAACCTGAATCTGAG-3') AS (5'-CCCCGAGTGAATCTTTCTACGC-3')
Hsp90	S (5'-TCCTTCGGGAGTTGATCTCTAATGC-3') AS (5'-GAATTTTGAGCTCTTTACCACTGTCCAA-3')
p23	S (5'-ACCAGTTCGCCCGTCCC-3') AS (5'-CCTTCGATCGTACCACTTTGCAGA-3')
dyskerin	S (5'-CCTCGGCTGTGGACCGG-3') AS (5'-AAATAATTACTTCCGCATCCGCCA-3')
β-actin	S (5'-TCACCCACACTGTGCCCATCTA-3') AS (5'-CAGCGGAACCGCTCATTGCCAATGG-3')

S, sense; AS, antisense.

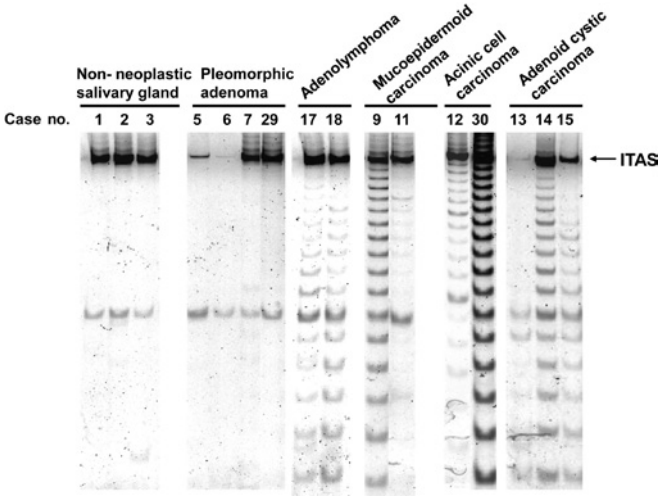

Figure 1. Telomerase activity in salivary gland tissues. Telomerase activity was measured by the TRAP assay using 0.1 μg protein of each tissue extract. ITAS, position of internal PCR control band.

at 94˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min and finally at 72˚C for 5 min. For Hsp90, p23 and dyskerin the thermal cycle was 33 cycles at 94˚C for 1 min, 55˚C for 1 min, 72˚C for 1 min and finally at 72˚C for 5 min. The primer sequences are shown in Table I. PCR products were analyzed by 1.5% agarose gel electrophoresis and sequenced to verify their identity. The quantification of EGFR mRNA expression level was carried out using a real-time fluorescence detection method. The fluorescence was detected by the laser detector of the ABI prism 7700 sequence detection system (Perkin-Elmer, Foster City, CA, USA) and detection was carried out by measuring the binding of a fluorescence dye, SYBR-Green I, to double-stranded DNA. The reaction mixture contained 1.0 μg cDNA, 10 μl SYBR-Green PCR Master mix (Toyobo) and 10 pmol of each pair of oligonucleotide primers. The primer sequences were: EGFR (HER1); 5'-GAGAGGAGAACTGCCAGAA-3' (sense) and 5'-GTAGCATTTATGGAGTG-3' (antisense). The PCR program was as follows: initial melting at 95˚C for 30 sec followed by 40 cycles at 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 60 sec. The quantification of mRNA expression relative to an internal control, β-actin, was performed by the ΔCt method (23).

Immunohistochemistry. Immunohistochemical staining was performed by the immunoperoxidase technique following antigen retrieval with microwave treatment (500 W, 10 min) in citrate buffer at pH 6.0. After blocking peroxidase activity using 3% H_2O_2-methanol for 10 min, specimens were blocked with phosphate-buffered saline (PBS) containing 5% normal horse serum (Vector Laboratories, Inc., Burlingame, CA, USA). Anti-Ki-67 monoclonal antibody (MIB-1) was obtained from Medical and Biological Laboratories (Tokyo, Japan). After 6 h of incubation at room temperature with primary antibody, specimens were rinsed briefly with PBS and incubated with secondary antibody (anti-mouse IgG antibody; Medical and Biological Laboratories, Tokyo, Japan) (diluted 1:200) for 1 h at room temperature. The cell nuclei stained by Ki-67 were calculated for each section in x200 microscopic fields. All cell nuclei staining brown above the background, regardless of intensity, were considered positively stained. The Ki-67 labeling index (LI) was defined as the number of salivary epithelial cells immunostained by Ki-67 per 500 salivary epithelial cells in each case.

Statistical analysis. Statistical analysis was performed using the Student's t-test or Spearman's rank correlation test. $P<0.05$ was regarded as statistically significant.

Results

Telomerase activity of salivary gland carcinomas. Fig. 1 shows the results of the telomerase assay of the non-neoplastic salivary glands and salivary gland tumors. The overall results are shown in Table II. Of the 24 malignant tumors, 15 samples (4 each of high-grade and low-grade mucoepidermoid carcinomas, 4 adenoid cystic carcinomas and 3 acinic cell carcinomas) exhibited strong telomerase activity, while 9 samples (7 low-grade mucoepidermoid carcinomas and 2 adenoid cystic carcinomas) showed weak activity (Table II). Of the benign tumors, 4 adenolymphomas were telomerase-positive, while all 8 pleomorphic adenomas were negative (Table II). The 6 non-neoplastic salivary glands were telomerase-negative. The mean level of telomerase activity was higher in malignant tumors (3.18±2.71) than in benign tumors (0.042±0.01) (Student's t-test, $p<0.05$). In the overall malignant tumors, telomerase activity was not correlated with clinicopathological factors, such as age, gender and tumor type. In mucoepidermoid carcinomas, the high-grade cases and 4 of the 11 low-grade cases showed high telomerase activity (Table II). Furthermore, telomerase activity was found to be significantly higher in high-grade mucoepidermoid carcinomas compared to that in low-grade mucoepidermoid carcinomas (Student's t-test, $p<0.05$).

Telomerase activity correlates with the expression of hTERT/EGFR mRNA in salivary gland carcinomas. mRNA expression of telomerase components hTERT, hTR, hTEP1, Hsp90,

Table II. Telomerase activity and Ki-67 labeling index (LI) in salivary gland tumors.

Histological diagnosis	Telomerase activity[a]			Ki-67 LI
	Strong	Weak	Negative	
Non-neoplastic salivary gland	0	0	6	0.8±0.5
Benign tumor				
Pleomorphic adenoma	0	0	8	2.8±1.8
Adenolymphoma	4	0	0	4.4±1.1
Malignant tumor				
Mucoepidermoid carcinoma				
High-grade	4	0	0	13.6±21.1
Low-grade	4	7	0	2.9±1.3
Adenoid cystic carcinoma	4	2	0	6.9±2.4
Acinic cell carcinoma	3	0	0	10.3±7.2

[a]Samples showing <3 sequential bands were judged as negative. We calculated the ratio of the density of the total number of ladder bands to the ITAS signal. When the ratio was <3.0, telomerase activity was classified as weak and when the ratio was ≥3.0, it was classified as strong.

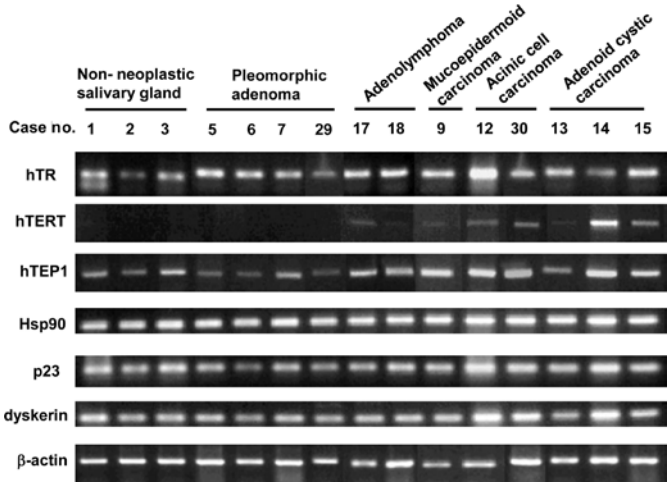

Figure 2. mRNA expression of telomerase components in salivary gland tissues. Six telomerase components (hTERT, hTR, hTEP1, Hsp90, p23 and dyskerin) were examined by RT-PCR. β-actin was used as a positive control.

p23 and dyskerin was determined by RT-PCR analysis in 15 cases, including non-neoplastic salivary glands and salivary gland tumors (Fig. 2). All 6 malignant tumors exhibited an increased mRNA expression of hTERT. Although 2 adenolymphomas revealed hTERT mRNA expression, neither the 4 pleomorphic adenomas nor the non-neoplastic salivary glands revealed hTERT expression. Densitometric scanning was performed on each band of PCR products and the signal intensity was normalized to an internal control (β-actin). A significant correlation was observed between telomerase activity and the mRNA expression of hTERT (Spearman's rank correlation test, $p<0.05$) (Fig. 3). However, no correlation was found between telomerase activity and other telomerase components (hTEP1, Hsp90, p23 and dyskerin). Furthermore,

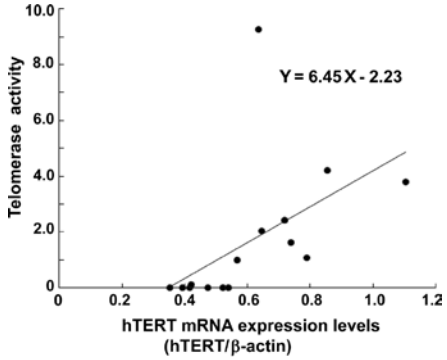

Figure 3. Correlation between the telomerase activity and hTERT mRNA expression in salivary gland tissues. A significant correlation was noted between telomerase activity and mRNA expression of hTERT (Spearman's rank correlation test, $p<0.05$).

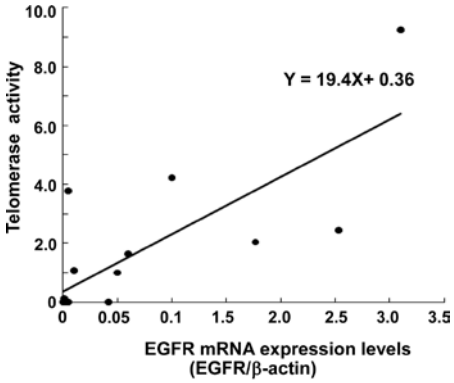

Figure 4. Correlation between the telomerase activity and EGFR mRNA expression in salivary gland tissues. A significant correlation was found between telomerase activity and mRNA expression of EGFR (Spearman's rank correlation test, $p<0.01$).

a significant correlation was found between telomerase activity and mRNA expression of EGFR in the 15 cases examined in the rank correlation test for mRNA expression of telomerase components (Spearman's rank correlation test, $p<0.01$) (Fig. 4).

Ki-67 LI increase in salivary gland carcinomas harboring high telomerase activity. Ki-67 LI in salivary gland tissues is shown in Table II. Ki-67 LI in each malignant histological type, with the exception of low-grade mucoepidermoid carcinoma, was found to be higher compared to that in each benign histological type and in the non-neoplastic salivary gland cases. The index of malignant tumors (8.43±4.6) was significantly higher than that of the non-neoplastic salivary glands (0.8±0.5) (Student's t-test, $p<0.05$). Although most of the malignant cases which had high telomerase activity revealed increased levels of Ki-67 LI, no significant correlation was found between telomerase activity and Ki-67 LI (Spearman's rank correlation test, $p>0.05$).

Discussion

In this study, telomerase activity was observed in all of the malignant tumors. The levels of telomerase activity were higher in malignant tumors than in non-neoplastic salivary glands or benign tumors. Moreover, the 4 high-grade mucoepidermoid

carcinomas exhbited strong telomerase activity, while 7 of the 11 low-grade mucoepidermoid carcinomas revealed weak telomerase activity. A statistical correlation was observed between telomerase activity and the histological grade of the mucoepidermoid carcinomas. Previously, Liao *et al* reported that low-grade mucoepidermoid carcinomas showed negative telomerase activity (24). These results indicate that weak telomerase activity reflects a low malignancy of certain mucoepidermoid carcinomas and suggest the possibility that tumor cells involved have been in the non-proliferative stage of growth arrest or terminal differentiation, in which case telomerase activity would be greatly reduced. Of the 6 adenoid cystic carcinomas, 4 cases exhibited strong telomerase activity and 2 cases showed weak activity. Although the main reason for certain malignant salivary tumors exhibited decreased telomerase activity remains unclear, these results raise the possibility that the number of tumor cells examined was below the threshold required for the detection of telomerase activity. In one case of adenoid cystic carcinoma tissue the weak telomerase activity was composed of extensive fibrous tissue with a limited epithelial component. In such cases, the activity of telomerase would have been greatly reduced if tumor cells had been in a non-proliferative condition.

Among the benign tumors, telomerase activity was observed in the 4 adenolymphomas, while the 8 pleomorphic adenomas exhibited none (Table II). The type of cell revealing this activity was not examined; however, the adenolymphoma cases revealed a considerably higher Ki-67 LI in lymphoid tissue than in epithelial tumor tissue (data not shown). In addition, strong telomerase activity was found in one acinic cell carcinoma case (no. 30) harboring infiltrating lymphocytes (Fig. 1). It is well known that lymphoid tissue shows strong telomerase activity (25). Taken together, it is most likely that lymphocytes involved in tissues exhibited the telomerase activity in the above cases.

mRNA expression of telomerase components in salivary gland tissues has been scarcely investigated (22,26). In the present study, hTERT mRNA was highly expressed in malignant tumors and correlated with the telomerase activity. Nakayama *et al* indicated a similar correlation between the activity of telomerase and the expression of hTERT mRNA in hepatocellular carcinomas by more precise semi-quantification using cultured carcinoma cells as the standard (6). These results suggest that detection of hTERT mRNA is sensitive for telomerase as well as the determination of telomerase activity using TRAP assay. mRNA expression of other telomerase components (hTR, hTEP1, Hsp90, p23 and dyskerin) was expressed, not only in non-neoplastic tissues, but also in benign/malignant tumors (Fig. 2). These findings are in accordance with the results of previous studies of telomerase components in other malignant tumors (14,26,27).

EGFR is a transmembrane glycoprotein that belongs to the erbB family (ErbB1/HER1, ErbB2/HER2, ErbB3 and ErbB4) of tyrosine kinase receptors as the signal transduction initiator (28). Activation of the EGFR signaling pathway has been linked to tumor cell proliferation, survival, angiogenesis and metastasis (18). Overexpression of EGFR has been reported in a number of human cancers (19,28-30). Recently, Heeg *et al* reported that EGFR leads to the phosphorylation of hTERT and regulates telomerase activity through the PI3K/AKT signaling pathway (31). Combined EGFR, cyclin D1 and hTERT protein expression was correlated with an aggressive phenotype in squamous cell carcinoma of the larynx (32). In the present study, a significant correlation was found between the expression of EGFR mRNA and telomerase activity. Collectively, these results indicate that EGFR plays a significant role in regulating the telomerase activity of salivary gland tumors. It is assumed that EGFR is a key determinant of telomerase activity in human malignant tumors.

In conclusion, the results of this study suggest that telomerase activity and mRNA expression of hTERT are useful markers for detecting malignant cells in salivary gland carcinomas. In addition, EGFR expression may also be capable of determining malignancy in salivary gland carcinomas, although numerous further studies are required to clarify the pivotal role of EGFR in regulating telomerase activity.

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