RESEARCH COMMUNICATION

Increased Expression of the PRL-3 Gene in Human Oral Squamous Cell Carcinoma and Dysplasia Tissues

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Abstract

Phosphatase of regenerating liver (PRL) belongs to a class of the protein tyrosine phosphatase family, which is known so far to consist of 3 members, PRL-1, PRL-2, and PRL-3. The aim of this study was to uncover the role of PRL genes in development of oral malignancy. We analyzed expression levels of the 3 PRL genes in 50 human oral squamous cell carcinomas (OSCCs), 11 dysplasia and 12 normal mucosa tissues by a real-time RT-PCR method. PRL-3 but not PRL-1 or PRL-2 expressions were significantly higher in OSCC and dysplasia than in normal mucosa tissues. Additionally, PRL-3 expressions were significantly higher in OSCC tissues harboring dominant-negative p53 or recessive p53 mutation than in those harboring wild-type p53. These results suggest that PRL-3 plays a role in oral cancer development and can be useful as a marker of pre-malignant and malignant lesion of oral mucosa.

Keywords: PRL - oral squamous carcinoma - dysplasia - p53

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Introduction

Head and neck cancer is the 6th most common cancer and there has been little improvement in patient morbidity in the past 50 years (Hunter et al., 2005). Squamous cell carcinomas (SCCs) are the most common form of head and neck cancer. Oral SCCs (OSCCs) often develop in a normal–dysplasia–carcinoma sequence. Long-term follow-up studies showed that 11–36% of oral epithelial dysplasias transformed into SCC and that dysplasias were generally associated with a risk for malignant transformation (Sudbo et al., 2001; Silverman et al., 1984). Therefore, more reliable biological markers than those presently known are required for earlier diagnosis, prognosis and follow-up.

The p53 gene is the most frequent target of genetic alterations, being mutated in half of human cancers (Sigal and Rotter, 2000; Iwakuma et al., 2005). We previously reported that the p53 gene mutations occurred at high frequency (in almost 80% of OSCCs)(Kashiwazaki et al., 1997). In the functional interactions of mutated p53 with the remaining wild-type (WT) p53 allele, the p53 mutations are classified into two types, recessive (R) and dominant-negative (DN) mutations. DN p53 mutants inactivate the endogenous WT p53 protein in a DN fashion by forming heterotetramer complex (Milner and Medcalf, 1991; Milner et al., 1991). We previously reported that DN p53 mutation is a risk factor for metastatic recurrence in patients with OSCCs (Hassan et al., 2008). Immunohistochemical analyses and various mutation analyses of the DNA-binding domain also demonstrate that p53 mutation is a useful marker for predicting prognosis of patients with OSCC (Yamazaki et al., 2003; De Vicente et al., 2004; Marx, 2007).

Protein tyrosine phosphorylation is well known to play key roles in regulating functions of diverse proteins that control numerous essential events in eukaryotes, such as transcriptional regulation, apoptosis, cell cycle progression, protein degradation, and protein trafficking (Hunter, 2000; Lyon et al., 2002; Hoffman et al., 2004). Protein tyrosine phosphorylation occurs basically through catalyses by protein tyrosine kinases (PTK) and protein tyrosine phosphatases (PTP). Abnormal balance between PTK and PTP activities causes aberrant tyrosine phosphorylation, which is frequently linked to malignant phenotypes of cancer (Hunter, 2000; Lyon et al., 2002; Hoffman et al., 2004). Phosphatase of regenerating liver (PRL) is a newly identified class of the protein

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tyrosine phosphatase family, which is known so far to consist of 3 members, PRL-1, PRL-2, and PRL-3 (Cates et al., 1996; Zeng et al., 1998). Although PRLs are expressed normally in the skeletal muscle and brain at high levels, in the heart at moderate levels, and in many other tissues at low levels, aberrant expressions of PRLs have been identified in a variety of cancer cell lines and tissues (Zeng et al., 1998; Diamond et al., 1994; Matter et al., 2001; Bardelli et al., 2003). For example, PRL-1 is overexpressed in pancreatic cancer and melanoma cell lines (Wang et al., 2002; Han et al., 2002); upregulated expression of PRL-2 is observed in prostate cancer cell lines and cancer tissues (Wang et al., 2002): PRL-3 is upregulated in colon, gastric and liver cancer tissues (Kato et al., 2004; Peng et al., 2004; Miskad et al., 2004; Wu et al., 2004). Further, recent interest in PRLs shows their roles in invasion and metastasis. Overexpression of PRL-3 is related to metastasis of human colon cancers and gastric cancers (Saha et al., 2001; Bardelli et al., 2003; Miskad et al., 2007). Transduction of PRL-1 or PRL-3 gene into Chinese hamster ovary cells increased their motile, invasive and metastatic abilities (Zeng et al., 2003), and mouse B16 melanoma cells expressing PRL-3 showed highly motile and metastatic abilities compared to the control cells (Wu et al., 2004). These findings strongly indicate that aberrant expressions of PRLs are involved in carcinogenesis and malignant progression of a variety of cancers. Interestingly, recent reports demonstrated that PRL-1 and PRL-3 were identified as one of the target genes of p53 (Min et al., 2009; Basak et al., 2008; Min et al., 2010).

These led us to examine the relationships between the expression levels of PRLs and p53 statuses in OSCC, dysplasia or normal mucosa tissues.

Materials and Methods

Clinical Specimens

We used 50 oral SCC tissues, 11 dysplasia tissues (clinically diagnosed as leukoplakia), and 12 normal tissues from 68 patients who had undergone surgery at the Hokkaido University Hospital from January 2000 to September 2004. Three of 50 SCC tissues and 3 of 11 dysplasia tissues were originated from the same patient who had multiple lesions. One to five bulk tissue samples of about 5 mm size were immediately cut from the oral tissues resected by a standard surgical procedure. The tissue samples were snap frozen in liquid nitrogen and stored at −80°C until use. This study was approved by the Ethics Committee of Hokkaido University, and informed consent was obtained from all the patients.

RNA Extraction and cDNA Preparation

Total RNA was extracted from the clinical specimens by using Trizol (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. For real-time PCR, 1 μg of total RNA was subjected to cDNA synthesis in 100 μl of reaction mixture containing Taq Man RT buffer (Applied Biosystems, Foster City, CA), 5.5 mM MgCl2, 500 μM dNTP, 2.5 μM random hexamers, 0.4 U/μl RNAase inhibitor, 1.25 U/μl MultiScribeTM reverse transcriptase. The reverse transcription reaction was performed sequentially for 10 min at 25°C, for 30 min at 48°C, and for 5 min at 95°C.

Quantitative Real-Time RT-PCR

Quantitative RT-PCR assays were carried out by using ABI PRISM 7900HT (Applied Biosystems) with SYBR-green fluorescence. Real-time PCR amplification was performed in 20 μl of reaction mixture containing 2 μl of cDNA sample, 10 μl of QuantiTect™ SYBR Green PCR Master Mix (Qiagen, Valencia, CA) and with specific primer sets. The sense/antisense primers were designed as follows: PRL-1, 5'-CCGGCTGTATGATTAGGCCACAA-3'/5'-GCAGTAATCTCCACTGCCCTTCA-3'; PRL-2, 5'-GTCCAGCCCCTGTGGAGAT-3'/5'-ACTCGAACCAAAGTCGTCACTCC-3'; PRL-3, 5'-GCGTGTGTGTGAAGTGACCTAT-3'/5'-GCCAGTCTTCCACTACCTTGC-3'; β-actin, 5'-TTGCCGACAGGATGCAGAA-3'/5'-GCCGATCCACACGGAGTACT-3'. PCR was carried out by starting with a 15-min hot start at 95°C, followed by a denaturation step at 94°C for 15 s, an annealing step at 60°C for 30 s, and an extension step at 72°C for 1 min for 40 cycles. Dissociation curve analysis (95°C for 15 s, 60°C for 15 s, and 95°C for 15 s) was performed at the end of the 40 cycles to verify the identity of the PCR product. Data were analyzed by using Sequence Detector Systems version 2.0 software (Applied Biosystems). Quantification was done by using the standard curve method. Finally we represented relative gene expression levels as the ratio of the target PRL gene to the internal reference gene (β-actin) expression based on the initial copy number calibrated along the standard curve.

Yeast p53 functional assay

The yeast functional assay was performed according to our previous method (Flaman et al., 1995; Tada et al., 1996). Colorimetric evaluation of yeast colonies (red or white colonies) was done after 48 h culture. To confirm mutations, we collected the pSS16 plasmids containing a mutant p53 from red yeast colonies, and then transfected the plasmids into XL-1 blue E- coli by electroporation. The plasmids were recovered, purified, and sequenced with BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster city, CA) on ABI PRISMS 3100-avant genetic analyzer (Applied Biosystems, Foster city, CA).

Transdominance assay

The DN potential of the detected p53 mutant was tested using a yeast-based transdominance assay as described previously (Hassan et al., 2008). Briefly, yIG397 was transformed with both a plasmid with wild-type p53 and a plasmid with the mutant p53 that had been sequence-verified. For each transformation, 50 μl of yeast suspension was mixed with 100 ng of pTSHP53 (Trp 1 marker), 100 ng of mutant p53-containing pSS16 (Leu 2 marker), 50 μg of sonicated single-stranded salmon sperm DNA and 300 μl of LiOAc containing 40% polyethylene glycol 4000. The mixture was incubated at 30°C for 30 min and heat-shocked at 42°C for 15 min. Yeast was then

plated on SD medium minus leucine and tryptophan, but with a limited amount of adenine (5 μg/ml). The plates were then incubated for 48 hr in a 30°C-humidified atmosphere. Double-transformant clones (Leu+, Trp+) giving rise to white (Ade+) or pink/red (Ade-) colonies were interpreted as expressing recessive and DN mutant, respectively.

Statistical Analysis

The relationship between each PRL gene expression and each clinicopathological parameter was determined by the Mann-Whitney U-test. The statistical software package applied was StatView 5.0 for Macintosh (SAS Institute, Cary, NC). A value of $p < 0.01$ was considered statistically significant.

Results

Expression of the PRL gene in normal mucosa, dysplasia and squamous cell carcinoma tissues

Expression levels of PRL-3 were significantly different among normal, dysplasia and SCC tissues whereas those of PRL-1 and PRL-2 were not (Figure 1a). The expression levels of the PRL-3 gene in both SCC and dysplasia tissues were significantly high compared to those in normal mucosa tissues ($p < 0.01$, Mann-Whitney U-test). There were no significant differences in the expression levels of PRL-3 between SCC and dysplasia tissues.

Expressions of PRL genes in different disease stages

We next compared the expression levels of PRL genes among different disease stages. As shown in Figure 1b, there was no difference in the expression levels of each PRL gene among dysplasia, SCC stage I, stage II, stage III and stage IV although those of PRL-3 were significantly higher in tissues of each disease stage than in the normal tissues.

Table 1. p53 Status of the Samples Examined

	Wild type	No expression	Recessive mutant type	Dominant-negative mutant type
SCC	12	3	24	11
Dysplasia	10	0	0	1
Normal	12	0	0	0

Expressions of PRL genes in SCC tissues with lymph node metastasis and those without it

To find a particular PRL gene associated with metastasis, we compared the expression levels of each PRL gene between SCC with lymph node metastasis and SCC without it. There was no correlation between the expression levels of any PRL gene and the metastatic status.

Expression of PRL genes in SCC tissues harboring wild type p53 and those harboring mutant p53

As it is known that PRLs mutually interact with p53 in the regulation of their expressions at transcriptional and post-translational levels (Basak et al., 2008; Min et al., 2010), we examined the relations between the expression levels of PRL genes and p53 mutation status. The latter was

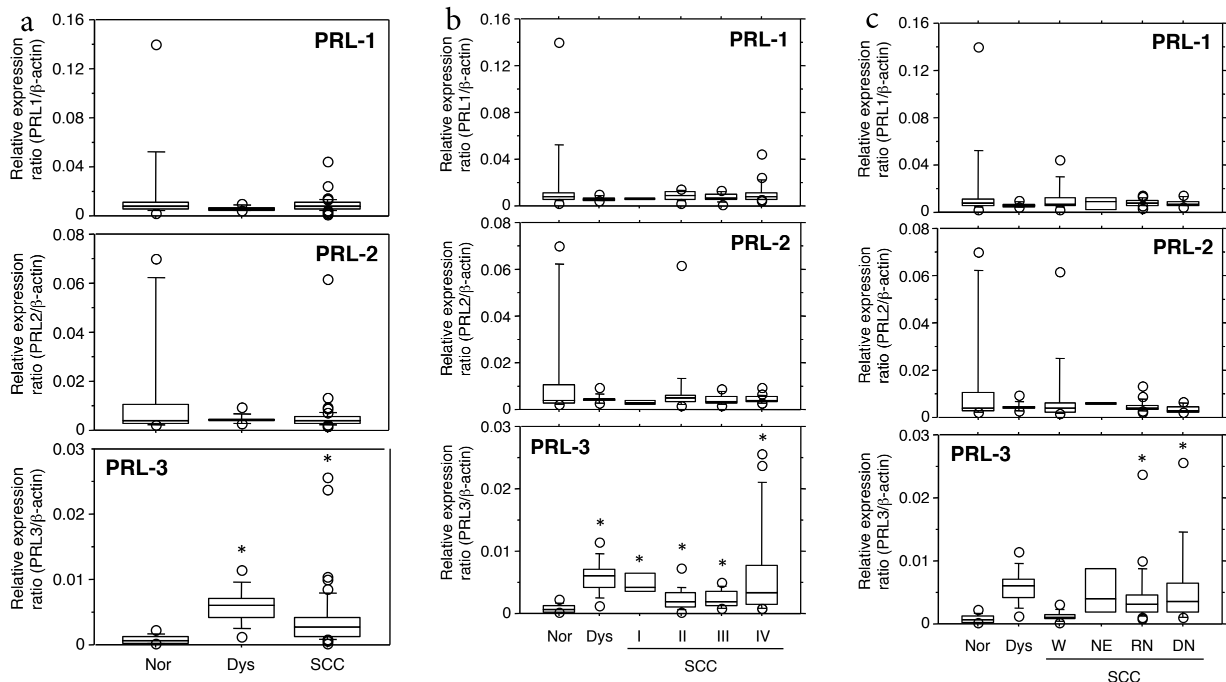

Figure 1. Expression levels of PRL genes. a) Comparison among normal mucosa, dysplasia and squamous cell carcinoma (SCC); b) Comparison of the three, SCCs being classified in 4 stages; c) Comparison of the three, SCCs being classified according to p53 statuses. Nor: normal mucosa tissues (n = 12); Dys: dysplasia tissues (n = 11); SCC: squamous cell carcinoma tissues (n =50: stage I (4), II (15), III (14), IV (17)); W: wild type p53 (n = 12); NE: no expression of p53 mRNA (n = 3); RN: recessive mutant p53 (n = 24); DN: dominant negative mutant p53 (n = 11). The distribution of the relative ratio (PRL/b-actin) was summarized by using boxplots. The central box in each plot shows the interquatile (25th to 75th percentile) range. The line in the box shows the median. The whiskers (vertical bars) were drawn to the 90th and 10th percentiles. Extreme values higher than the 90th percentile or lower than the 10th percentile were marked with circles individually. *$p < 0.01$ compared to the normal mucosa tissues (a and b) or SCC tissues harboring wild type p53 (c) (p-values were calculated by the Man-Whitney U –test).

evaluated by a yeast functional assay which provides both functional and sequence information of p53 mutations. As a resultant, the expression levels of PRL-3 in SCC tissues harbouring mutant p53 (regardless of dominant or recessive negative mutation) were significantly higher than those in SCC tissues harboring wild type p53 ($p < 0.01$) (Figure 1c). Meanwhile, dysplasia tissues which harbored wild type p53 except one sample showed high expressions of PRL-3 at a similar level to the SCC tissues with mutant p53.

Discussion

In this study, we examined the expression levels of PRL-1, PRL-2 and PRL-3 genes in 50 cases of SCC, 10 cases of dysplasia and 12 cases of normal mucosa tissues by a quantitative RT-PCR method. We found that the expression levels of PRL-3 in SCC and dysplasia tissues were significantly higher than those in normal mucosa tissues and that there was no difference in expression levels of PRL-1 and PRL-2 among SCC, dysplasia and normal mucosa tissues. Comparing the expression levels of PRLs among SCC tissues in 4 different disease stages, or between SCC tissues with lymph node metastasis and those without it, we did not find any difference in the expression levels of PRLs. Thus, among 3 PRL genes, only PRL-3 is likely to be related to malignancy of oral mucosa. It is well known that increased expressions of PRL-3 correlate with metastatic ability in several kinds of cancers including colorectal, breast, gastric and esophageal cancers (Peng et al., 2004; Miskad et al., 2004; Wu et al., 2004; Zeng et al., 2003; Radke et al., 2006; Ooki et al., 2010). As above mentioned, there was no difference in the expression levels of PRL-3 among SCC tissues in the different disease stages, or between SCC tissues with metastasis and without it. In OSCC, therefore, dysregulated expressions of PRL-3 may play an important role in carcinogenesis, especially development of pre-malignant lesion (dysplasia) rather than invasion and metastasis. As OSCC often develops through dysplasia, the presence of dysplasia can be recognized as a risk of development of SCC. PRL-3 may be a useful molecular marker for early detection of dysplasia, which should help to improve prognosis of OSCC patients.

What is the function of PRL-3 of which expression was elevated in dysplasia and SCC? We speculate a potent function of PRL-3 in premalignant and malignant lesion as follows: Common properties of the cells in dysplasia and SCC are their fitness to proliferate, survive and lose cell polarity. And such cell properties are promoted through activation of PI3K/Akt pathway (Cully et al., 2006). A recent report showed that PRL-3 down-regulated PTEN expression, which resulted in activation of signaling pathway through PI3K and Akt (Wang et al., 2007). Further, by immunohistochemical analysis, the cells with phosphorylated Akt (active Akt) proteins were observed in oral epithelial dysplasia and early OSCC tissues but not in normal mucosa tissues (Watanabe et al., 2009). Taken together with these findings, it is possible that PRL-3-positive cells in premalignant and SCC tissues have the advantage of cell proliferation and survival or loss of cell polarity through the activation of PI3K/Akt pathway.

We had another interesting result in this study: the OSCCs harboring p53 mutant gene expressed PRL-3 at a high level compared to those harboring wild type p53. The p53 protein responds to various kinds of stress such as DNA damages, hypoxia and aberrant growth signals, and transactivates the target genes for cell cycle arrest, apoptosis and DNA repair (Harris and Levine, 2005). In normal cells in the absence of stress, wild type p53 protein is promptly degraded by MDM2 which functions as an E3 ubiquitin ligase. And p53 transcriptionally activates the expression of MDM2 in a negative feedback loop (Wu et al., 1993). Generally, the mutant p53 proteins often become stable and accumulate in tumor cells (Cadwell and Zambetti, 2001; Sigal and Rotter, 2000). Since MDM2 retains the ability to bind and degrade mutant p53, the inability to activate the transcription of Mdm2 is shown to be associated with the stability of mutant p53 proteins (Midgley and Lane, 1997). Min et al. (2010) demonstrated that overexpression of PRL-3 induced PIRH2 mRNA through the transactivation of EGR1 and increased phosphorylated MDM2. Both PIRH2 and phosphorylated MDM2 enhance ubiquitination and proteosome-mediated degradation of p53 protein. Thus, we speculate that the expression of PRL-3 in OSCC cell harboring mutant p53 is elevated in order to degrade the abnormally accumulated p53 proteins. However, such a speculation cannot explain the high expression of PRL-3 in dysplasia tissues because they harbored wild type p53 except one sample. There is a possibility that a quite different event occurs between SCC and dysplasia tissues in the relationship of PRL-3 and p53 status.

In conclusion, our study suggests that PRL-3 may be involved in the development of pre-cancerous lesion (dysplasia) and malignant transformation of oral mucosa tissues. PRL-3 could be a valuable therapeutic target against conversion of premalignant tissues (dysplasia) into malignant tissues (SCC) although additional investigations are required before we apply it to clinical trials.

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100.0
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Newly diagnosed without treatment

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