DNA Hypermethylation Status of Multiple Genes in Papillary Thyroid Carcinomas

Eiwa Ishida^a Mitsutoshi Nakamura^a Keiji Shimada^a Tomonori Higuchi^a
Keisuke Takatsu^c Katsunari Yane^b Noboru Konishi^a

Departments of ^aPathology and ^bOtolaryngology, Nara Medical University School of Medicine, Nara, and
^cDepartment of Molecular Biology, Institute of Gerontology, Nippon Medical School, Kashiwara, Japan

Key Words

Chronic inflammation • Homozygous deletion • Methylation • Papillary thyroid carcinoma

Abstract

Objective: The aim of this study was hypermethylation of multiple genes for papillary thyroid carcinomas (PTCs). **Methods:** We examined 39 lesions using methylation-specific PCR to assess hypermethylation in genes, including *p16^{INK4a}*, *p14^{ARF}*, *RB1*, *p27^{Kip1}* and *O⁶-MGMT*. Homozygous deletions of *p16^{INK4a}* and *p14^{ARF}* were investigated by differential PCR, all with reference to clinicopathological factors. **Results:** We found methylation of *p16^{INK4a}* in 35.9% (14/39); *p14^{ARF}* in 2.6% (1/39); *RB1* in 23.1% (9/39); *p27^{Kip1}* in 15.4% (6/39), and *O⁶-MGMT* in 15.4% (6/39). Hypermethylation of at least one of these genes was apparent in 66.7% (26/39). Homozygous deletions of *p14^{ARF}* and *p16^{INK4a}* were detected in 7.7 (3/39) and 2.6% (1/39), respectively. In cases with *p16^{INK4a}* alterations, tumors were significantly increased. A history of chronic thyroid disease and lymphocytic infiltration was significantly associated with *p14^{ARF}* alterations, without regional lymph node metastases. **Conclusions:** Our data suggest that alterations in *p16^{INK4a}*, mainly hypermethylation, may be linked to tumor growth but not tumor development, while alterations in *p14^{ARF}* may contribute to the induction of chronic inflammation-related PTCs.

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Introduction

The papillary thyroid carcinoma (PTC) is the most frequent malignancy originating in the thyroid gland. With respect to gene alterations in carcinogenesis, PTCs have exceedingly low rates of loss of heterozygosity, with no specific region displaying a particularly high prevalence compared with follicular carcinomas [1], but somatic rearrangement of the *sRET* proto-oncogene has been detected in 25–35% of PTCs in the Western World [2, 3], and frequencies varied in Japanese cases (3–36%) [4–6]. Recently, *BRAF* mutations, i.e. alternative molecular events, have been detected in 0–83% of PTCs, [7–10]. Finally, activated MAP kinase signaling caused by *RET* rearrangement, *BRAF* or *RAS* mutations, or other molecular alterations is thought to be essential for the induction of PTC [11]. However, the frequencies of gene alterations have been shown to differ in different ethnicities, reflecting different lifestyles, including nutrients such as iodine, and other mechanisms of PTC development and progression need to be identified.

The roles of DNA methylation in carcinogenesis have been highlighted for various types of cancers. Promotor hypermethylation of CpG islands has been shown to be an epigenetic change resulting in loss of function of responsive genes involved in cell cycle regulation and DNA repair. In PTCs, hypermethylation at exon 1 of *p16^{INK4a}* was detected in 6 (33.3%) of 20 primary tumors, indicat-

ing that the function of this gene may be lost during disease progression [12]. However, the knowledge on hypermethylation in PTCs is still insufficient.

In this study, a total of 39 tumors were evaluated using methylation-specific PCR (MSP) to assess hypermethylation in multiple genes implicated in regulatory processes, including *p16^{INK4a}*, *p14^{ARF}*, *RBI*, *p27^{Kip1}* and *O⁶-MGMT*. We also investigated homozygous deletions of *p16^{INK4a}* and *p14^{ARF}*, and protein expression by immunohistochemistry. The results showed statistically significant correlations between alterations in specific genes and certain clinicopathological features of the tumors.

Materials and Methods

Tumor Samples and DNA Extraction

Samples of 39 PTCs, consisting of conventional PTCs with well-differentiated histology, were obtained from the Department of Otolaryngology, Nara Medical University Hospital. Detailed clinicopathological data, e.g. age, sex, past history of chronic thyroid disease, tumor size, presence of regional lymph node metastasis, distant metastasis and clinical stage, were collected and summarized in table 1. The patients comprised 9 males and 30 females, ranging in age from 12 to 79 years, with a mean of 54.1 years. Two had a past history of chronic thyroiditis, and 2 suffered from Graves' (Basedow's) disease. According to the TNM classification, 9 (23.1%) presented with stage I, 12 (30.8%) with stage II, 17 (43.6%) with stage III and 1 (2.6%) with stage IV lesions.

The tumor samples were all fixed in 10% buffered formalin and embedded in paraffin, cut at 4 μ m and stained with hematoxylin and eosin (HE) for histopathological evaluation, with especial focus on lymphocytic infiltration. DNA was extracted by methods described previously [13].

Methylation-Specific PCR

MSP is sufficiently sensitive to detect a single methylated allele among 1,000 unmethylated ones [14] and, therefore, may be the most appropriate methodology for identifying DNA methylation patterns within CpG islands of *p16^{INK4a}*, *p14^{ARF}*, *RBI*, *p27^{Kip1}* and *O⁶-MGMT*. MSP distinguishes unmethylated from methylated alleles based on sequence changes produced after bisulfite treatment of DNA, which converts only unmethylated cytosine to uracil, and subsequent PCR using primers designed for either methylated or unmethylated DNA. It was here performed using sodium bisulfite modification with a 'CpGenome' DNA modification kit (Intergen, Oxford, UK) according to the manufacturer's protocol with additional minor modification [15, 16]. The primer sequences and the detailed MSP conditions have been reported previously [14, 17–22]. The regions analyzed for *p16^{INK4a}*, *p14^{ARF}* and *O⁶-MGMT* were promoter regions of the transcriptional start site [14, 17, 23]. For *RBI* and *p27^{Kip1}*, the regions analyzed were promoter regions of the translational start site and exon 2, respectively [20, 22]. Control methylated (Intergen) and unmethylated DNA from blood samples of normal volunteers were also treated with bisulfite as positive and negative controls. Amplified products were subsequently electrophoresed on 2% agarose gels and visualized by ethidium bromide staining.

Differential PCR for *p14^{ARF}* and *p16^{INK4a}* Deletions

To detect homozygous deletions, differential PCRs were carried out. Duplex PCR was performed to generate a 149-bp fragment covering exon 1 β of the *p14^{ARF}* gene, and a 204-bp exon fragment of *p16^{INK4a}* 1 α , together with a 160-bp fragment of the *GAPDH* gene and a 187-bp fragment of β -actin as internal controls, mainly to confirm the integrity of the template and also to assess contamination of samples by normal cells. Both sets of primers, for the control locus and the potentially deleted locus, were included in the PCR reaction mixture. DNA was amplified with 30 and 29 cycles of PCR for the *p16^{INK4a}* and *p14^{ARF}* genes, respectively. The primer sequences and detailed PCR conditions have been described previously [16, 24]. PCR products were analyzed on 8% acrylamide gels and photographed using a DC290 Zoom Digital Camera (Eastman Kodak, Rochester, N.Y., USA). Densitometry was performed using Kodak Digital Science ID Image Analysis software (version 3.5.2; Eastman Kodak). Samples presenting <20% of the control signal were considered homozygously deleted [16]. To assess the sensitivity of differential PCR for homozygous deletions, we performed a titration experiment with various ratios of normal DNA and DNA from A172 glioma cells with homozygous co-deletion of the *p16^{INK4a}* and *p14^{ARF}* genes. The chosen threshold value of 0.2 allows the detection of homozygous deletions in the presence of approximately 30% normal DNA for *p16^{INK4a}* and 25% for *p14^{ARF}* [16].

Immunohistochemistry

Protein expression was assessed immunohistochemically, using a polyclonal antihuman *p14^{ARF}* antibody (FL-132; SC1661; Santa Cruz Biotechnology, Santa Cruz, Calif., USA) and monoclonal antibodies to *p16^{INK4a}* (F-12; SC1661; Santa Cruz Biotechnology) and *pRB* (clone G3-245; PharMingen, San Diego, Calif., USA). Details on the conditions have been reported previously [25]. For *p14^{ARF}*, *p16^{INK4a}* and *pRB* immunostaining, reactivity was recorded as negative when <5% of cancer cells demonstrated staining, in agreement with a previous study [21].

Statistical Methods

Statistical analyses were performed using StatView-J 5.0 to analyze correlations between clinicopathological factors and genetic alterations. Tumor size and stage were divided into two groups, T1/2 and T3/4, and SI/II and SIII/IV, respectively. Associations between tumor variables were assessed by Fisher's exact test. Significance was defined as $p < 0.05$.

Results

Gene Methylation in PTC

Table 1 summarizes the methylation status for the five genes analyzed in this study. We found methylation of *p16^{INK4a}* in 35.9% (14/39); *p14^{ARF}* in 2.6% (1/39); *RBI* in 23.1% (9/39); *p27^{Kip1}* in 15.4% (6/39), and *O⁶-MGMT* in 15.4% (6/39). Representative methylation patterns are illustrated in figure 1. Hypermethylation of at least one of the genes was detected in 66.7% (26/39) of the samples. Methylated and unmethylated DNAs showed the expect-

Table 1. Clinicopathological factors and genetic and epigenetic alterations in the 39 PTCs

Case No.	Age years	Sex	CTD	Tumor	Node	Metas-tases	EX	Stage	LI	<i>p14^{ARF}</i>	<i>p16^{INK4a}</i>	<i>RBI</i>	<i>p27^{Kip1}</i>	<i>O⁶-MGMT</i>
1	29	F	–	T2	–	–	–	I	–	–	–	–	–	–
2	12	F	–	T3	+	–	–	I	–	–	M	–	–	–
3	71	M	U	T4	+	–	+	III	–	–	M	–	–	–
4	79	F	–	T2	+	–	+	III	–	–	–	–	–	–
5	38	F	–	T2	+	–	–	I	–	–	M	–	M	–
6	46	M	–	T3	–	–	–	II	–	–	M	M	–	M
7	30	F	–	T2	+	–	+	I	+	–	M	M	–	–
8	69	F	–	T2	+	–	+	III	–	–	–	–	–	–
9	78	M	–	T4	+	+	–	IV	–	–	M	–	–	–
10	79	F	–	U	+	–	U	III	–	–	D	–	M	–
11	56	F	–	T2	+	–	+	III	+	–	M	–	–	–
12	66	F	–	T2	+	–	+	III	–	–	–	–	–	M
13	68	F	–	T2	+	–	+	III	–	–	–	–	–	–
14	48	F	–	T2	+	–	–	III	–	–	–	M	–	–
15	64	F	–	T2	–	–	–	II	–	–	–	–	M	–
16	51	M	–	T3	+	–	–	III	–	–	–	–	–	–
17	66	M	–	T2	+	–	–	III	–	–	–	M	–	–
18	77	F	–	T2	+	–	+	III	–	–	–	–	M	–
19	63	F	–	T3	+	–	+	III	–	–	M	–	–	–
21	49	F	–	T2	–	–	–	II	+	–	–	–	–	–
22	50	F	–	T2	+	–	–	III	–	–	–	–	–	–
24	56	F	–	T2	+	–	–	III	–	–	–	–	–	–
25	30	F	GD	T1	–	–	–	I	+	–	–	–	–	–
26	73	F	–	U	+	–	U	III	–	–	–	–	–	M
28	66	F	–	T2	+	–	–	III	–	–	–	M	M	–
29	29	F	–	T2	+	–	–	I	–	–	–	M	–	–
30	32	M	–	T1	+	–	–	I	–	D	–	–	–	M
31	44	M	–	T2	+	–	–	I	–	–	–	M	–	–
33	64	F	CT	T2	–	–	–	II	+	M	M	–	–	M
34	58	F	–	T4	+	–	+	III	–	–	M	–	–	–
38	46	M	–	T3	–	–	–	II	–	D	M	–	–	–
41	18	F	–	T2	+	–	–	I	–	–	M	M	–	–
42	49	F	–	T2	–	–	–	II	+	–	–	–	–	–
44	75	F	GD	T2	–	–	–	II	+	D	–	–	–	–
46	45	F	–	T2	–	–	–	II	–	–	M	–	–	–
48	60	M	–	T3	–	–	–	II	–	–	M	M	–	–
53	65	F	–	T3	–	–	–	II	–	–	–	–	–	–
54	58	F	CT	T2	–	–	–	II	+	–	–	–	M	M
55	51	F	–	T2	–	–	–	II	–	–	–	–	–	–

CTD = Chronic thyroid disease; GD = Graves' disease; CT = chronic thyroiditis; EX = extension beyond the glands; LI = lymphocytic infiltration; M = methylated; D = deletion; U = unknown.

ed fragment sizes of 150 and 151 bp for *p16^{INK4a}*; 122 and 132 bp for *p14^{ARF}*; 81 and 93 bp for *O⁶-MGMT*; 163 and 163 bp for *RBI*, and 195 and 212 bp for *p27^{Kip1}*, respectively.

p14^{ARF} and p16^{INK4a} Deletions

As shown in table 1, homozygous deletions in *p14^{ARF}* and *p16^{INK4a}* were detected in 3 (7.7%) and 1 (2.6%) of the

39 samples, respectively. Representative deletion patterns are illustrated in figure 2.

Immunohistochemistry

Nuclear immunoreactivity for *p14^{ARF}*, *p16^{INK4a}* and *pRB* was significantly correlated with a lack of hypermethylation or homozygous deletion (fig. 3).

Fig. 1. Methylation-specific PCR for CpG islands in *p16^{INK4a}*, *p14^{ARF}*, *RBI*, *p27^{Kip1}* and *O⁶-MGMT* genes in PTCs. A 25-bp DNA ladder (Invitrogen, Carlsbad, Calif., USA) is shown on the left as a molecular weight marker. The presence of a visible PCR product in lane U indicates an unmethylated gene: the presence of a product in lane M indicates a methylated gene. Case numbers are indicated above each gel. Cases 2, 3 and 5 for *p16^{INK4a}*, case 33 for *p14^{ARF}*, cases 6, 7 and 14 for *RBI*, cases 5 and 10 for *p27^{Kip1}*, and cases 6 and 12 for *O⁶-MGMT* are methylated. NC = Negative control DNA from normal blood sample; PC = positive control for methylated DNA.

Fig. 2. Differential PCR for assessment of *p14^{ARF}* and *p16^{INK4a}* homozygous deletions in PTCs. Cases 30, 38 and 44 have *p14^{ARF}* homozygous deletions, and case 10 has a *p16^{INK4a}* homozygous deletion. Cases 8, 9 and 46 have a normal gene status. NC = Negative control DNA from a normal blood sample.

Clinicopathological Factors and Gene Alterations

We examined each tumor sample for potential causal relationships by comparing genetic and epigenetic alterations with clinicopathological parameters. As shown in table 2, tumors with *p16^{INK4a}* alterations, mainly hyper-

methylation, were significantly larger ($p = 0.0023$). In cases with a previous history of chronic thyroid disease, lymphocytic infiltration was a significant finding ($p = 0.0009$), together with *p14^{ARF}* alterations ($p = 0.0475$) and infrequent metastases to regional lymph nodes ($p = 0.0136$).

Fig. 3. With p14^{ARF} immunohistochemistry, case 2 without p14^{ARF} alteration shows positive staining (a), and case 44 with p14^{ARF} deletion is negative (b). With p16^{INK4a} immunohistochemistry, case 1 without p16^{INK4a} alteration is positive (c), and case 2 with p16^{INK4a} methylation is negative (d). With pRB immunohistochemistry, case 5 without RB1 methylation demonstrates focally positive staining (e), and case 41 with RB1 methylation is negative (f).

Table 2. Correlations between clinicopathological factors and gene alterations in PTCs

Factors	<i>p14^{ARF}</i>	<i>p16^{INK4a}</i>	<i>RB1</i>	Overall status for alteration	Tumor (T1/2)	Node	Metastases	LI
<i>Tumor</i>								
T1/2	3/27	6/27	7/27	17/27	–	17/27	0/27	–
T3/4	1/10	8/10	2/10	8/10	–	6/10	1/10	–
p value	>0.999	0.0023	>0.999	0.445	–	>0.999	0.270	–
<i>Node</i>								
+	1/25	10/25	7/25	19/25	–	–	1/25	–
–	3/14	5/14	2/14	8/14	–	–	0/14	–
p value	0.123	>0.999	0.445	0.287	–	–	>0.999	–
<i>Metastases</i>								
+	0/1	1/1	0/1	1/1	–	–	–	–
–	4/38	14/38	9/38	26/38	–	–	–	–
p value	>0.999	0.385	>0.999	>0.999	–	–	–	–
<i>LI</i>								
+	2/8	3/8	1/8	5/8	8/8	2/8	0/8	–
–	2/31	12/31	8/31	22/31	19/31	23/31	1/31	–
p value	0.180	>0.999	0.653	0.683	0.079	0.0161	>0.999	–
<i>CTD</i>								
+	2/4	1/4	0/4		4/4	0/4	0/4	4/4
–	2/34	13/34	9/34	23/34	23/34	24/34	1/34	4/34
p value	0.0475	>0.999	0.555	>0.999	0.553	0.014	>0.999	0.0009
<i>Sex</i>								
Males	2/9	5/9	4/9	8/9	3/9	6/9	1/9	0/9
Females	2/30	10/30	5/30	19/30	24/30	19/30	0/30	8/30
p value	0.223	0.266	0.170	0.228	0.0053	>0.999	0.231	0.160

LI = Lymphocytic infiltration; CTD = chronic thyroid disease. Significant p values are shown in bold.

Discussion

Activated MAP kinase signaling caused by RET rearrangement, BRAF or RAS mutations, or other molecular alterations, is thought to be essential for the genesis of PTC [11]. However, the frequencies of the above-described gene alterations vary according to ethnicity. For example, rearrangements of the *RET* proto-oncogene in PTCs are relatively frequent [2, 3], although prevalence varies considerably (3–36%) in Japan [4–6], and other mechanisms of tumor development and PTC progression need to be clarified. The role of DNA hypermethylation in tumor suppressor or DNA repair genes in carcinogenesis has been highlighted. Therefore, we aimed to examine the methylation status of five implicated genes and homozygous deletions of *p14^{ARF}* and *p16^{INK4a}* and to correlate them with clinicopathological factors.

Cell proliferation is regulated by a family of CDKs, and *p16^{INK4a}* is a CDKI which specifically inhibits CDK4/6 and consequently cyclin D-dependent phosphorylation of Rb, leading to less transcription of E2F-responsive genes necessary for S-phase entry [26]. Deregulation of the *p16^{INK4a}* gene product through loss of heterozygosity, promoter hypermethylation and/or mutations in the *INK4a/ARF* locus is a common phenomenon in the transformation process, which is often found in more aggressive tumors [27]. In this study, the frequency of *p16^{INK4a}* hypermethylation of PTC was found to be 38.5%, in line with a previous study [12]. Interestingly, in the 15 cases with *p16^{INK4a}* alterations, consisting of 14 with hypermethylation and only 1 with homozygous deletions, tumors were significantly larger (T3 and T4), suggesting that *p16^{INK4a}* alterations in PTCs are associated with tumor growth or progression, but not tumor development. There was no association between *p16^{INK4a}* alterations

and clinical stage in this study. For the TNM classification of PTC, age is thought to be an important factor; patients below 45 years of age without distant metastases are grouped in stage I, irrespective of the tumor size and the presence of lymph node metastases.

pRB (*RB1* gene products) is a negative regulator of cell proliferation, and in a hypophosphorylated state, pRb exerts its antiproliferative function by sequestering and altering the function of the E2F family of transcription factors that control the expression of a bank of genes essential for cells to progress from G1 to the S phase [27]. The importance of *RB1* in cancer development is well established, and most human cancers show somatic alterations, either in pRB or other components of the pathway [28]. To our knowledge, we were the first to report *RB1* hypermethylation in PTCs, and a relatively high frequency was detected (23.1%), though previous immunohistochemical studies of RB expression in PTCs gave conflicting results [29, 30]. In our previous study, a clear correlation between *RB1* alterations and immunohistochemical expression was noted [15]. The present findings suggest that the *RB1* gene might play a role in thyroid tumorigenesis, although no significant association between *RB1* gene methylations and clinicopathological factors was revealed. An inverse correlation between *p16^{INK4a}* and *RB1* expression was found, demonstrating that each of the alterations can lead to cancer formation [31]. With respect to the association between *p16^{INK4a}* alterations and cancer growth, further studies including other mechanisms of pRB loss are required to establish the hypothesis that alterations in the *p16^{INK4a}/RB1* pathway induce cancer growth in PTCs.

p14^{ARF} plays a major role in the *p53* pathway by binding specifically to MDM2, resulting in stabilization of both *p53* and MDM2 [32]. *p27^{Kip1}* encodes a cyclin kinase inhibitor, which acts as a potent tumor suppressor gene in a variety of human cancers [33]. *O⁶-MGMT* encodes a DNA repair protein, and prevents mutations and cell death that result from aberrant alkylation of DNA [34]. With regard to hypermethylation of *p14^{ARF}*, *p27^{Kip1}* and *O⁶-MGMT* in PTCs, the frequencies found here were low (2.6, 15.4 and 15.5%, respectively), and there was no link with clinicopathological factors, indicating that inactivation of these genes is not a major incidence in PTC tumorigenesis. Thyroid neoplasms, including PTCs, exhibit significantly decreased *p27^{Kip1}* expression [35], especially in nonmetastasizing PTCs [36], but this is presumably due to other mechanisms.

Our cases with a previous history of chronic thyroid disease demonstrated significant lymphocytic infiltra-

tion in otherwise normal parenchyma adjacent to the cancers. Of particular interest, there was a significant association between chronic thyroid disease and *p14^{ARF}* alterations or lack of regional lymph node metastasis. Two of the 4 cases with *p14^{ARF}* alterations had a past history of chronic thyroid disease. In general, gastric cancers exhibit a high level of aberrant CpG island methylation, and gastritis with marked infiltration of mononuclear cells also displays a number of alterations in genes like *p14^{ARF}*, suggesting that chronic inflammation may be closely associated with increased methylation in nonneoplastic gastric mucosa [37]. Similarly, higher frequencies of *p14^{ARF}* hypermethylation have been reported in ulcerative colitis-associated colorectal carcinomas and nonneoplastic ulcerative colitis mucosa than in normal colonic mucosa [38]. Although our study included only a small number of cases with chronic thyroid disease, *p14^{ARF}* alterations are involved in chronic inflammation-related PTCs. An association between lymphocyte infiltration and tumor progression has also been reported [39–41], and lymphocytes may help to interrupt tumor growth immunologically in the absence of regional lymph node metastasis.

Additionally, immunohistochemically 14 cases (35.9%) expressed RET, showing clear nuclear and cytoplasmic staining, in contrast to a previous study [42], with no significant association with changes at the gene level and clinicopathological factors (data not shown). Polyclonal antibody against RET recognizes both rearranged and mutated RET oncogene proteins, and a previous report demonstrated a significant correlation between immunohistochemical RET overexpression and *RET* rearrangement, which was confirmed by RT-PCR [43]. A recent report showed that positive staining does not necessarily mean the presence of a rearrangement [44], and associations between *RET* rearrangement and clinicopathological factors or gene alterations require further evaluation.

Recent reports have indicated that *BRAF* mutations can be detected in 0–80% of PTCs, as alternative events to *RET* rearrangement [7–10]. Others have reported early *RASSF1A* hypermethylation and mutual exclusion with *BRAF* mutations in PTCs, and it is conceivable that *BRAF* or *Ras* mutations, *RET* rearrangements and *RASSF1A* hypermethylation could complete the panel of genetic and epigenetic alterations responsible for PTC generation [45]. Our results indicate that of the *p16^{INK4a}* alterations, mainly hypermethylation may be associated with tumor progression, and regarding chronic inflammation-related PTCs, *p14^{ARF}* alteration may be a key issue.

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