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Genetic Alterations of *RD^{INK4/ARF}* Enhancer in Human Cancer Cells

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Abstract

Recent identification of an enhancer element, *RD^{INK4/ARF}* (*RD*), in the prominent *INK4/ARF* locus provides a novel mechanism to simultaneously regulate the transcription of *p15^{INK4B}* (*p15*), *p14^{ARF}*, and *p16^{INK4A}* (*p16*) tumor suppressor genes. While genetic inactivation of *p15*, *p14^{ARF}*, and *p16* in human tumors has been extensively studied, little is known about genetic alterations of *RD* and its impact on *p15*, *p14^{ARF}*, and *p16* in human cancer. The purpose of this study was to investigate the potential existence of genetic alterations of *RD* in human cancer cells. DNAs extracted from 17 different cancer cell lines and 31 primary pheochromocytoma tumors were analyzed for deletion and mutation of *RD* using qPCR and direct DNA sequencing. We found that *RD* was deleted in human cancer cell lines and pheochromocytoma tumors at frequencies of 41.2% (7/17) and 13.0% (4/31), respectively. While some of these *RD* deletion events occurred along with deletions of the entire *INK4/ARF* locus, other *RD* deletion events were independent of genetic alterations in *p15*, *p14^{ARF}*, and *p16*. Furthermore, the status of *RD* was poorly associated with the expression of *p15*, *p14^{ARF}*, and *p16* in tested cancer cell lines and tumors. This study demonstrates for the first time that deletion of the *RD* enhancer is a prevalent event in human cancer cells. Its implication in carcinogenesis remains to be further explored.

INTRODUCTION

The *INK4/ARF* (*CDKN2A*) locus plays a central role in tumor suppression as reflected by the fact that this locus is inactivated in nearly 50% of all human cancers [1, 2]. This locus of ~50 kb encodes three distinct tumor suppressors; namely, *P16^{INK4A}* (*P16*), *P15^{INK4B}* (*P15*), and *P14^{ARF}* (Figure 1). *P16* and *P15* specifically inhibit cyclin D-dependent kinase 4/6 (CDK4/6)-mediated phosphorylation of pRB (the retinoblastoma susceptible gene product) and the subsequent E2F-mediated transactivation of genes required for entry into the S phase; *P14^{ARF}* negatively modulates the E3 ubiquitin ligase activity of MDM2, thus activating P53-mediated apoptosis or cell cycle arrest. While the *p15*, *p14^{ARF}*, and *p16* genes are driven by their respective individual promoters [2], accumulating evidence indicates that there are locus control region mechanisms able to modulate these three genes simultaneously [1]. One such mechanism involves a DNA element located upstream of the

p15 gene (Figure 1) [3, 4]. This regulatory element, *RD^{INK4/ARF}* (hereafter, *RD*), is conserved among mammalian *INK4/ARF* loci and exhibits a global impact on enhancing the concomitant expression of *p15*, *p14^{ARF}*, and *p16*. Moreover, CDC6, an essential DNA replication modulator frequently over-expressed in a number of human cancers [1], is able to bind to *RD* and recruit histone deacetylase 1 (HDAC1) and histone deacetylase 2 (HDAC2) to the *INK4/ARF* locus, resulting in heterochromatinization and transcriptional silencing [3]. Further studies have demonstrated that such CDC6-mediated repression of the *INK4/ARF* locus involves physical interactions between CDC6 and Bmi1, a Polycomb (PcG) protein that functions to generate and recognize histone modifications in genomic domains with clusters of genes. Upon senescence, JMJD3 (KDM6B, a histone demethylase) and MLL (a histone methyltransferase) are recruited to the *INK4/ARF* locus, provoking the dissociation of PcG proteins from *RD* and the transcription of *p16* [5]. The *RD*/CDC6 interaction provides a novel mechanism that is important in cell cycle control and carcinogenesis since its perturbation by *RD* alterations and/or increased expression of CDC6 may down-regulate both the Rb and P53 pathways. While genetic alterations of *p16* (as well as *p15* and *p14^{ARF}*) in human cancer cells and tumors have been extensively investigated [1, 2], little is known about changes in the genetic status of *RD* in human cancer. In this study, we investigated the genetic status of *RD* and its correlation with the expression of *p16* (as well as *p15* and *p14^{ARF}*) in 17 human cancer cell lines and a cohort of 31 human pheochromocytoma primary tumors.

MATERIALS AND METHODS

Cell Culture

All cell lines used in this study are listed in Table 1. TE-1177, SCC-83-01-82, and SCC-83-01-82CA were kindly provided by Dr. S.M. D'Ambrosio of The Ohio State University [6]; UM-SCC-22A was obtained from the University of Michigan. These four cell lines were cultured in Advanced D-MEM/F-12 medium (Life Technologies/Invitrogen, Carlsbad, CA) containing 5% fetal bovine serum (FBS; Invitrogen) in a 90% relative humidity incubator at 37°C supplied with 5% CO₂ [6]. The other cell lines were purchased from American Type Culture Collection (ATCC; Manassas, VA) and cultured according to the provider's recommendations.

Detecting Genetic Alterations of *RD* in Cancer Cells

Deletion, methylation, and point mutations of *p16*, *p15*, and *p14^{ARF}* were evaluated as previously reported with primers and probes listed in Table 2 [7–12]. The genetic status of *RD* was investigated as follows.

RD Point Mutations

The *RD* fragment was amplified by PCR using the following primers: 5'-CAA TTG CCC TCC TGT TAA GAC-3' (forward) and 5'-CCC ACT TTG TCA GGT ATC TTA TTT-3' (reverse), and analyzed by electrophoresis (Figure 1A). After purification, both strands of *RD* fragments were sequenced to identify potential mutations.

As shown in Figure 2A, *RD* was homozygously deleted in UM-SCC-22A and PANC-1. Thus, UM-SCC-22A (*RD*^{-/-}) together with TE-1177 (normal oral epithelial cell line, *RD*^{+/+}) were used in the qPCR-based genomic deletion assay.

***RD* Genomic Deletion**

Homozygous and hemizygous deletions of *RD* were determined using multiplex qPCR on a Cepheid Smart Cycler as previously described [13]. The primers and probes for *RD* and β -actin (*ACTB*, endogenous control) are listed in Table 2. All experiments were performed in triplicate.

Samples containing mixed genomic DNAs from TE-1177 (*RD*^{+/+}) and UM-SCC-22A (*RD*^{-/-}) at various ratios (100:0, 75:25, 50:50, 25:75, 0:100) were analyzed and the resulting quantification cycle value (Cq) values were normalized using the following equation [14]:

$$\Delta\Delta Cq = (Cq_{RD}^{\text{Sample}} - Cq_{RD}^{\text{TE-1177}}) - (Cq_{ACTB}^{\text{Sample}} - Cq_{ACTB}^{\text{TE-1177}})$$

The Cq values were then plotted against known ratios of *RD* (in exponential form) to get a calibration line with a correlation coefficient of -0.996 (Figure 2B), indicating that the dosage of *RD* in a sample can be accurately measured using this technique [15]. Subsequently, the gene dosage of *RD* in different cancer cell lines and tumors was determined and the results were interpreted as follows [15]: dosage <25%, homozygous deletion (*RD*^{-/-}); dosage between 25% and 75%, hemizygous deletion (*RD*^{+/-}); and dosage >75%, *RD* wild type (*RD*^{+/+}).

***RD* methylation status**

While methylation is a primary mechanism in transcriptionally inactivating genes, we analyzed the *RD* sequence with two widely used programs, CpG Island Searcher [16] and EMBOSS CpGPlot [17], and no potential CpG island was identified. This is not unexpected since the *RD* sequence is 63.8% A/T-rich. Hence, *RD* methylation was not further investigated in this study.

Quantitatively Determining the Expression of *p15*, *p16*, *p14*^{ARF}, and *CDC6* in Cancer Cells

Extraction of total RNA and first-strand cDNA synthesis were performed as previously described [6]. Quantitative RT-qPCR reactions were carried out using a StepOne Real-Time PCR System (Applied Biosystems) and pre-validated TaqMan gene expression assays as listed in Table 2. Human hypoxanthine phosphoribosyltransferase (*HPRT1*) was used as an endogenous control for gene expression. Each gene (*p16*, *p15*, *p14*^{ARF}, *CDC6*, and *HPRT1*) was amplified separately, and all experiments were performed in triplicate.

The relative gene expression level of a target was determined using a comparative Cq method, in which data from cancer cells were normalized to that from TE-1177 [6]:

$$\Delta\Delta Cq = (Cq_{\text{target}} - Cq_{HPRT1})_{\text{cancer cell}} - (Cq_{\text{target}} - Cq_{HPRT1})^{\text{TE-1177}} \text{ Relative gene expression} = 2^{-\Delta\Delta Cq}.$$

A two-fold increased (> 2) or decreased (< 0.5) value was considered significant for mRNA over-expression or down-regulation, respectively.

Western Blot

Cell pellets were washed three times in ice-cold PBS and then lysed with RIPA buffer [20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na₂EDTA, 1 mM EGTA, 1% NP-40, 1% sodium deoxycholate, 2.5 mM sodium pyrophosphate, 1 mM β -glycerophosphate, 1 mM Na₃VO₄, 1 mM PMSF, and 1 μ g/mL of leupeptin; Cell Signaling Technologies; Danvers, MA] on ice for 20 min. Subsequently, cell lysates were clarified by centrifugation at 4°C, 13,000 rpm for 5 min and the concentration of total proteins in the supernatants was determined using the BCA assay (Thermo Fisher Scientific/Pierce, Rockford, IL). The samples were subjected to SDS-PAGE (10% or 15%) at 50 μ g of total proteins per lane and then transferred to nitrocellulose membrane and immunoblotted with the following antibodies: mouse anti- β actin monoclonal antibody (sc-56459; Santa Cruz Biotechnology; Santa Cruz, CA), rabbit anti-P14^{ARF} polyclonal antibody (sc-8340; Santa Cruz Biotechnology), rabbit anti-P15 polyclonal antibody (sc-613; Santa Cruz Biotechnology), mouse anti-P16 monoclonal antibody (sc-1661; Santa Cruz Biotechnology); rabbit anti-CDC6 monoclonal antibody (C42F7; Cell Signaling Technologies). The concentration of each antibody was used as suggested by the suppliers. Immunodetection was performed with the Enhanced Chemiluminescence Kit for Western blotting (GE Healthcare Biosciences, Piscataway, NJ).

Analyzing Pheochromocytoma Tumors

Pheochromocytomas are adrenal gland tumors that have been shown to have molecular defects in cell cycle associated genes, including *p16* [13]. A cohort of 31 human pheochromocytoma tumors and seven histologically normal specimens were obtained from The Ohio State University Medical Center under the guidance of the Institutional Review Board as previously reported [13]. Genomic DNA was extracted from paraffin-embedded specimens using a ChargeSwitch gDNA Kit (Life Technologies/Invitrogen) and subjected to assays to detect the genetic status of *RD* and *p16* [13]. Protein expression of P16 was evaluated by immunohistochemistry (IHC)-based tissue microarrays using a primary antibody against human P16 protein (16P04; Cell Marque; Rockland, CA).

RESULTS AND DISCUSSION

The Genetic Status of the *INK4/ARF* Locus in Human Cancer Cells

We first extracted genomic DNAs from 18 human cell lines (one normal oral epithelial cell line, 17 neoplastic cell lines) and investigated potential genetic abnormalities of *RD* including homozygous/hemizygous deletions and point mutations. In parallel, genetic statuses of *p15*, *p14^{ARF}*, and *p16* in these cell lines were evaluated as previously described [7–12]. Table 1 summarizes the genetic statuses of *RD*, *p15*, *p14^{ARF}*, and *p16* in these cell lines. While four cell lines, SCC-9, UM-SCC-22A, PANC-1, and MIA PaCa-2, harbored homozygous deletions of *RD*, hemizygous deletions of *RD* were found in SCC-15, SCC-4, and SCC-25 cells. Interestingly, no point mutations of *RD* were identified in any of these 17 tumor cell lines, demonstrating that hemizygous (3/17) and homozygous (4/17) deletions are

the major mechanisms to inactivate *RD* at the DNA level. Of the four *RD*^{-/-} cell lines, UM-SCC-22A, PANC-1, and MIA PaCa-2 also harbored homozygous deletions of *p15*, *p14*^{ARF}, and *p16*, indicating that the entire *INK4/ARF* locus was deleted in these cell lines. In contrast, SCC-9 harbored homozygous deletions of *RD*, *p15*, and *p14*^{ARF}, but retained intact *p16*, suggesting that the deletion event in SCC-9 occurs at the upstream of the *p16* gene. Out of the three *RD*^{+/-} cell lines, SCC-4 had intact *p15*, *p14*^{ARF}, and *p16* genes, indicative of *RD*-specific genetic alterations in this cell line; SCC-15 and SCC-25 had methylated *p16* and homozygous deletion of *p16*, respectively, whereas their *p15* and *p14*^{ARF} genes remained intact, suggesting that there are two independent inactivating events targeting *RD* and *p16*, respectively, in SCC-15 and SCC-25.

In addition, genetic alterations of *p15*, *p14*^{ARF} or *p16* were observed in some *RD*^{+/+} cell lines. For example, CAL-27 and PC3 (both *RD*^{+/+}) had promoter hypermethylation in *p15* and *p16*, respectively, whereas DU145 had a point mutation (G → T) in exon 2 of *p16*, which leads to mutated P16 (Asp74Tyr) and P14ARF (Arg88Leu) proteins [18]. These findings support the notion that molecular alterations of *RD* and *p16* (as well as *p15* and *p14*^{ARF}) represent independent mechanisms of regulation.

Expression of *p15*, *p14*^{ARF}, and *p16* in Human Cancer Cells

We then determined the expression levels of *p15*, *p14*^{ARF}, and *p16* in these cell lines to evaluate how the genetic status of *RD* influences the transcription of these *INK4/ARF* genes (Figure 3). Since binding of CDC6 directly down-regulates the enhancer activity of *RD*, we also assessed the expression level of *CDC6* in these cell lines. As expected, no expression of *p15*, *p14*^{ARF}, and *p16* was detected in cell lines with homozygous deletions of the entire *INK4/ARF* locus (UM-SCC-22A, PANC-1, and MIA PaCa-2). Similar results were observed with SCC-9, which has homozygous deletions of *RD*, *p15*, and *p14*^{ARF} but intact *p16*, suggesting that the transcription of *p16* in this cell line is silenced through yet-to-be identified mechanisms. As for the other 13 cell lines, the overall expression patterns of *p15*, *p14*^{ARF}, and *p16* are different from each other. The *p16* gene was over-expressed in six cell lines (HeLa, SiHa, C-33A, COLO320, CAL-27, and DU145) and normally expressed in five cell lines (PC3, SW480, SCC-4, SCC-83-01-82, and SCC-83-01-82CA). The expression of *p16* was nominally detected in SCC-15 and SCC-25 since the *p16* gene was either methylated (SCC-15) or homozygously deleted (SCC-25). Notably, even though methylation of the *p16* promoter was identified in PC3 cells [18], the expression of *p16* remained comparable to that in TE-1177. Overall, these results indicate that unless the *p16* gene is homozygously deleted or highly silenced by methylation, it tends to be over-expressed or normally expressed at the mRNA level in most of cancerous or precancerous cancer cell lines. These findings are consistent with previous studies showing that *p16* (wild type or with point mutations) was over-expressed prevalently in human tumors, such as prostate, lung, brain tumors, and oral squamous cell carcinomas [1, 19]. Similarly, *p14*^{ARF} was over-expressed in all tested cell lines except UM-SCC-22A, PANC-1, MIA PaCa-2, SCC-9 (with homozygous deletions of the entire *INK4/ARF* locus) and SCC-25. In SCC-25, *p14*^{ARF} was expressed at a level comparable with that in TE-1177. Interestingly, both *p16* and *p14*^{ARF} were over-expressed in DU145, a cell line harboring mutated P16 and P14^{ARF} proteins. In contrast, while a moderately increased expression of *p15* was found in COLO320 cells, the

expression levels of *p15* were significantly reduced or unchanged (in comparison with TE-1177) in nine and three cell lines, respectively, indicating that the expression of *p15* is down-regulated in the majority of cancerous or precancerous cell lines. While *CDC6* was expressed in HeLa, SCC-83-01-82CA, SW480, and SCC-25 at a level comparable to that in TE-1177, its over-expression was observed in the remaining 13 cell lines.

As for each individual cell line, the expression of *p15*, *p14^{ARF}*, and *p16* appears to be differential. For example, in SCC-4 cells (*RD^{+/-}*), *p15*, *p14^{ARF}*, and *p16* were down-regulated, over-expressed, and normally expressed, respectively. In comparison, SCC-15 (*RD^{+/-}*) had normally expressed, over-expressed, and lowly-expressed *p15*, *p14^{ARF}*, and *p16*, respectively. Similar observations were obtained among *RD^{+/+}* cell lines. For example, *p15*, *p14^{ARF}*, and *p16* were down-regulated, over-expressed, and normally expressed, respectively, in SCC-83-01-82 cells.

We continued to evaluate the expression of *CDC6*, *P16*, *P14^{ARF}*, and *P15* at the protein level in these cell lines using Western blot analyses. As shown in Figure 4, *CDC6* was highly expressed in all tested cell lines relative to the TE-1177 control cell line. *P14^{ARF}* was highly expressed in CAL-27 (*RD^{+/+}*), SCC-4 (*RD^{+/-}*), SCC-15 (*RD^{+/-}*), SCC-83-01-82, SCC-83-01-82CA (*RD^{+/+}*), but not detected in SCC-9 (*RD^{-/-}*) and UM-SCC-22A (*RD^{-/-}*), in which the *p14^{ARF}* gene was homozygously deleted. *P16* was highly expressed in CAL-27, SCC-83-01-82, and SCC-83-01-82CA, all of which retain intact *p16* gene. In contrast, *P16* protein was not detected in SCC-4, SCC-9, SCC-15, and UM-SCC-22A. While the *p16* gene is homozygously deleted in UM-SCC-22A, it is transcriptionally silenced in SCC-15 (through methylation) and SCC-9 (through unknown mechanisms). In SCC-4, *p16* was expressed at the mRNA level, but not at the protein level, indicating that post-transcriptional mechanisms are involved in the regulation of *P16* [1]. As for *p15*, we failed to detect *P15* protein in all tested cell lines (data not shown). Such inability to detect *P15* protein in our Western blot assay could be attributed to the following reasons: (1) the expression of *P15* in these cell lines is below the sensitivity of our Western immunoblot assay and (2) *P15* protein was degraded following cell lysis and sample preparation. Overall, these Western blot results are consistent with our previous observations with the gene expression: *P14^{ARF}* and *P16* are differentially expressed and the genetic status of individual genes, not the status of *RD*, appears to be associated with their expression.

Genetic Alterations of *RD* in Human Pheochromocytoma Tumors

We further analyzed the genetic status of *RD* in a cohort of human pheochromocytoma tumors, of which the genetic status of *p16* and the expression level of *P16* protein were determined previously [13]. As listed in Table 3, homozygous and hemizygous deletions of *RD* were identified in one and three tumors, respectively, while no “normal” specimens harbored *RD* alterations. The overall frequency of *RD* alterations in this cohort of pheochromocytomas is 13.0% (4/31). In comparison, deletions (hemizygous and homozygous), point mutation, and aberrant CpG methylation of *p16* were found in 9, 1, and 11 tumors with an overall frequency of 67.7% (21/31). In the group of four pheochromocytomas with *RD* alterations, one tumor (T21) harbored homozygous deletions of both *RD* and *p16*, and another tumor (T5) had hemizygous deletions of both *RD* and *p16*,

indicating that alterations of *RD* and *p16* in these two tumors may result from a single molecular event in the *INK4/ARF* locus. The remaining pheochromocytomas with *RD* alterations, T1 and T18L, harbored hemizygotously deleted *RD*; however, the status of *p16* in these two tumors were different: *p16* was methylated in T18L, but remained intact in T1. Hence, these results suggest that *RD* alteration is independent of, or at least not always concomitant with *p16* alteration in pheochromocytoma tumors.

It is also worthwhile to note that P16 protein was expressed at low levels or even undetected in all but one pheochromocytomas (30/31, 96.8%), which is different from our previous finding that *p16* was over-expressed at the mRNA level in the majority of 17 tested neoplastic cell lines. Such inconsistency could be attributed to the following reasons: (1) the transcriptional levels of *p16* mRNA and protein expression levels of P16 are not necessarily stoichiometrically coupled, since a number of confounding cellular processes can mediate the stabilities (expression) of *p16* mRNA and protein in cancer cells [1] and (2) the microenvironment surrounding a tumor tissue *in vivo* is different from that defined *in vitro* for cultured cells. Importantly, the only tumor (T16L) exhibiting highly expressed P16 had an intact *RD*, but a point mutation in *p16* leading to a partial loss of P16 function [13]. Since no genetic alteration of *RD* was identified in the majority of low-expressing P16 tumors, there does not appear to be a strong association between the *RD* status and the expression of P16 in pheochromocytoma tumors.

Here, we present our studies on the prevalence of *RD* alterations in human cancer cells. In a panel of 17 human cell lines derived from different types of cancers, up to 41.2% (7/17) demonstrated homozygous (4/17) or hemizygous (3/17) *RD* deletions. We also showed that in a cohort of 31 human pheochromocytoma tumors, three tumors harbored hemizygous deletions of *RD* and one tumor had homozygous deletions of *RD*. These results reveal that deletion of *RD* occurs in human cancer cells and tumor tissues with a considerable incidence (11/48, 22.9%). As described earlier, some *RD*-altering events identified in this study were global and involved the entire *INK4/ARF* locus, while other *RD*-altering events were localized specifically to the *RD* region. Moreover, some cancer cell lines and tumors had intact *RD*, but harbored genetic alterations (i.e., deletion, methylation, and point mutations) in one or more of the *p15*, *p14^{ARF}*, and *p16* genes. These results support a model in which some *RD* alterations are independent of genetic alterations of the *p15*, *p14^{ARF}*, and *p16* tumor suppressor genes. Notably, point mutations (not detected) and methylation (not investigated) are not supported as significant mediators of *RD* functional activities. However, hemizygous and homozygous deletions of *RD* were identified both in diverse human cancer cell lines and pheochromocytoma tumor tissues, but not in non-malignant cells *in vitro* or non-tumor human tissue samples. These findings suggesting that deletion is the primary mechanism to functionally inactivate *RD* activities at the genomic level. In contrast, a spectrum of genetic alterations of *p15*, *p14^{ARF}*, and *p16* were present in these cell lines and tumor specimens, including not only deletion, but also methylation and point mutations. Specifically, both methylation and deletion are major mechanisms to inactivate *p16* in pheochromocytomas as evidenced by their incidences of 29.0% (10/31) and 35.5% (11/31), respectively. This difference in “inactivating” mechanism between *RD* and three *INK4/ARF* genes could be partially ascribed to the A/T-rich nature of *RD*, which is distinct from the G/C-rich nature of *p15*, *p14^{ARF}*, and *p16* genes [1, 2]. To our knowledge, this is the

first report on genetic alterations of *RD* in human cancer cells. However, it is important to note that how genetic alterations of *RD* contribute to the regulation of tumor suppressors P15, P14^{ARF}, and P16 as well as carcinogenesis remains to be further investigated. The small sample size of either the *in vitro* studies (n = 17) or clinical pheochromocytoma samples (n = 31) impairs our effort to definitively correlate the status of *RD* with the expression of *p15*, *p14^{ARF}*, and *p16*. Furthermore, *RD*-mediated regulation of *p15*, *p14^{ARF}*, and *p16* directly or indirectly involves a number of regulatory proteins, including CDC6, Bmi1, HDAC1, and HDAC2. While *CDC6* is highly expressed in most of tested cancer cell lines regardless of the status of *RD*, the expression (and the functioning) of Bmi1 as well as other factors may further contribute to the differential expression of *p15*, *p14^{ARF}*, and *p16* in these cell lines. From this perspective, it is of significance to investigate the status of *RD*, its regulatory factors (including CDC6, Bmi1, HDAC1, and HDAC2), and its downstream effectors (*p15*, *p14^{ARF}*, and *p16*) in a large cohort of patient-matched normal and tumor specimens in regard to the potentials of *RD* in mediating both pRb and P53 tumor suppressive pathways. Indeed, such a study in a cohort of human squamous cell carcinomas of the head and neck is ongoing in our laboratory.

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Figure 1.

The schematic structure of the *INK4/ARF* locus. Promoters for *p15*, *p14^{ARF}*, and *p16* genes are indicated by horizontal arrows. Boxes represent exons, and the empty circle represents the regulatory element *RD*. The minus sign indicates negative regulation. This figure was adapted from Ref. [4].

A **B**

Figure 2. qPCR assays to detect genetic alterations of *RD*. (A) Amplification of *RD* from selected cell lines. *FPR1* (N-formyl peptide receptor) was used as an endogenous control [20]. The PCR

products of *RD* and *FPR1* are 406 and 500 bp, respectively. (B) The calibration curve for qPCR-based analyses of *RD* deletion. Multiplex qPCR for *RD* and *ACTB* was performed with a series of mixtures of genomic DNAs from TE-1177 (*RD*^{+/+}) and UM-SCC-22A (*RD*^{-/-}) cells at various ratios. The resulting Cq values were normalized as described in Materials and Methods Section. The Cq values were plotted against the relative ratio of normal *RD* in the mixtures (in exponential form) to get a calibration curve with a linear correlation coefficient of -0.996.

Figure 3. Quantitative determination of *CDC6*, *p16*, *p14^{ARF}*, and *p15* mRNA expression in human cell lines. Brown, *CDC6*; orange, *p16*; lime green, *p14^{ARF}*; dark green, *p15*. *HPRT1* was used as an endogenous control, and TE-1177 cells were used to represent the reference baseline state for normalization [6]. Quantitative RT-PCR-based assays were conducted in triplicate. Values (y-axis) represent fold-changes (in the log form) calibrated to the expression levels of indicated genes in TE-1177. Error bars represent standard deviations. Dashed lines represent twofold increases (upper) and decreases (lower) in gene expression. ND, not detected.

Figure 4.

Western Blot Analyses. Soluble proteins (50 mg) from selected cell lines were subjected to SDS-PAGE analysis, and after electro-transfer to nitrocellulose membrane, the proteins were probed using primary antibodies against β-actin (sc-56459; Santa Cruz Biotechnology), CDC6 (C42F7; Cell Signaling Technologies), P16 (sc-1661; Santa Cruz Biotechnology), and P14^{ARF} (sc-8340; Santa Cruz Biotechnology). Immunodetection was performed with the Enhanced Chemiluminescence Kit for Western blotting (GE Healthcare Bioscience). TE-1177 was the reference cell line. β-actin was the internal reference protein.

Table 1

The Genetic Status of the INK4/ARF Locus and mRNA Expression of p15, p14ARF, p16, and CDC6 in Human Cancer Cell Lines

Cell line	Source	Genetic Status ^(a)			mRNA Expression ^(b)					
		RD	p15	p14ARF	p16	p15	p14ARF	p16	CDC6	
TE-1177	Oral (tonsil)	+/+	+/+	+/+	+/+	Control	Control	Control	Control	Control
SCC-83-01-82	Oral	+/+	+/+	+/+	+/+	Low	High	Normal	High	High
SCC-83-01-82CA	Oral	+/+	+/+	+/+	+/+	Low	High	Low	Normal	Normal
UM-SCC-22A	Oral (hypopharynx)	-/-	-/-	-/-	-/-	NE	NE	NE	NE	Normal
CAL-27	Oral (tongue)	+/+	M	+/+	+/+	NE	High	High	High	High
SCC-15	Oral (tongue)	+/+	+/+	+/+	+/+	Normal	High	Low	High	High
SCC-4	Oral (tongue)	+/+	+/+	+/+	+/+	Low	High	Normal	Normal	Normal
SCC-9	Oral (tongue)	-/-	-/-	-/-	+/+	NE	NE	NE	High	High
SCC-25	Oral (tongue)	+/+	+/+	+/+	-/-	Low	Normal	NE	NE	Normal
HeLa	Cervical	+/+	+/+	+/+	+/+	Low	High	High	High	Normal
C-33A	Cervical	+/+	+/+	+/+	+/+	Low	High	High	High	High
SiHa	Cervical	+/+	+/+	+/+	+/+	Low	High	High	High	High
MIA PaCa-2	Pancreatic	-/-	-/-	-/-	-/-	NE	NE	NE	NE	High
PANC-1	Pancreatic	-/-	-/-	-/-	-/-	NE	NE	NE	NE	High
PC3	Prostate	+/+	+/+	+/+	M	High	High	High	High	High
DU145	Prostate	+/+	+/+	R88L	D74Y	Normal	High	High	High	High
COLO320	Colorectal	+/+	+/+	+/+	+/+	Low	High	Normal	High	High
SW480	Colorectal	+/+	+/+	+/+	+/+	Low	High	Normal	Normal	High

^(a) The genetic status of a target sequence: +/+, wild type; +/-, hemizygous deletion; -/-, homozygous deletion; M, methylation.

^(b) The mRNA expression levels of p15, p14ARF, p16, and CDC6 were quantitatively determined using TaqMan assays, in which TE-1177 was the control cell line, and HPRT1 was the reference gene for normalization. Results were interpreted as follows: increase of two or more than two-fold, high; decrease of two or more than two-fold, low; increase or decrease by less than two-fold, normal; NE, not expressed.

Table 2**PCR Primers and Probes**

DNA Fragment Analysis		
RD	F	5'-CAATTGCCCTCCTGTTAAGAC-3'
	R	5'-CCCACCTTGTGTCAGGTATCTTATTT-3'
FPR1 ^(a)	F	5'-CTCACCACAGTGCTTGCTGC-3'
	R	5'-CTTACCTGGACTGTTACCAG-3'
p16-1α	F	5'-CGGCTGCGGAGAGGGGAGAC-3'
	R	5'-CAGCGCCCGCACCTCCTCTA-3'
p14-1β	F	5'-GGAGGCGGCGAGAACAT-3'
	R	5'-GGGCCTTTCCTACCTGGTCTT-3'
p16-2a	F	5'-GGGCTCTACACAAGCTTCCTT-3'
	R	5'-AGCCAGGTCCACGGGCAGGTACA-3'
p16-2b	F	5'-GGGAGGGCTTCCTGGACAC-3'
	R	5'-TTTGAAGCTCTCAGGGTACA-3'
p16-3	F	5'-GCCTGTTTCTTTCTGCCCTCTG-3'
	R	5'-CGAAAGCGGGTGGGTTGT-3'
p15-1	F	5'-GCGTCTGGGGGCTGCGGAATG-3'
	R	5'-CCTCGCCAACGTAGACTCCTGTA-3'
p15-2	F	5'-CCCGGCCGCGCATCTCCCATAC-3'
	R	5'-GTTGTGGGCTGGGGAACC-3'
DNA Deletion Analysis (Multiplex PCR)		
RD	F	5'-TTATGCAGTTCCTCACCAAAGTTT-3'
	R	5'-GGATAGGAAGAGAACCGCAAGTT-3'
	P	5'-FAM-AGGCAACAAATCCA-TAMRA-3'
p16	F	5'-GGCTCTACACAAGCTTCCTTTCC-3'
	R	5'-TCATGACCTGCCAGAGAGAACA-3'
	P	5'-FAM-CCCCACCTGGCTCTGACCA-TAMRA-3'
p14ARF	F	5'-GCGCATTCGGCACTTGTT-3'
	R	5'-CTGGGCTAGAGACGAATTATCTGTT-3'
	P	5'-FAM-TGTTTGGTGTGATTTCGT-MGB-3'
p15	F	5'-GGCCGGAGGTGTGCATT-3'
	R	5'-CTCTTTAGGATTTTGTGCTGGGTAAA-3'
	P	5'-FAM-CACGCGTAAACAG-MGB-3'
ACTB ^(b)	F	5'-CGTAGCACAGCTTCTCCTTAATGTC-3'
	R	5'-AGCGCGGCTACAGCTTCA-3'
	P	5'-TET-ATTTCCCGCTCGGCCGTGGT-TAMRA-3'
DNA Methylation Analysis (Methylation-Specific PCR)		
p15	CpG Wiz p15 amplification kit (Chemicon, S7802)	

p16	CpG Wiz p16 amplification kit (Chemicon, S7800)
p14ARF	F 5'-TTTTTGGTGTTAAAGGGTGGTGTAGT-3' (unmethylated)
	R 5'-CACAAAAACCCTCACTCAC AACAA-3' (unmethylated)
	F 5'-GTGTTAAAGGCGGCGTAGC-3' (methylated)
	R 5'-AAAACCCTC ACTCGCGACGA-3' (methylated)

Gene Expression Analysis (TaqMan Assays)

HPRT1 ^(c)	Hs99999909_m1
CDC6	Hs00154374_m1
p14ARF	Hs00924091_m1
p15	Hs00394703_m1
p16	Hs00233365_m1

ACTB, β -actin; FPR1, N-formyl peptide receptor gene [20]

F, forward primer; R, reverse primer; P, probe

^(a) DNA Fragment Analysis control

^(b) Deletion Analysis control

^(c) Gene Expression Analysis control

Table 3

Genetic Analyses of *RD* in Human Pheochromocytoma Tumor Specimens

Specimen(<i>a,b</i>)	Expression of P16(<i>b</i>)	<i>p16</i> (<i>c</i>)	<i>RD</i>	Specimen(<i>a,b</i>)	Expression of P16(<i>b</i>)	<i>p16</i> (<i>c</i>)	<i>RD</i>
T1	Low	+/+	+/+	T18R	Negative	-/-	+/+
T2	Negative	+/+	+/+	T18L	Low	M	+/+
T3	Low	+/+	+/+	T19	Negative	M	+/+
T4	Negative	+/+	+/+	T20	Negative	+/+	+/+
T5	Low	+/+	+/+	T21	Low	-/-	-/-
T6	Negative	+/+	+/+	T22	Negative	+/+	+/+
T7	Low	+/+	+/+	T23	Negative	+/+	+/+
T8	Low	+/+	+/+	T24R	Low	+/+	+/+
T9R	Low	+/+	+/+	T24L	Low	M	+/+
T9L	Negative	M	+/+	T25	Low	M	+/+
T10	Low	M	+/+	T26	Low	M	+/+
T11	Low	-/-	+/+	T27	Low	M	+/+
T12	Negative	+/+	+/+	C3	High	+/+	+/+
T13	Negative	+/+	+/+	C4	Low	+/+	+/+
T14	Low	M	+/+	C9R	High	+/+	+/+
T15	Negative	M	+/+	C9L	High	+/+	+/+
T16R	Low	+/+	+/+	C22	High	+/+	+/+
T16L	High	A20P	+/+	C25	High	+/+	+/+
T17	Negative	M	+/+	C27	Low	+/+	+/+

(*a*) T, tumor specimen; C, normal specimen [13]; R, right-sided tumor; L, left-sided tumor

(*b*) Determined by immunohistochemistry (IHC)-based tissue microarray [13] Based on the percentage of positive nuclear P16 staining by IHC, the samples were categorized as P16-negative (complete absence of nuclear immunostaining), P16-low (1–25% of positive nuclei) or P16-high (>25% of positive nuclei).

(*c*) The genetic status of a target sequence: +/+, wild-type; +/-, hemizygous deletion; -/-, homozygous deletion; M, methylation.