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Identification of *PAK4* as a putative target gene for amplification within 19q13.12-q13.2 in oral squamous-cell carcinoma

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Amplification of chromosomal DNA is thought to be one of the mechanisms activating cancer-related genes in tumors. To identify the most likely target for amplification in the region 19q13.12-q13.2, detected previously in SKN-3 cells by a genome-wide screening of DNA copy-number aberrations in a panel of oral squamous-cell carcinoma (OSCC) cell lines, we determined the extent of the amplicon, analyzed a panel of cell lines for the expression of candidate genes within the amplicon, and then evaluated growth-suppressive effects by knocking down genes of interest. Reported information about the function and/or expression of each gene, remarkable overexpression in SKN-3 cells and relatively frequent overexpression in additional OSCC lines compared with an immortalized normal oral epithelial cell line, and expression level–dependent proliferation-promoting activity led us to conclude that the *p21-activated kinase 4* (*PAK4*) gene was the most likely target. An immunohistochemical analysis of primary tumors from 105 cases of head and neck SCC including 50 cases of OSCC demonstrated the overexpression of PAK4 to be significantly associated with a poorer prognosis. These findings reveal that the PAK4 overexpression through amplification or other mechanisms promotes the proliferation and/or survival of OSCC cells, and that PAK4 might be a good diagnostic and/or therapeutic target. (*Cancer Sci* 2009; 100: 1908–1916)

Oral cancer, predominantly oral squamous-cell carcinoma (OSCC), is the most common head and neck neoplasm (head and neck SCC, HNSCC), affecting 270 000 people worldwide each year.[1] Despite recent progress in the diagnosis of and therapeutic modalities for OSCC, the prognosis remains unimproved, reflecting the ineffectiveness of current treatment regimens.[1] A more comprehensive understanding of the molecular pathogenesis of OSCC is urgently needed to identify new targets and strategies for treatment, and the ability to recognize early OSCCs and/or pre-malignant lesions may provide insights into the best phase for chemoprevention.[2] Although OSCC is believed to arise through the accumulation of genetic and epigenetic alterations impairing the function of tumor-suppressor genes or provoking the functions of oncogenes,[3,4] the genes associated with oral carcinogenesis either directly or indirectly still remain an enigma.

DNA amplification, one of the mechanisms for activating oncogenes or genes promoting growth, metastasis, or resistance to drugs and radiation, has been found in a broad spectrum of tumors.[5] Several localized amplicons containing known oncogenes, e.g. *ERBB2* (17q), *MYC* (8q24), and *MYCN* (2p24), have been shown to have clinical significance as diagnostic and prognostic markers and therapeutic targets.[6] Recently advanced genomic screening strategies for copy-number aberrations (CNAs), including array-based comparative genomic hybridization (array-CGH), have revealed various novel amplified regions, which may contain as yet uncharacterized driver oncogenes, with greater accuracy and a higher spatial resolution than conventional CGH.[7,8] During our previous screening of CNAs in OSCC by array-CGH using in-house bacterial artificial chromosome (BAC)-based arrays against a panel of OSCC cell lines, we identified several remarkably novel amplified regions.[9]

In this study, we focused on a 2.5-Mb-amplicon at 19q13.12-13.2, a novel amplified region detected in the OSCC cell line SKN-3.[9] Although similar amplicons have been observed in other types of tumors, including pancreatic, ovarian, and breast cancers,[10–12] 19q13.12-13.2 is one of the smallest and is unlikely to contain *AKT2*, the most likely target of amplification at 19q13 reported to date. Here, we present the results of a comprehensive evaluation of the 19q13.12-13.2 amplicon in OSCC cell lines, including detailed analyses of copy-numbers and expression levels as well as loss-of-function screening using synthetic siRNA technology. Through this approach, we identified *PAK4*, encoding a protein belonging to the group-II p21 protein (Cdc42/Rac)-activated kinase (PAK) family of serine/threonine kinases with significant roles in many fundamental cellular functions and the development of cancer and metastasis,[13] as one possible target for amplification in SKN-3 cells.

Materials and Methods

Cell lines and tissue samples. A total of 22 OSCC cell lines (Ca9-22, HO-1-u-1, HO-1-N-1, HOC-313, HOC-815, HSC-2, HSC-3, HSC-4, HSC-5, HSC-6, HSC-7, KON, KOSC-2, KOSC-3, NA, OM-1, OM-2, Sa3, SAS, SKN-3, TSU, and ZA), which were established from surgically resected tumors in Tokyo Medical and Dental University or purchased from the Japanese Collection of Research Bioresources (Osaka, Japan) and maintained in appropriate media supplemented with 10% FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL), were used for this study. The immortalized normal oral epithelial cell-derived cell line, RT7, kindly provided by Dr Nobuyuki Kamata (Hiroshima University, Hiroshima, Japan), was maintained in keratinocyte serum-free medium containing epidermal growth factor and bovine pituitary extract (Invitrogen, Carlsbad, CA, USA).

Surgically resected specimens used for the immunohistochemical analysis were obtained from 105 patients who had

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Table 1. Association between clinicopathological factors and PAK4 immunoreactivity in primary HNSCC

		n	PAK4 immunoreactivity*		
			Positive (%)	Negative (%)	*P*-values†
Total		105	70 (66.6)	35 (33.3)	
Age (years)					
	>60	63	41 (65.1)	22 (34.9)	0.6726
	≤60	42	29 (69.0)	13 (31.0)	
Gender					
	Male	79	55 (69.6)	24 (30.4)	0.2631
	Female	26	15 (57.7)	11 (42.3)	
Differentiation					
	Well	37	28 (75.7)	9 (24.3)	0.1486
	Mod-poor	68	42 (61.8)	26 (38.2)	
Stage					
	I–II	71	51 (71.8)	20 (28.2)	0.1048
	III–IV	34	19 (55.9)	15 (44.1)	
TNM classification					
T category					
	T1+T2	63	36 (57.1)	27 (42.9)	**0.0112**
	T3+T4	42	34 (81.0)	8 (19.0)	
N category					
	N0	46	27 (58.7)	19 (41.3)	0.126
	N1–3	59	43 (72.9)	16 (27.1)	
M category					
	M0	80	50 (62.5)	30 (37.5)	0.1052
	M1	25	20 (80.0)	5 (20.0)	
Localization					
	Oral	50	33 (66.0)	17 (34.0)	0.4966
	Oropharynx	13	7 (53.8)	6 (46.2)	
	Hypopharynx	42	30 (71.4)	12 (28.6)	

Note: Statistically significant values are in boldface type.
*PAK4 expression was evaluated by immunohistochemical staining as described in 'Materials and Methods'.
†*P*-values are from χ^2 or Fisher's exact tests, and were statistically significant when <0.05 (two-sided).
HNSCC, head and neck squamous-cell carcinoma.

undergone curative resection of HNSCC, excluding the larynx, at the National Defense Medical College Hospital during the period from 1989 to 2000 with written consent from the parents of each patient in the formal style and after approval by the local ethics committee. The primary locus of tumors was the oral cavity in 50 cases, oropharynx in 13, and hypopharynx in 42. Hospital records of clinical and survival data were available for all patients. Clinical and pathologic examinations were performed according to the general rules for clinical studies on head and neck cancer.[14] Disease stage was defined in accordance with the TNM classification.[15] Patient characteristics are listed in Table 1. The median follow-up period for the surviving patients with HNSCCs was 48 months (ranging from 1 to 134.3 months).

Fluorescence *in situ* hybridization (FISH). Metaphase chromosomes were prepared from normal male lymphocytes and from each OSCC cell line. FISH analyses were performed as described previously,[14] using BACs as probes.

High-density oligonucleotide array-CGH. A genome-wide analysis of CNAs was performed using the Human Genome CGH Microarray Kit 244K (Agilent Technologies, Santa Clara, CA, USA) according to the directions provided by the manufacturer. The hybridized arrays were scanned using an Agilent scanner (Agilent Technologies). The CGH Analytics program version 3.4.40 (Agilent Technologies) was used to export the array CGH data for usage in other analytical programs.

Quantitative real-time genomic PCR and RT-PCR. Quantitative real-time PCR experiments were performed with an ABI Prism 7900 sequence-detection system (Applied Biosystems, Foster City, CA, USA) as described previously[16] using CYBR Green or with an ABI PRISM 7500 sequence detection System (Applied Biosystems) using TaqMan Gene Expression Assays (Applied Biosystems) according to the manufacturer's instructions. The genomic copy-number of a gene of interest in each sample was normalized by dividing it by the corresponding internal control (*COL7A1*) value, and recording as a copy-number ratio. mRNA expression levels of genes of interest were normalized against a housekeeping gene, *ACTB*, as an internal control to collect the relative expression data. Each assay was performed in triplicate for each sample. Primer sequences designed by ourselves are shown in Supporting Table S1.

Western blotting. The anti-PAK4 antibody was purchased from Cell Signaling Technology (Beverly, MA, USA), anti-FLAG tag and anti-β-actin antibodies from Sigma (St. Louis, MO, US), and anti-p21 antibody from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Cells were lysed, and lysates were analyzed as described elsewhere.[9]

Loss-of-function by siRNA and cell proliferation analysis. Loss of function screening was done using siRNAs purchased from Santa Cruz Biotechnology (*PAK4*-siRNA, sc-39060; *PSMC4*-siRNA, sc-43851; and *MAP3K10*-siRNA, sc-39111). A control siRNA for the firefly *luciferase* gene (CGUACGCGGAAUACUUCGA, *Luc*-siRNA) was synthesized by Sigma. Each siRNA (10 nM) was transfected into cells using Lipofectamine RNAiMAX (Invitrogen). The knockdown of target genes was confirmed by quantitative real-time RT-PCR and/or western blotting. Numbers of viable cells were assessed 24–72 h after transfection using a colorimetric water-soluble tetrazolium salt (WST) assay (Cell counting kit-8; Dojindo, Kumamoto, Japan).[9] The cell cycle was evaluated 72 h after transfection by FACS as described.[9]

Colony-formation assay. Plasmids expressing FLAG-tagged wild-type PAK4 (pCMV-3Tag1A-PAK4WT) and constitutive active (CA) and kinase inactive (KI) mutants of PAK4 (pCMV-3Tag1A-PAK4CA and pCMV-3Tag1A-PAK4KI, respectively) were obtained by cloning the full coding sequences for wild-type PAK4 and the CA and KI mutant forms, in which serine 474 and lysines 350/351were mutagenized to glutamate and alanines, respectively,[17] in-frame into the vector pCMV-3Tag1A (Stratagene, La Jolla, CA, USA) along with the FLAG epitope. Each construct or the empty vector was introduced into cells as described previously.[9] The expression of PAK4 protein in transfected cells was confirmed by western blotting. After 3 weeks of incubation with appropriate concentrations of G418, cells were fixed with 70% ethanol and stained with crystal violet.

Mutation analysis. We looked for mutations in *PAK4* by means of direct sequencing, using primers designed for genomic sequences around exons encoding the protein-binding domain, catalytic domain, and activating domain of the PAK4 (exons 4, 6, and 9; Supporting Table S1).

Immunohistochemistry. Indirect immunohistochemistry was done with formalin-fixed, paraffin-embedded tissue sections. Antigens were retrieved by microwave pretreatment in 5N-NaOH buffer for 10 min at 95°C. After blocking in hydrogen peroxide and in 2% normal swine serum, the slides were incubated with an anti-PAK4 antibody (1:100 dilution) overnight at 4°C and then reacted with a Histofine simple stain, MAX PO(G) (Nichirei, Tokyo, Japan). Antigen-antibody reactions were visualized with 0.2% diaminobenzidine tetrahydrochloride and hydrogen peroxide. The slides were counterstained with Mayer's hematoxylin. Formalin-fixed paraffin-embedded SKN-3 cells over-expressing *PAK4* mRNA and HSC-7 and SAS cells expressing low levels of *PAK4* mRNA were used as positive and negative controls, respectively. The specificity of the antibody was also verified by western blotting. The percentage of the total cell population that expressed PAK4 was evaluated for each case at ×200 magnification. Expression of PAK4 was graded as either positive (≥10% of tumor cell cytoplasm showing immunopositivity) or negative (<10% of tumor cell cytoplasm showing immunopositivity or no staining).

Statistical analysis. Differences between subgroups were tested with the Mann–Whitney *U*-test. For multiple group comparisons, the one-way ANOVA, followed by Scheffé's post-hoc test, was used. Correlations between PAK4 expression in primary tumors and the clinicopathologic variables pertaining to the corresponding patients were analyzed for statistical significance with the χ^2 or Fisher's exact tests. For the analysis of survival, Kaplan–Meier survival curves were constructed for groups based on univariate predictors and differences between the groups were tested with the log-rank test. Multivariate survival analysis was performed using the likelihood ratio test of the Cox proportional-hazards model. Differences were assessed with a two-sided test and considered significant at the $P < 0.05$ level.

Results

Detailed mapping of the 19q13.12-13.2 amplicon in SKN-3 cells. Using high resolution array-CGH with in-house BAC array, we recently found the region 19q13.12-13.2 to be amplified in one of 18 OSCC cell lines, SKN-3.[9] To map this region in detail, we first performed FISH using 14 sequential BAC clones (RP11-44B13, 115H16, 587I9, 91H20, 140E1, 118P21, 446K10, 787F3, 256O9, 841P21, 343J4, 67A5, 246P10, and 384E6; Fig. 1a) as probes distributing across approximately 3.5 Mb and found remarkable amplification with a homogenously staining region (HSR), on the metaphase chromosomes of SKN-3 cells (Fig. 1a,b). This amplicon is bounded by two BACs, RP11-587I9 and RP11-67A5, whereas three BACs, namely RP11-44B13, RP11-115H16, and RP11-384E6, showed lower copy-number gain comparatively (Fig. 1b and data not shown), helping us to map the amplicon to a 2.5-Mb region (Fig. 1a). Since the oncogene *AKT2*, a putative and well-characterized target for amplification within 19q13.12-13.2 in other tumors,[10–12] is located at the edge of this amplicon in SKN-3 cells (Fig. 1a,b), we examined whether *AKT2* is really included in this amplicon as a potential driver gene. High-density oligonucleotide array-CGH and quantitative genomic PCR analyses demonstrated that most but not the entire *AKT2* gene was included within the amplicon (Fig. 1c,d). This result was confirmed by the relatively low level of *AKT2* mRNA in SKN-3 cells compared with other OSCC cell lines without the19q13.12-13.2 amplicon (Supporting Fig. S1). Conversely, the defined 19q13.12-13.2 amplicon in SKN-3 contains 66 transcripts (Supporting Table S2), including 49 known genes and 17 possible transcripts encoding hypothetical or predicted proteins, between *ZNF573* and *CNTD2* based on the human genome databases (http://www.ncbi.nlm.nih.gov/ and http://genome.ucsc.edu/), prompting us to explore novel targets for amplification other than the *AKT2* gene.

Screening and selection of putative target genes located within the 19q13.12-13.2 amplicon. To select putative targets from among the 66 genes located within the 19q13.12-13.2 amplicon in SKN-3 cells, we first excluded 17 hypothetical or predicted genes (Supporting Table S2, Fig. 2a). We next searched two comprehensive expression databases (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=unigene and http://www.lsbm.org/database/index.html) and known functional information in human genome databases and previous reports (http://www.ncbi.nlm.nih.gov/sites/entrez?db=OMIM and http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed), and selected 31 genes out of 49, which showed a tumor-associated expression pattern and/or had a potential function in carcinogenesis (Supporting Table S2).

Based upon the hypothesis that putative targets for amplification could be highly expressed in cells with high-level amplification and also show increased expression in cancer cells compared with cells from normal tissue,[8] we next performed quantitative RT-PCR analyses of all 31 selected genes to compare the mRNA levels in a panel of 22 OSCC cell lines within those in the control RT7 cell line (Fig. 2b and Supporting Fig. S1). In the quantitative analysis, we found three patterns (patterns A, B, and C) of expression among the 31 genes (Fig. 2b, Supporting Fig. S1, and Supporting Table S2). We paid particular attention to 17 genes in patterns A and B, which showed high mRNA levels in SKN-3 cells along with other OSCC lines to some extent compared with RT7 cells, whereas we excluded 14 genes which did not fulfill this criterion (pattern C in Fig. 2b and Supporting Fig. S1). Among these 17 genes, *PAK4*, *PSMC4*, and *MAP3K10*, were overexpressed frequently in OSCC cell lines having no amplification (>5/21 cell lines) and expressed at the highest levels in the amplified SKN-3 cells (pattern A in Fig. 2b). The expression pattern of PAK4 protein, for which a specific antibody is available for western blotting, correlated with that of the mRNA in a panel of OSCC lines (Fig. 2c). On the other hand, 14 genes were overexpressed in SKN-3 cells, but not in most of the other OSCC cell lines, compared with RT7 cells (pattern B in Fig. 2b and Supporting Fig. S1), suggesting those genes to be given less priority in our selection of targets.

Based on the series of *in silico* and experimental analyses described above, we chose three known genes, *PAK4*, *PSMC4*, and *MAP3K10*, as candidate targets for 19q13.12-13.2 amplification in the SKN-3 cell line.

Effect of knockdown of candidate targets on proliferation of OSCC cells. To identify which of the amplified targets are functionally important in OSCC, we silenced the expression of each gene using siRNA in OSCC lines. We first evaluated the effect of siRNA for each gene on cell viability in SKN-3 cells with high-level amplification/overexpression of those genes. Treatment with the gene-specific siRNA successfully down-regulated the

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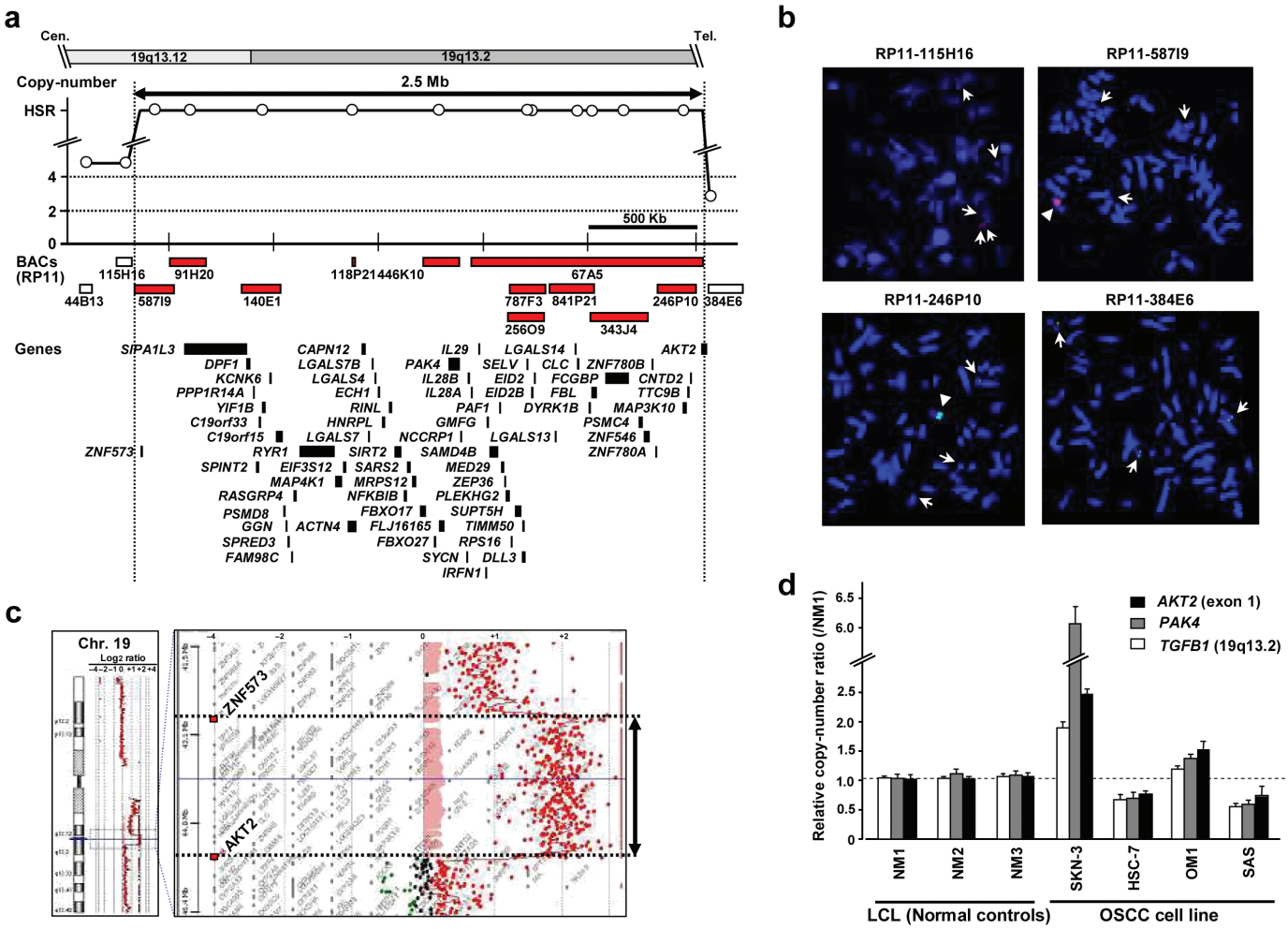

Fig. 1. Genetic aberrations at 19q13.12-13.2 in the oral squamous-cell carcinoma (OSCC) cell line SKN-3. (a) Amplicon map of genomic profiles of 19q13.12-13.2 amplification in the SKN-3 cell line. Bacterial artifical chromosome (BAC) clones used for FISH and their copy-numbers are indicated as bars and circles, respectively: those within the homozygously staining region (HSR) and outside of the HSR in SKN-3 cells are shown with red and white bars/circles, respectively. Sixty-six transcripts located within the peak of 2.5-Mb amplification showing an HSR pattern in SKN-3 cells and *AKT2* are indicated as closed bars. All transcripts located around the amplicon are positioned according to the human genome database (http://genome.ucsc.edu/, March 2006). (b) Representative images of the FISH analysis of metaphase chromosomes prepared from SKN-3 cells. BAC clones located around the amplicon were used as probes (green or red signals, arrows and arrowheads). RP11-115H16 (red) and RP11-384E6 (green) BAC clones showed five and three signals (arrows), respectively, in SKN-3 cells. Notably, both RP11-587I9 (red) and RP11-246P10 (green) BAC clones showed remarkable amplification with a HSR pattern (arrowheads) besides three signals (arrows) in SKN-3 cells. (c) Changes in copy number around 19q13.12-13.2 in SKN-3 cells determined using high-density oligonucleotide array-CGH (Agilent Human Genome CGH Microarray Kit 244K; Agilent Technologies Santa Clara, CA, USA). This array-CGH analysis clearly showed that all of *ZNF573*, but only part of *AKT2* (3′ side), is located within the amplicon. (d) Representative results of quantitative real-time genomic PCR for the *AKT2* (exon1), *PAK4*, and control *TGFB1* (19q13.2) genes. Relative genomic copy-numbers of genes were calculated by dividing the value in each sample by that in normal control leukocytes (NM1) after normalization to the corresponding internal control (*COL7A1*) value.

mRNA expression of each gene compared with control siRNA (*Luc*-siRNA, Fig. 3a).

Down-regulation of *PAK4*, *PSMC4*, and *MAP3K10* expression resulted in a significant reduction in the viability of SKN-3 cells (Fig. 3a). On the other hand, down-regulation of *PAK4* expression did not remarkably reduce proliferation in HSC-7 cells, one of the cell lines showing a lower expression level of this gene, whereas down-regulation of *PSMC4* and *MAP3K10* expression significantly suppressed the proliferation of HSC-7 cells (Fig. 3b). As in SKN-3 cells, in addition, downregulation of *PAK4* expression in NA cells, which show relatively high levels of *PAK4* expression without amplification, reduced proliferation (Supporting Fig. S2), suggesting that *PAK4*, but not *PSMC4* or *MAP3K10*, have a proliferation-promoting effect in an expression level–dependent manner in OSCC cells.

We further characterized the proliferation-inhibiting effect of the knockdown of *PAK4* in SKN-3 cells. In a FACS analysis of SKN-3, treatment with siRNA for *PAK4* resulted in an accumulation of cells in G_0-G_1 phase compared with control *Luc*-siRNA-treated counterparts (Fig. 3c). Consistent with the result of the FACS analysis, p21 protein expression was highly induced after treatment with *PAK4*-siRNA compared with control *Luc*-siRNA (Fig. 3c).

To confirm the proliferation-promoting effect of the ectopic overexpression of PAK4 in OSCC cells and clarify whether it depends on the kinase activity of PAK4, we carried out a colony-formation assay by transfecting the expression construct of the WT, CA mutant (S474E), or KI mutant (K350A/K351A) of PAK4 into cells having relatively low levels of PAK4. Ectopic expression of wild-type and mutant PAK4 with a FLAG-tag in

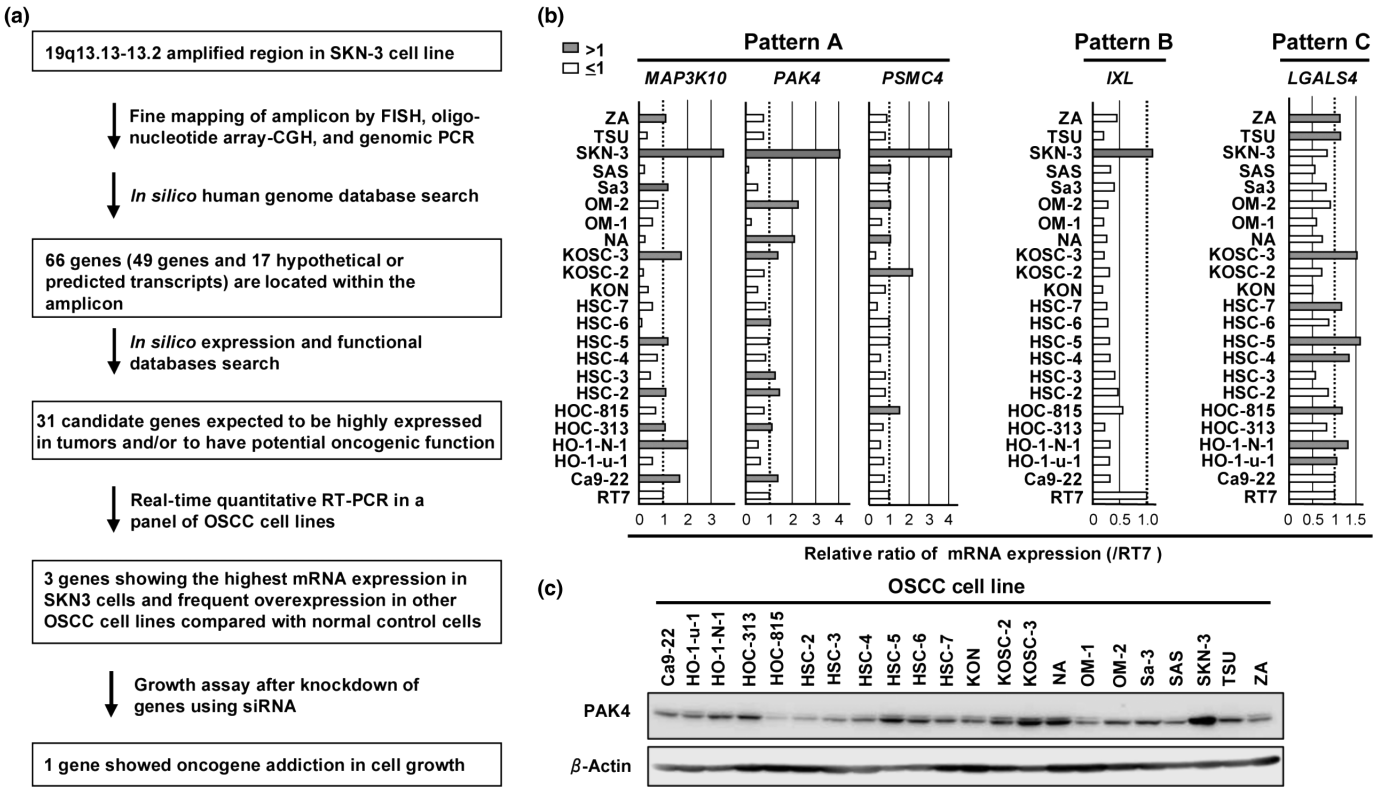

Fig. 2. (a) Schematic diagram of our systematic approach to the identification of novel targets for 19q13.12-13.2 amplification. (b) Representative results of quantitative real time RT-PCR to examine the expression levels of genes within the 13.12-13.2 amplicon of SKN-3 among a panel of oral squamous-cell carcinoma (OSCC) cell lines normalized to the immortalized normal oral epithelial cell line RT7. *ACTB* was used as an internal control. Gray and white bars indicate cell lines with increased expression and with no or decreased expression of each gene compared with RT7, respectively. *MAP3K10*, *PAK4*, and *PSMC4* were overexpressed frequently in OSCC cell lines having no amplification (>5/21 cell lines) along with the highest expression in the amplified SKN-3 cells (pattern A). *IXL* was overexpressed in SKN-3 cells but not in most of the other OSCC cell lines compared with RT7 (pattern B, see Supporting Fig. S1a). *LGALS4* did not fulfill criteria for pattern A and pattern B (pattern C, see Supporting Fig. S1b). (c) Western blot analysis of the PAK4 protein level in a panel of OSCC cell lines.

these cell lines was verified by western blotting using an epitope tag-specific antibody (Fig. 3d). The KI mutant showed lower PAK4 levels in both cell lines, although no difference in the mRNA expression of ectopically expressed PAK4 was detected among constructs by RT-PCR (data not shown). WT PAK4 and the CA mutant produced remarkably more colonies than did the empty plasmid and KI-mutant form of PAK4 in both cell lines (Fig. 3d).

In an analysis in a panel of OSCC cell lines, no mutation was detected within sequences around exons encoding the protein-binding domain, catalytic domain, and activating domain of the PAK4 (data not shown).

Clinicopathological association of PAK4 overexpression in primary HNSCCs. To clarify the clinicopathological significance of PAK4 protein levels in primary HNSCCs including OSCCs, we evaluated the expression of PAK4 protein by immunohistochemistry in 105 primary tumors, including 50 OSCCs and 55 oropharyngeal or hypopharyngeal SCCs.

To confirm the specificity of the immunoreactivity of the anti-PAK4 antibody, we first stained SKN-3 cells (Fig. 4a) as well as HSC-7 and SAS cells (Fig. 4b) as positive and negative controls, respectively. Consistent with the results of RT-PCR and western blotting, strong PAK4 immunoreactivity was observed in SKN-3 cells, whereas weak or almost no immunoreactivity was detected in HSC-7 and SAS cells.

In primary tissues, normal oral and pharyngeal squamous epithelia showed almost no PAK4 immunoreactivity (Fig. 4c and data not shown). On the other hand, immunoreactivity was observed in 70 of 105 primary HNSCCs. In all of the PAK4-positive cases, the immunoreactivity was detected in the cytoplasm of cancer cells. Notably, higher levels of PAK4 protein in primary HNSCCs were frequently observed at the invasive front of the tumors (Fig. 4d) or at the center of the tumor mass (Fig. 4e), indicating potential roles in the progression of tumor cell proliferation or in tumor cell survival, respectively. Among various clinicopathological characteristics (Table 1), PAK4 expression status was significantly associated with category T of the TNM classification: HNSCCs lack PAK4 expression in localized cases (T1 and T2), but exhibit frequent PAK4 expression in diffuse cases (T3 and T4; $P = 0.0112$, χ^2-test). However, the PAK4 protein expression in each tumor was not associated with other characteristics including the age and gender of patients, differentiation status, category N, category M, stage in the TNM classification, or location of tumors. Kaplan–Meier survival estimates showed that PAK4 immunoreactivity in tumor cells was significantly associated with a worse overall survival in all cases ($P = 0.0164$, log-rank test; Fig. 4f). The prognostic capability of PAK4 was further compared with clinicopathological variables, such as age, differentiation status, and tumor stage using the Cox proportional hazard regression model (Table 2). Multivariate analysis using a stepwise Cox regression procedure revealed that the PAK4 expression status and tumor stage were identified as independently selected predictive factors for overall survival in both forward and backward procedures ($P = 0.024$ and 0.006, respectively).

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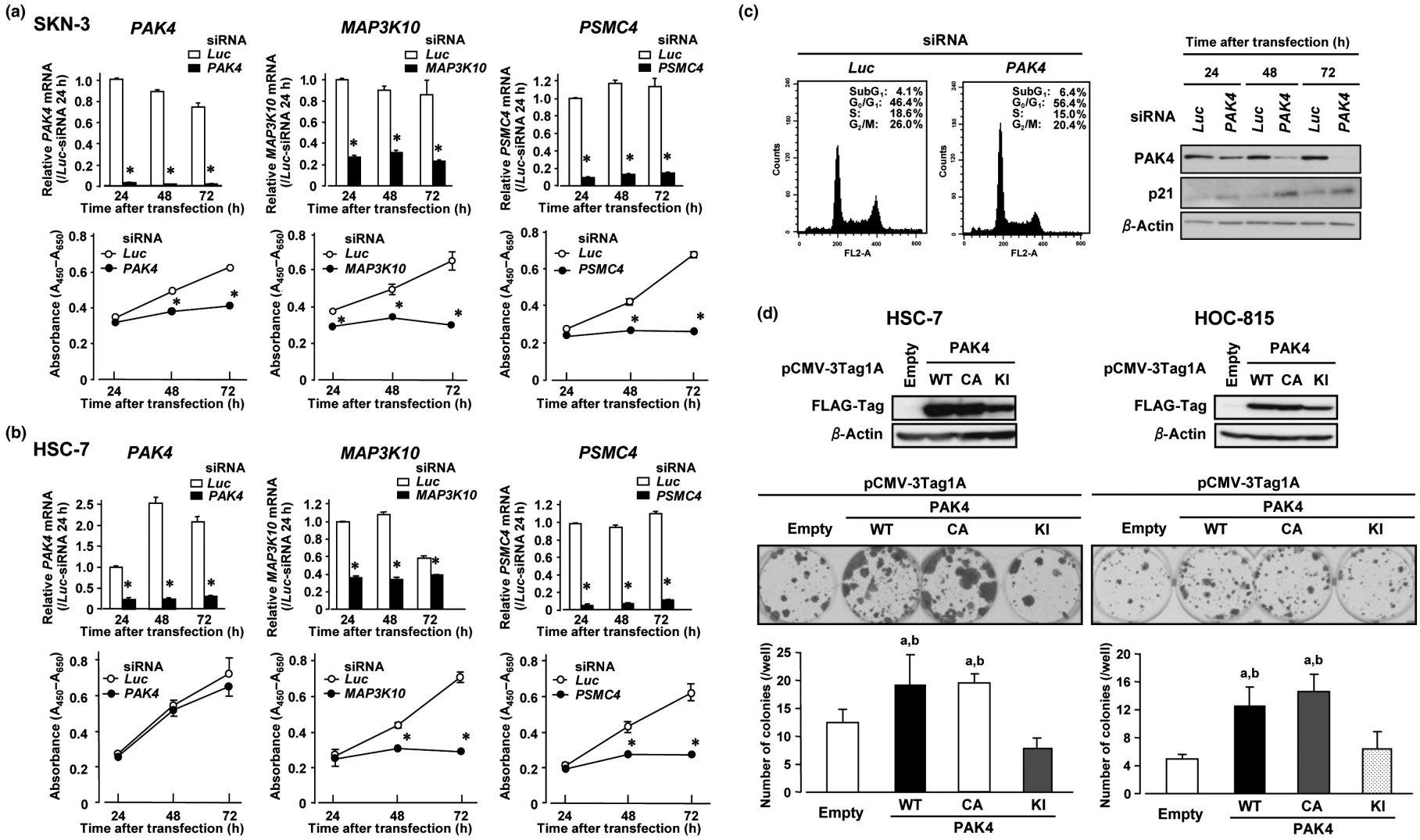

Fig. 3. (a) Representative results of the expression of *PAK4*, *MAP3K10*, and *PSMC4* mRNA (upper) and cell proliferation (lower) in SKN-3 cells treated with gene-specific siRNAs or control *luciferase* siRNA (*Luc*-siRNA) for 24–72 h. For the mRNA expression analysis, *ACTB* was used as an internal control, and relative levels were calculated by dividing each value by that in SKN-3 cells treated with *Luc*-siRNA for 24 h. The numbers of viable cells after transfection of each siRNA were assessed at the indicated times by the water-soluble tetrazolium salt (WST) assay. The data presented are the means ± SD for quadruplicate experiments. Statistical analysis used the Mann–Whitney *U*-test: $*P < 0.05$. (b) Representative results of the expression of *PAK4*, *MAP3K10*, and *PSMC4* mRNA (upper) and cell proliferation (lower) in the HSC-7 cells treated with gene-specific siRNAs or control *luciferase* siRNA (*Luc*-siRNA) for 24–72 h. See legend for Figure 3(a) for interpretation. (c) Effect of *PAK4*-siRNA or *Luc*-siRNA treatment on the population in each phase of the cell cycle assessed by FACS (upper) and protein levels of p21(lower) in SKN-3 cells. FACS was performed 72 h after treatment with *PAK4*-siRNA or control *Luc*-siRNA. Cell lysates were collected 24–72 h after transfection of each siRNA, and subjected to western blotting with appropriate antibodies. (d) Colony-formation assays using the HSC-7 and HOC-815 cell lines with relatively weak PAK4 expression. Cells were transiently transfected with FLAG-tagged constructs containing empty vector (pCMV-3Tag1A-empty), wild-type PAK4 (pCMV-3Tag1A-PAK4WT), a constitutive active mutant (pCMV-3Tag1A-PAK4CA, S474E), or a kinase-inactive mutant (pCMV-3Tag1A-PAK4KI, K350A/K351A) of PAK4, and selected with appropriate concentrations of G418 for 3 weeks. Top, western blotting prepared with 20 μg of protein extract and anti-FLAG tag-specific antibody. Middle, 3 weeks after transfection and subsequent selection of drug-resistant colonies, the colonies formed by wild-type or constitutive active PAK4-transfected cells, but not by kinase inactive PAK4-transfected cells, were more numerous than those formed by empty vector-transfected cells. Bottom, quantitative analysis of colony formation (colonies >2 mm were counted). Columns, means of three separate experiments, each performed in triplicate; bars, SD (histogram). Differences among multiple comparisons were analyzed by one-way ANOVA with subsequent Scheffé's tests: a, $P < 0.05$ *versus* empty vector; b, $P < 0.05$ *versus* pCMV-3Tag1A-PAK4KI.

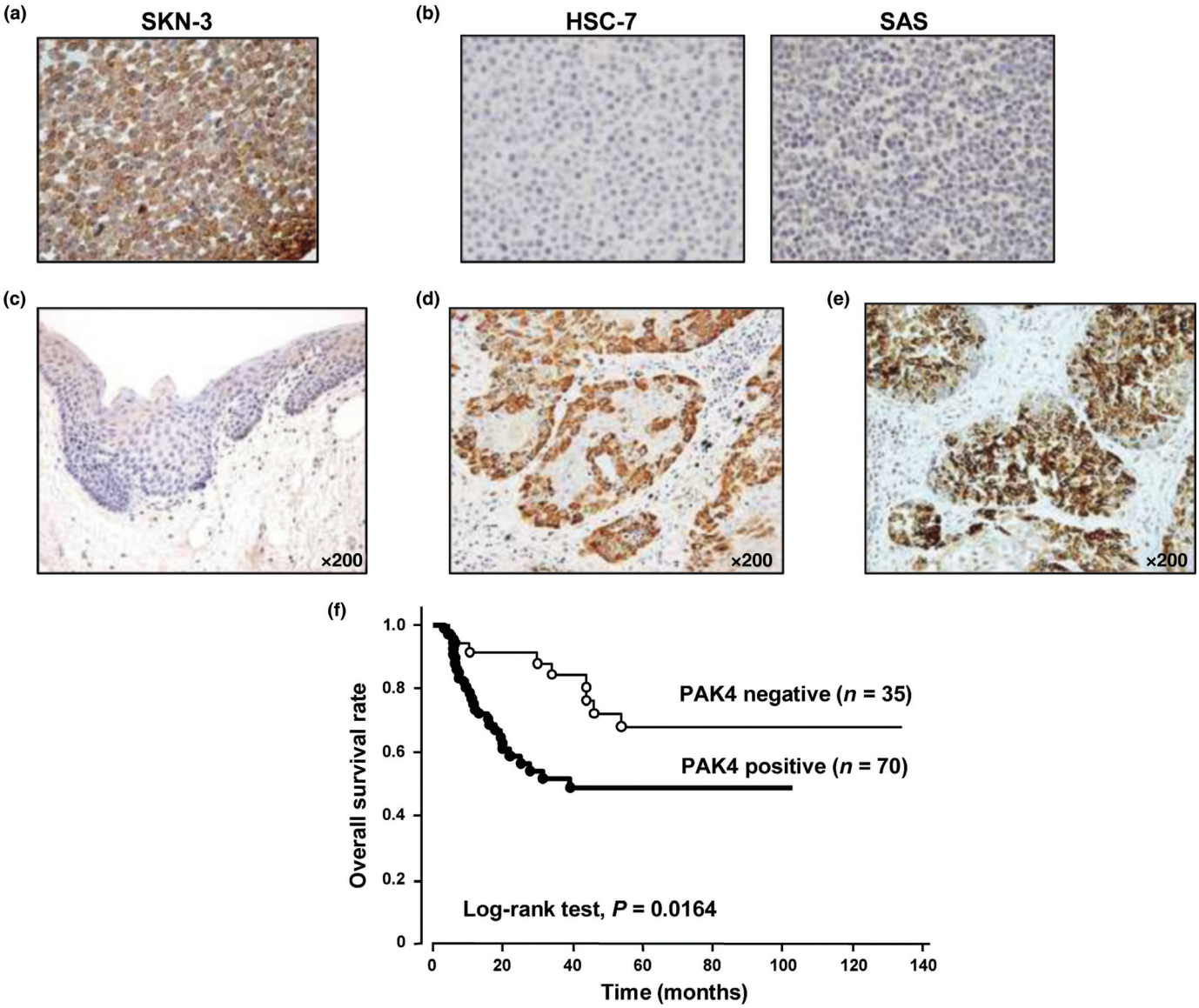

Fig. 4. (a,b) Immunohistochemical staining of PAK4 in oral squamous-cell carcinoma (OSCC) cell lines. Formalin-fixed and paraffin-embedded cell pellets of OSCC cell lines with high (SKN-3, a) and weak (HSC-7 and SAS, b) PAK4 expression were used as positive and negative controls for immunohistochemical staining, respectively. (c–e) Immunohistochemical staining of PAK4 in normal oral epithelia (c) and tumor samples (d,e) of head and neck squamous-cell carcinoma (HNSCC). In each staining, OSCC cell lines with high and weak PAK4 expression were used as positive and negative controls, respectively. (f) Kaplan–Meier curves for overall survival rates of patients with primary HNSCC negative or positive for PAK4 protein. Patients with positive PAK4 expressed in tumors had a significantly worse prognosis than those with no expression ($P = 0.0164$, log-rank test).

Table 2. Cox proportional hazard regression analysis for overall survival

Factor		Univariate		Multivariate†
		Hazard ratio (95% confidence interval)	*P*-values*	*P*-values*
Age (years)				
	>60 *versus* ≤60	1.341 (0.715–2.514)	0.835	X
Differentiation				
	Poor–moderate *versus* well	2.111 (0.998–4.465)	0.051	X
Stage				
	III+IV *versus* I+II	3.413 (1.504–7.752)	**0.003**	**0.006**
PAK4 expression‡				
	Positive *versus* negative	2.451 (1.151–5.208)	**0.02**	**0.024**

Note: Statistically significant values are in boldface type.
*P-values are from two-sided tests and were statistically significant when <0.05.
†Forward- and backward-stepwise analyses were used for multivariate analysis.
‡PAK4 expression was evaluated by immunohistochemical analysis as described in 'Materials and Methods'.

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Discussion

In a previous study using an in-house BAC array, we identified several remarkable changes including amplifications and homozygous deletions in OSCC cell lines.[9] In this study, we focused on one amplified region within 19q13.12-13.2. Amplification of 19q13 has been reported in various tumors including OSCC.[4] Although many possible targets for amplification in this region, such as *AKT2*, *MLL4*, *CCNE1*, *SERTAD1*, *DYRK1B*, *PAF1*, *IXL*, and *ACTN4*,[10,18–24] have been reported in various tumors, potential targets in OSCC remain unknown. In this study, we identified *PAK4* as a novel target for amplification within 19q13.12-13.2 in OSCC, because (a) increased expression of PAK4 was observed in an OSCC cell line with remarkable amplification of this region (SKN-3) as well as in OSCC lines without amplification; (b) knockdown of PAK4 expression inhibited cell proliferation in an expression level–dependent manner and ectopic overexpression of PAK4 induced cell proliferation; and (c) PAK4 protein overexpression was frequently observed in primary tumors from cases of HNSCC, including OSCC, and correlated with a worse overall survival rate.

Among previously reported targets of amplification at 19q13,[10,18–24] *AKT2*, *MLL4*, *CCNE1*, and *SERTAD1* were not included within the 2.5-Mb amplicon detected in SKN-3 cells, indicating that other genes contribute to the pathogenesis of at least some OSCCs. Indeed, our expression analysis demonstrated a low mRNA level of *AKT2* in a panel of OSCC lines compared with the normal oral epithelia-derived cell line. Among *DYRK1B*, *PAF1*, *IXL*, and *ACTN4* located within the 19q13.12-13.2 amplicon of the SKN-3 cell line, *DYRK1B* mRNA was not expressed in SKN-3 cells or other OSCC cell lines (Supporting Table S2), and *PAF1*, *IXL*, and *ACTN4* mRNAs were infrequently overexpressed in OSCC cell lines other than SKN-3 (Fig. 2), suggesting that they are also likely to be excluded as potential candidates for tumor-promoting genes activated in OSCC. Taken together, none of the eight reported genes around 19q13.12-13.2 was included among possible targets for amplification in OSCC.

Among 63 transcripts located within the 2.5-Mb amplicon in the SKN-3 cell line, we chose three genes as possible targets for amplification in OSCC through our systematic approach using public databases as well as expression analyses. Among the three candidates, knockdown of *MAP3K10* and *PSMC4* inhibited cell proliferation regardless of their expression level in OSCC cell lines, whereas knockdown of *PAK4* inhibited cell proliferation in an expression level–dependent manner, suggesting that a recently reported widespread and important phenomenon, termed 'oncogene addiction',[25] is applicable to *PAK4* in OSCC. After knockdown of *PAK4*, G_1 arrest with an increase in p21 expression was observed in the SKN-3 cell line, suggesting overexpressed PAK4 to promote the proliferation of OSCC cells at least partly by accelerating G_1-S transition. In the immunohistochemical analysis of primary HNSCCs, a higher level of PAK4 protein was sometimes observed at the invasive front of the tumors, indicating potential roles in tumor cell proliferation. Combined with the correlation between PAK4 immunoreactivity and a poorer overall survival rate in HNSCC even after stratification with other clinicopathological factors by multivariate analysis, the findings suggest that the proliferation-promoting effect of PAK4 is critical in some lineages of HNSCC even in the earlier stage and that PAK4 expression status may be a prognosticator in cases of HNSCC, although further study using an independent cohort will be needed to test this hypothesis.

PAK4 is a member of the group II PAK serine/threonine kinases originally identified as a protein that promotes filopodial development in response to activated Cdc42.[26,27] PAK4 is highly expressed during embryogenesis but expressed at low levels in most adult tissues,[28] and *PAK4* mRNA levels are increased in a wide range of cancer cell lines and primary tumors.[29,30] Those findings suggest that high levels of PAK4, which most likely plays a critical role in the rapid cell proliferation in embryogenesis, may no longer be needed in adult tissues, and improperly expressed or activated PAK4 may promote cell survival, uncontrolled proliferation, and tumorigenesis.[29,30] Indeed, it was reported that PAK4 plays a vital role in Ras-driven, anchorage-independent cell proliferation, a hallmark of oncogenesis.[17] PAK4 was also reported to phosphorylate Raf1 and activates the ERK pathway downstream of Raf, thus playing an additional role in the regulation of cell proliferation and differentiation that is under the control of ERK/MAPK signaling,[31] although our preliminary experiment detected no alteration in the level of activated ERK (data not shown). Overexpression of wild-type or activated PAK4 protects cells from apoptosis induced by UV irradiation or serum withdrawal.[32] In addition, overexpression of PAK4 was shown to lead to the formation of tumors in athymic mice *in vivo*, whereas deletion of PAK4 inhibited tumorigenesis.[30] However, it was unknown whether overexpressed wild-type or mutated PAK4 directly or indirectly promotes cell proliferation in human cancer. Our results showing that (a) no mutation was detected within the coding sequence with the functional motif of PAK4 in OSCC cell lines, although its proliferation-promoting activity seems to depend on the kinase activity; (b) p21 protein levels were increased after knockdown of PAK4; and (c) frequently observed PAK4 immunoreactivity in primary HNSCCs correlated with a poor prognosis, together with previous reports, suggest that the overexpression of wild-type PAK4 through various mechanisms, including amplification, has the potential to produce conditions favorable for oncogenic transformation through its kinase activity–dependent ability to promote uncontrolled cell proliferation and/or survival. Although PAKs including PAK4 provide compelling evidence for targeting due to their implications in cancer, availability of crystal structures, unique movements of the catalytic motifs, and inhibitor profiling, no specific inhibitor for group-II PAKs has been approved in drug development.[13,27] Development of a PAK4-specific inhibitor will provide new avenues for understanding the physiological and pathophysiological functions as well as an opportunity for PAK4-targeted anticancer therapy.

In summary, our detailed characterization of the 19q13.12-13.2 amplicon detected in an OSCC cell line and sequential analyses of genes within the amplicon revealed that *PAK4* is a novel target for amplification/activation. Since knockdown of *PAK4* inhibited proliferation in OSCC cells overexpressing this gene and expression status was correlated with tumor size and overall survival in primary cases of HNSCC including OSCC, PAK4 may play a critical role in the proliferation and survival of at least a subset of HNSCCs, and thereby may be a novel prognosticator as well as an attractive therapeutic target. Finally, this study shows that the combination of copy-number and expression analyses together with targeted siRNA screening provides an efficient approach for the identification of novel putative amplification targets, which are correlated with the pathogenesis of human cancer.

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References

1 Parkin DM, Bray F, Ferlay J, Pisani P. Global cancer statistics, 2002. *CA Cancer J Clin* 2005; **55**: 74–108.
2 Brinkman BM, Wong DT. Disease mechanism and biomarkers of oral squamous cell carcinoma. *Curr Opin Oncol* 2006; **18**: 228–33.
3 Ha PK, Califano JA. Promoter methylation and inactivation of tumour-suppressor genes in oral squamous-cell carcinoma. *Lancet Oncol* 2006; **7**: 77–82.
4 Martin CL, Reshmi SC, Ried T *et al*. Chromosomal imbalances in oral squamous cell carcinoma: examination of 31 cell lines and review of the literature. *Oral Oncol* 2008; **44**: 369–82.
5 Schwab M. Oncogene amplification in solid tumors. *Semin Cancer Biol* 1999; **9**: 319–25.
6 Savelyeva L, Schwab M. Amplification of oncogenes revisited: from expression profiling to clinical application. *Cancer Lett* 2001; **167**: 115–23.
7 Pinkel D, Segraves R, Sudar D *et al*. High resolution analysis of DNA copy number variation using comparative genomic hybridization to microarrays. *Nat Genet* 1998; **20**: 207–11.
8 Inazawa J, Inoue J, Imoto I. Comparative genomic hybridization (CGH)-arrays pave the way for identification of novel cancer-related genes. *Cancer Sci* 2004; **95**: 559–63.
9 Suzuki E, Imoto I, Pimkhaokham A *et al*. PRTFDC1, a possible tumor-suppressor gene, is frequently silenced in oral squamous-cell carcinomas by aberrant promoter hypermethylation. *Oncogene* 2007; **26**: 7921–32.
10 Cheng JQ, Godwin AK, Bellacosa A *et al*. AKT2, a putative oncogene encoding a member of a subfamily of protein-serine/threonine kinases, is amplified in human ovarian carcinomas. *Proc Natl Acad Sci USA* 1992; **89**: 9267–71.
11 Kallioniemi A, Kallioniemi OP, Piper J *et al*. Detection and mapping of amplified DNA sequences in breast cancer by comparative genomic hybridization. *Proc Natl Acad Sci USA* 1994; **91**: 2156–60.
12 Bellacosa A, de Feo D, Godwin AK *et al*. Molecular alterations of the AKT2 oncogene in ovarian and breast carcinomas. *Int J Cancer* 1995; **64**: 280–5.
13 Eswaran J, Soundararajan M, Knapp S. Targeting group II PAKs in cancer and metastasis. *Cancer Metastasis Rev* 2009; **28**: 209–17.
14 Japan Society for Head and Neck Cancer. *General Rules for Clinical Studies on Head and Neck Cancer*, 3rd edn. Tokyo: Kanahara, 2001.
15 Sobin LH, Wittekind C. In: *International Union Against Cancer (UICC). TNM Classification of Malignant Tumours*, 6th edn. New York: Wiley–Liss, 2002; 19–52.
16 Inoue J, Otsuki T, Hirasawa A *et al*. Overexpression of PDZK1 within the 1q12-q22 amplicon is likely to be associated with drug-resistance phenotype in multiple myeloma. *Am J Pathol* 2004; **165**: 71–81.
17 Callow MG, Clairvoyant F, Zhu S *et al*. Requirement for PAK4 in the anchorage-independent growth of human cancer cell lines. *J Biol Chem* 2002; **277**: 550–8.
18 Huntsman DG, Chin SF, Muleris M *et al*. MLL2, the second human homolog of the Drosophila trithorax gene, maps to 19q13.1 and is amplified in solid tumor cell lines. *Oncogene* 1999; **18**: 7975–84.
19 Richter J, Wagner U, Kononen J *et al*. High-throughput tissue microarray analysis of cyclin E gene amplification and overexpression in urinary bladder cancer. *Am J Pathol* 2000; **157**: 787–94.
20 Tang TC, Sham JS, Xie D *et al*. Identification of a candidate oncogene SEI-1 within a minimal amplified region at 19q13.1 in ovarian cancer cell lines. *Cancer Res* 2002; **62**: 7157–61.
21 Deng X, Ewton DZ, Li S *et al*. The kinase Mirk/Dyrk1B mediates cell survival in pancreatic ductal adenocarcinoma. *Cancer Res* 2006; **66**: 4149–58.
22 Moniaux N, Nemos C, Schmied BM *et al*. The human homologue of the RNA polymerase II-associated factor 1 (hPaf1), localized on the 19q13 amplicon, is associated with tumorigenesis. *Oncogene* 2006; **25**: 3247–57.
23 Kuuselo R, Savinainen K, Azorsa DO *et al*. Intersex-like (IXL) is a cell survival regulator in pancreatic cancer with 19q13 amplification. *Cancer Res* 2007; **67**: 1943–9.
24 Kikuchi S, Honda K, Tsuda H *et al*. Expression and gene amplification of actinin-4 in invasive ductal carcinoma of the pancreas. *Clin Cancer Res* 2008; **14**: 5348–56.
25 Weinstein IB. Cancer: addiction to oncogenes – The Achilles heal of cancer. *Science* 2002; **297**: 63–4.
26 Abo A, Qu J, Cammarano MS *et al*. PAK4, a novel effector for Cdc42Hs, is implicated in the reorganization of the actin cytoskeleton and in the formation of filopodia. *EMBO J* 1998; **17**: 6527–40.
27 Eswaran J, Soundararajan M, Kumar R, Knapp S. UnPAKing the class differences among p21-activated kinases. *Trends Biochem Sci* 2008; **33**: 394–403.
28 Qu J, Li X, Novitch BG *et al*. PAK4 kinase is essential for embryonic viability and for proper neuronal development. *Mol Cell Biol* 2003; **23**: 7122–33.
29 Qu J, Cammarano MS, Shi Q, Ha KC, de Lanerolle P, Minden A. Activated PAK4 regulates cell adhesion and anchorage-independent growth. *Mol Cell Biol* 2001; **21**: 3523–33.
30 Liu Y, Xiao H, Tian Y *et al*. The pak4 protein kinase plays a key role in cell survival and tumorigenesis in athymic mice. *Mol Cancer Res* 2008; **6**: 1215–24.
31 Cammarano MS, Nekrasova T, Noel B, Minden A. Pak4 induces premature senescence via a pathway requiring p16INK4/p19ARF and mitogen-activated protein kinase signaling. *Methods Mol Biol* 2005; **25**: 9532–42.
32 Gnesutta N, Qu J, Minden A. The serine/threonine kinase PAK4 prevents caspase activation and protects cells from apoptosis. *J Biol Chem* 2001; **276**: 14414–9.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Representative results of the quantitative real time RT-PCR analysis to examine the expression levels of genes within the 19q13.2-13.3 amplicon of SKN-3 among a panel of oral squamous-cell carcinoma (OSCC) cell lines normalized to the immortalized normal oral epithelial cell line, RT7. ACTB was used as an internal control. Gray and white bars indicate cell lines with increased expression and those with no or decreased expression of each gene compared with RT7, respectively. (a) Eleven genes showed 'pattern B' in a panel of OSCC cell lines. (b) Twelve genes showed 'pattern C' in a panel of OSCC cell lines.

Fig. S2. (a) Levels of PAK4 protein in SKN-3, NA, and HSC-7 cells treated with *PAK4*-specific siRNA (*PAK4*-siRNA) or control *luciferase* siRNA (*Luc*-siRNA) for 24–72 h. β-Actin was used as an internal control. SKN-3 amplifying *PAK4* and NA without *PAK4* amplification show remarkably high and intermediate expression of PAK4 protein, respectively, whereas HSC-7 shows very low level expression of PAK4 protein. PAK4 protein was efficiently downregulated by *PAK4*-siRNA treatment compared with *Luc*-siRNA treatment. (b) Levels of *PAK4* mRNA (left) and cell proliferation (right) in NA cells treated with *PAK4*-siRNA or control *Luc*-siRNA. See legend for Figure 3(a).

Table S1. Primers for PCR

Table S2. Summary of genes located within the 19q12 amplicon detected in SKN3 cells

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