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Transgenic Mice Expressing Constitutively Active Akt in Oral Epithelium Validate KLFA as a Potential Biomarker of Head and Neck Squamous Cell Carcinoma

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Absrtact. *Background: Head and neck squamous cell carcinoma (HNSCC) is a common human neoplasia, of poor prognosis and survival, which frequently displays Akt overactivation. Previously, we reported that mice expressing high levels of constitutively Akt activity (myrAkt) in oral epithelia develop lesions and tumors in the oral cavity. Materials and Methods: Functional genomics of primary keratinocytes from different transgenic mouse lines and immunostaining of mouse and human samples were performed in order to identify and validate putative biomarkers of oral cancer progression. Results: The expression of KLF4 was found to be increased only in tumor prone samples from mice bearing overactivation of Akt. Such increased expression was confirmed in oral dysplasias and tumors arising in those mice. Tissue microarray analysis of human samples confirmed the association between active Akt and increased KLF4 expression. Conclusion: These data support the notion that KLF4 is potentially a reliable marker of HNSCC, and that myrAkt transgenic mice are valuable tools for preclinical research of HNSCC.*

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Head and neck squamous cell carcinoma (HNSCC) is a common type of human cancer worldwide associated with alcohol and/or tobacco abuse (1). In spite of using new therapeutic approaches (2-5), the improvement in overall survival in patients with HNSCC is still low. Therefore new targeted therapies are required for the management of this disease. HNSCC results from the accumulation of numerous genetic and epigenetic alterations, which occur in a multistep process, affecting multiple biochemical pathways. The major pathways involved in HNSCC development include the pRb and p53-dependent pathways, epidermal growth factor receptor (EGFR), signal transducer and activator of transcription 3 (Stat3), nuclear factor κB (NFκB) and transforming growth factor β (TGFβ) (reviewed in (6-8)). These have provided several candidate genes of potential therapeutic relevance that are now being validated through *in vitro* analyses (6, 9, 10); however, these studies cannot recapitulate the complex nature of HNSCC tumors *in vivo*, and animal models of HNSCC will become essential tools for the evaluation of such therapeutic approaches. Nonetheless, there are few suitable genetically defined mouse models that fully recapitulate the molecular characteristics of human HNSCC in which to study the progression of this type of tumor under preclinical settings (6).

The Akt protein kinase regulates cell death and proliferation through phosphorylation of numerous targets and has been implicated in multiple human neoplasia (11). Several mouse models have recently shown that aberrant Akt signaling plays a predominant role in malignant transformation *in vivo*, either alone or in cooperation with other genetic alterations (12). We and others have provided evidence of the involvement of Akt

activation in the development and progression of HNSCC (13, 14); indeed, molecular alterations in the PI3K/Akt/PTEN signaling pathway are found in about 50% of HNSCC cases (15), indicating that this is a plausible target for the treatment of this disease (16). More recently, to assess the functions of deregulated Akt activity *in vivo*, we have generated transgenic mice expressing wild-type Akt or myrAkt (myristoilated Akt, making the kinase constitutively active) in the basal layer of stratified epithelium (17). Importantly, we observed that besides developmental defects in ectodermal organs (18), the myrAkt mice developed multiple pretumoral oral lesions and tumors that, when combined with the ablation of *Tp53* gene in the same cells, rapidly progress to overt oral tumors that phenocopy the molecular alterations previously found in human HNSCC (19). These characteristics make this model an excellent and unique preclinical tool for the therapeutic management of HNSCC at different steps. Here we use functional genomic approaches in these mice to analyze potential candidates of use as biomarkers of human HNSCC progression.

Materials and Methods

Mice and histological procedures. The generation of Bk5myrAkt and *Trp53$^{F/F}$*;K14cre mice and the protocols for genotyping have been previously described (17, 18, 20, 21). These mice were in an immunocompetent mixed C57/Bl6xDBAxFVB/n background. All the animal experiments were approved by the Animal Ethical Committee (CEEA) and conducted in compliance with Centro de Investigaciones Energéticas, Medioambientales y Tecnológicas (CIEMAT) Guidelines. For histological analysis, oral samples were fixed in formalin and embedded in paraffin prior to sectioning. Sections were stained and processed as described elsewhere (17-19). Antibodies were used as follows: anti-Ser473 phosphorylated Akt (Cell Signaling, Danvers, MA, USA) diluted 1/100; anti p53 (CM5 Novocastra, Newcastle, UK) diluted 1/500; anti p21 (Santa Cruz, Santa Cruz, CA, USA) diluted 1/100; anti-CycD1 (NeoMarkers, Fremont, CA, USA) diluted 1/100 and anti-KLF4 (Chemicon, Temecula, CA, USA) diluted 1/100. Biotin-conjugated secondary antibodies were purchased from Jackson ImmunoResearch, Palo Alyo, CA, USA and used at 1/1000. Signal was amplified using avidin-peroxidase (ABC elite kit; Vector, Burlingame, CA, USA) and peroxidase was visualized using diaminobenzidine as a substrate (DAB kit; Vector). Control slides were obtained by replacing primary antibodies with phosphate-buffered saline (PBS) or preimmune sera (data not shown). At least 5 different samples of each type (normal oral epithelium, dysplasia and squamous cell carcinoma, SCC) from at least five different mice of each genotype (only non-lesional oral epithelium was observed in control mice) were analyzed.

Affymetrix mouse gene chip 430A analysis. Total RNA from primary keratinocytes was extracted with Trizol (Gibco-BRL, Gaithersburg, MD USA) and purified using RNAeasy columns (Qiagen, Valencia, CA, USA) following manufacturers recommendations. The integrity of the RNA populations was tested in a Bioanalyzer (Agilent, Santa Clara, CA, USA) showing 28S/18S ratios above 1.7. Total RNA were hybridized at the Genomic Facility of the Centro de Investigación del Cáncer (Salamanca, Spain). We exported .cel files from Affymetrix GCOS software, and performed background subtraction with RMA (22) using the GEPAS analysis suit (23). The signal intensity values of each probe set were $\log_2$ transformed and standarized. Further analyses were performed using MeV software (24). Statistical *t*-test was used to select the genes with differential expression between the L84 primary keratinocytes and the others mouse genotypes ($p<0.002$). A total number of 121 significant probe sets were selected.

Western blot analysis. Western blot analyses of oral keratinocytes were performed as previously described (17, 18) using the antibodies given above.

Tissue microarray of human HNSCC. The construction of the tissue microarray containing human pretumoral and tumoral samples, and their histopathological characteristics have been previously described (25, 26). All specimens were obtained from patients diagnosed in the Hospital Universitario 12 de Octubre, Madrid, Spain. Informed consent was obtained from each patient and the study has been carried out with the correspondent ethical committee approval. Immunohistochemistry detection of Ser473 phosphorylated Akt and KLF4 was performed as for mouse samples. Statistical analyses were carried out with SSPS program, version 11. 5. (SSPS Inc, Chicago, IL, USA). Frequencies were compared by the χ^2 contingency test.

Results

The activation of Akt has been previously involved in the development and progression of HNSCC (13-15, 27). In agreement, we have observed that the expression of high levels of myrAkt, rendering constitutively high Akt activity in oral epithelia, leads to the formation of dysplastic lesions that eventually proceed to oral SCC (19). Of note, these alterations were not observed in mice expressing wtAkt (wtAkt line LA) or low levels of myrAkt (myrAkt line L60), associated with lower Akt activity. To identify putative genes involved in oral cancer progression, we performed global expression profiling of paired RNA samples from primary keratinocyte extracts of transgenic (myrAkt L60, myrAkt L84, and wtAktLA) and nontransgenic mice. The primary keratinocytes were selected to avoid the developmental defects observed in ectodermal tissues in these different mouse lines (18). As the pretumoral and tumoral lesions were only observed in myrAktL84 mice, the microarray data were processed to find specific clusters that included genes only upregulated in these primary keratinocytes compared with those derived from lines LA, L60 and controls (Figure 1A). Utilizing the Entrez Gene Database, we narrowed the dataset to those genes that were previously shown to be involved in cancer; this approach provides a list of 19 different genes (Table I). Most of them have been previously identified as being up-regulated in different types of cancer but their possible function in promoting carcinogenesis has not been evaluated. However, we also found up-regulation of

Table I. *Up-regulated genes in L84 primary keratinocytes.*

Probe set ID	Gene symbol	Gene title	*p*-Value	Fold change*
1416868_at	*Cdkn2c*	Cyclin-dependent kinase inhibitor 2C (p18, inhibits CDK4)	0.0013	4.38
1417040_a_at	*Bok*	Bcl-2-related ovarian killer protein	0.0013	3.42
1417394_at	*Klf4*	Kruppel-like factor 4 (gut)	0.0009	3.72
1418649_at	*Egln3 (PHD3)*	EGL nine homolog 3 (*C. elegans*)	0.0013	6.09
1419317_x_at	*Sprrl1 (Eig3)*	Small proline rich-like 1	0.0011	6.21
1419437_at	*Sim2 /// LOC547289 /// LOC547335*	Single-minded homolog 2 (Drosophila) /// similar to single-minded 2 protein /// similar to single-minded 2 protein	0.0002	5.93
1422470_at	*Bnip3 (Nip3)*	BCL2/adenovirus E1B 19 kDa-interacting protein 1, NIP3	0.0019	4.69
1422588_at	*Krt2-6b*	Keratin complex 2, basic, gene 6b	0.0015	5.65
1423542_at	*Klk7*	Kallikrein 7 (chymotryptic, stratum corneum)	0.0008	4.77
1424801_at	*Enah*	Enabled homolog (Drosophila)	0.0015	2.09
1425711_a_at	*Akt1*	Thymoma viral proto-oncogene 1	0.0009	4.28
1426047_a_at	*Ptprr (PTP-SL)*	Protein tyrosine phosphatase, receptor type, R	0.0004	3.88
1435133_at	*Ugcg (GCS)*	UDP-glucose ceramide glucosyltransferase	0.0019	2.74
1448756_at	*S100a9*	S100 calcium binding protein A9 (calgranulin B)	0.0012	5.99
1448886_at	*Gata3*	GATA binding protein 3	0.0018	2.43
1448982_at	*Prss18 (KLK6)*	Protease, serine, 18	0.0009	4.69
1450633_at	*Calm4 (Scarf)*	Calmodulin 4	0.0004	4.65
1448757_at	*Pml*	PML: promyelocytic leukemia	0.0006	5.13
1454159_a_at	*Igfbp2*	Insulin-like growth factor-binding protein 2	0.0002	4.87

* With respect to non-transgenic keratinocytes.

some genes that have been previously suggested as cancer-promoting agents. Among them we found *Pml*, in agreement with our previous data (19), which accounts for the induction of *p53* and premature senescence observed in tumoral samples derived from myrAkt L84 mice (19).

Interestingly, among the up-regulated ones, we also found Kruppel-like factor 4 (KLF4/GKLF/EZF). KLF4 is a transcription factor that can both activate and repress genes that are involved in cell-cycle regulation and differentiation in epithelium (28). Different data have indicated that KLF4 may have tumor-suppressive or oncogenic functions in a tissue-specific manner (29). We confirmed the increased expression of KLF4 in parallel with phosphorylated Akt in oral tissues of the myrAkt transgenic mice including oral dyplasia and SCC (Figure 1B-C´) by immunohistochemistry, and in primary keratinocytes by Western blot (Figure 1D). Of note, in oral dysplasia of the transgenic mice, the constitutive activation of Akt (Figure 1B) also induces *p53* expression (Figure 1B´), in agreement with the up-regulation of *Pml* (19).

Elevated KLF4 levels have also been linked to the early stages of human oral squamous-cell carcinomas development (30) and the ectopic KLF4 expression in transgenic mice has been shown to induce squamous epithelial dysplasia (31, 32). These data, together with our observations, may suggest that KLF4 could represent a potential biomarker associated with Akt activation for oral dysplasias and SCCs. In order to test this hypothesis, the expression of phosphorylated Akt was studied in parallel with KLF4 expression in tissue microarrays containing 84 HNSCCs and 59 oral dysplasias from human patients (25, 26). Of these, 59 dysplasias and 80 tumors were informative (Table II; examples of positive staining are provided in Figure 2). We found a significant number of dysplasias and tumors showing increased Akt activity, indicating that Akt activation is an early event during human HNSCC development in agreement with our

Table II. *Summary of tissue microarray analysis.*

Oral dysplasia	Akt-P		Total
	Negative	Positive	
KLF4			
Negative	10	7	17
Positive	7	35	42
Total	17	42	59

$p \leq 0.001$

HNSCC	Akt-P		Total
	Negative	Positive	
KLF4			
Negative	28	15	43
Positive	15	26	41
Total	43	41	80

$p \leq 0.027$

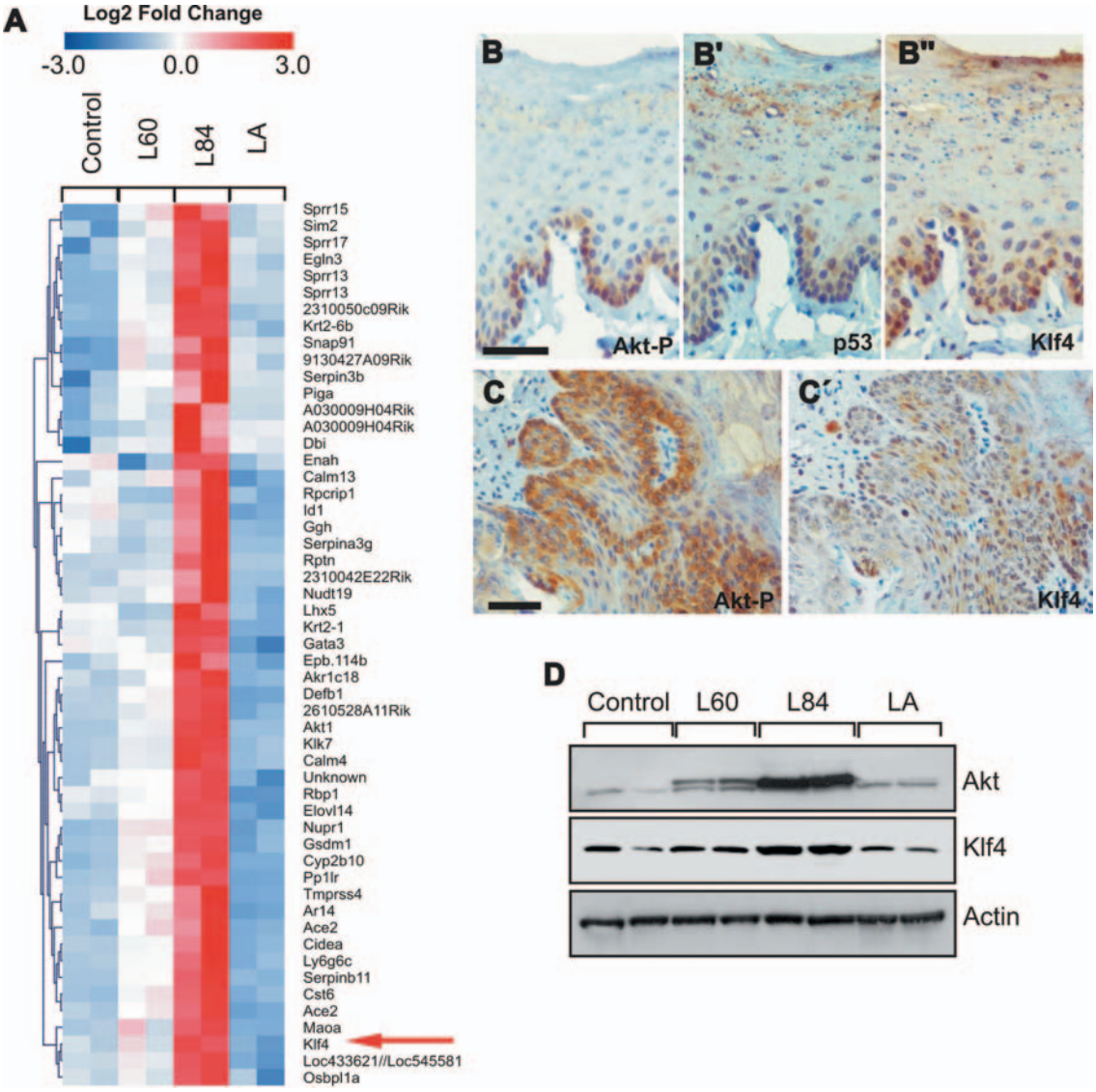

Figure 1. *Klf4 is up-regulated in myrAkt L84 mice. A, Heatmap of genes specifically upregulated in myrAktL84 primary keratinocytes. B-C, Examples of the immunohistochemical detection of phosphorylated Akt (B, C), p53 (B´) and Klf4 (B´´, ´) in oral dysplasia (B, B´, B´´) and oral SCC (C, C) in myrAkt L84 mice. Bars=200 μm. D, Western blot showing the expression of Akt1 and Klf4 in primary keratinocytes of the quoted lines. Actin was used as loading control.*

previous data (14, 17, 19). Similarly, the expression of KLF4 was prominent in both types of samples (Figure 2 and Table II) in agreement with others (30). Of note, there was a significant correlation between active, phosphorylated Akt and KLF4 expression in dysplasias ($p \leq 0.001$) and HNSCC ($p \leq 0.027$) (Table II). These data confirmed our observations in oral samples from myrAkt L84 mice and support the notion that KLF4 can be used as a surrogate marker of Akt activation during human oral carcinogenesis.

As mentioned above, *KLF4* can act as a tumor suppressor or an oncogene in a tissue-specific manner (29). KLF4 expression is induced during epithelial maturation *in vivo* (30, 33) and its repression in proliferating cells seems to occur through posttranscriptional mechanisms (31). Nonetheless, the ectopic expression of KLF4 is sufficient to drive skin hyperplasia, dysplasia and SCC in epidermis of transgenic mice (31, 32). One possible explanation for the dual role of *KLF4* as a tumor suppressor or an oncogene relies on the findings of Rowland *et al.* (34), which identified *KLF4* as a gene that can bypass ras-induced senescence, but whose expression in untransformed cells causes cell proliferation arrest (34). The mechanism

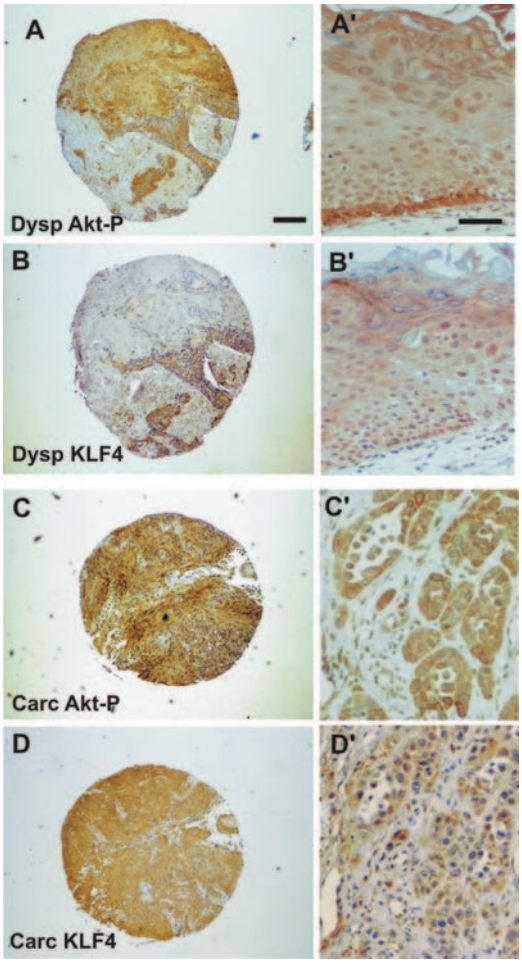

Figure 2. *Examples of tissue microarray analysis. Low magnification (A-D) and details (A´-D´) of human oral dysplasia (A-B´) and human HNSCC (C-D´) stained for phosphorylated Akt (A, A', C, C') and KLF4 (B, B', D, D´) Bar in A=mm; in B=200 μm.*

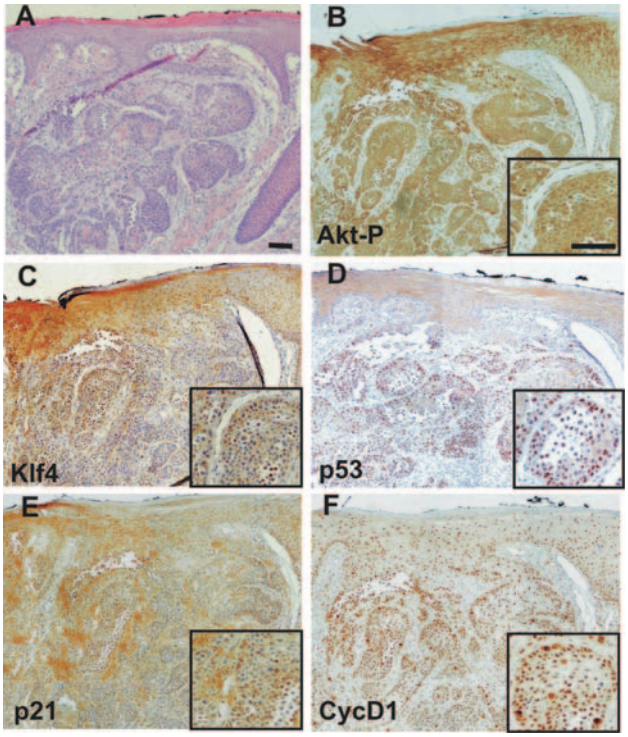

Figure 3. *Analyses of KLF4-related pathways in HNSCC. A, Example of hematoxylin-eosin-stained section of human oral SCC. B-F, Consecutive sections stained for phosphorylated Akt (B), KLF4 (C), p53 (D), p21 (E) and cyclin D1 (F). Insets in B, C, D, E, F show the same area of the tumor at higher magnification. Bars=200 μm.*

underlying these effects appears to be mediated by the different effects on p53 and p21 expression and requires cyclin D1 (34). Notably, in myrAkt transgenic mice, the progression to overt oral SCC is prevented by *p53* induction and thus premature senescence (19). Indeed, we observed the induction of *p53* (Figure 1B´) in the same cells that display induced KLF4 (Figure 1B´´) as a consequence of Akt activation (Figure 1B).

To explore this aspect in detail, human HNSCC previously characterized as displaying active Akt expression (14) were analyzed by immunohistochemistry for the expression of active Akt, p53, p21, cyclin D1 and KLF4 (Figure 3). We observed that tumoral cells expressing active Akt (Figure 3B) and subsequently KLF4 (Figure 3C), also displayed strong staining for p53 (Figure 3C), p21 (Figure 3D) and cyclin D1 (Figure 3E). Of note, p53-positive reactivity of human cancer samples has been correlated with inactivating mutations in this tumor suppressor gene. Nonetheless, as KLF4 transcriptionally represses *p53* gene expression regardless of its mutational status, these findings indicate that in human HNSCC samples, the induction of *KLF4* is not sufficient to completely abrogate *p53* expression. This may explain why, even upon *KLF4* induction, constitutive Akt expression in transgenic mice leads to premature senescence mediated by the induction of *p53* (Figure 1B and B´; see also (19). The observed induction of *p21*, which is in agreement with the data obtained in cultured cells (19, 34), might suggest reduced proliferation; however, the induction of cyclin D1, which is also mediated by Akt activation (14, 35) can counteract the possible antiproliferative effects of such *p21* induction.

Discussion

Molecular targeted therapies are promising in HNSCC management, and are now being validated through *in vitro* analyses (6, 9, 10). The relevance of Akt/PTEN pathway in these malignancies (8, 13-15, 27) supports its potential use

in such therapies (16, 36). Therefore, *in vivo* systems aimed at the analysis of these therapies are necessary. These approaches have been largely hindered by a lack of appropriate animal models mimicking these tumors at both the pathological and molecular levels. We have generated transgenic mice expressing constitutively active Akt in the basal layer of stratified epithelium, including that of the oral cavity (17, 18). These mice, besides displaying some developmental defects in ectodermal organs (18), also display spontaneous tumor development and increased sensitivity to chemical carcinogenesis protocols (17). However, in spite of the development of oral pretumoral lesions with a complete penetrance, few of these lesions progress to overt tumors due to the induction of *Pml* and, subsequently, of a p53-dependent premature senescence (19). Indeed, the somatic ablation of *Trp53* tumor suppressor gene in the same cells that express myrAkt leads to oral SCCs, which recapitulate the molecular features of human HNSCCs (19). These transgenic mice provide novel and plausible mouse models of human oral cancer, which, besides their possible use to test targeted therapies, may allow the consecutive steps involved in tumor initiation and progression to be studied and, consequently, novel biomarkers of progression of this disease to be identified.

Here, we used functional genomics to identify possible markers that may explain the tumor susceptibility in the oral cavity displayed by myrAkt L84 mice. We focused on those genes expressed selectively in myrAkt L84 primary keratinocytes because only in these transgenic mice did we find oral lesions (19). Moreover, we used primary cells to avoid the representation of genes involved in ectodermal development previously identified that can mask other possible pathways (18). This approach rendered a list of possible relevant genes that was further manually assessed in the search for genes previously involved in carcinogenesis. We thus obtain a list of 19 genes of potential use as possible biomarkers.

Due to its previous implication in human tumors (29, 37), here we focused our studies on KLF4. This transcription factor has been characterized as a tumor suppressor in some types of human gastrointestinal cancers (37-39), but it was also found to be overexpressed in human skin, oral cavity and breast cancers (30, 31, 40).

The use of tissue microarrays containing human pretumoral and tumoral samples not only allowed us to establish the relevance of Akt and KLF4 in this type of human pathologies, but also to determine a possible functional linkage between these two proteins. The possible molecular mechanisms of this functional connection are unknown but our data from myrAkt L84 mice may indicate that KLF4 is downstream of Akt signaling. However, there are few data about the transcriptional regulation of KLF4 (41, 42) that may explain this possibility and further studies are required. On the other hand, from our studies in human samples that KLF4 may lie upstream of Akt in human premalignat lesions can not be excluded.

The functions of *KLF4* as an oncogene or tumor suppressor have been correlated with its different activities in p53 and p21 expression. Indeed KLF4 represses p53, whereas it also activates p21 (29, 34). The repression of p53 may justify why KLF4 can also cause chromosomal instability (43, 44). We observed that KLF4 is coexpressed with p53, thus suggesting that in oral tumors, KLF4 is not sufficient to completely abolish the expression of p53. These data, suggesting that oncogenic activities of KLF4 are not solely mediated by p53 repression, are further supported by the functional cooperation between the ectopic expression of KLF4 and p53 absence observed in transgenic mice (32).

Collectively, our observations, besides reinforcing the possible use of myrAkt mice in preclinical intervention and prevention studies, also highlight its application in characterizing possible biomarkers, such as KLF4 reported here, and their related molecular mechanisms, for the analysis of HNSCC progression.

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