Lysyl Oxidase–Like 2 as a New Poor Prognosis Marker of Squamous Cell Carcinomas

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Abstract

Lysyl oxidase–like 2 (Loxl2) interacts with and stabilizes Snai1 transcription factor, promoting epithelial-mesenchymal transition. Either Loxl2 or Snai1 knock-down blocks tumor growth and induces differentiation, but the specific role of each factor in tumor progression is still unknown. Comparison of the gene expression profiles of the squamous cell carcinoma cell line HaCa4 after knocking-down Loxl2 or Snai1 revealed that a subset of epidermal differentiation genes was specifically up-regulated in Loxl2-silenced cells. In agreement, although both Loxl2- and Snai1-knockdown cells showed reduced in vivo invasion, only Loxl2-silenced cells exhibited a skin-like epidermal differentiation program. In addition, we show that expression of Loxl2 and Snai1 correlates with malignant progression in a two-stage mouse skin carcinogenesis model. Furthermore, we found that increased expression of both LOXL2 and SNAI1 correlates with local recurrence in a cohort of 256 human laryngeal squamous cell carcinomas. We describe for the first time that high levels of LOXL2 are associated with decreased overall and disease-free survival in laryngeal squamous cell carcinomas, lung squamous cell carcinoma, and lymph node–negative (N0) breast adenocarcinomas. Altogether, our results show that LOXL2 can be used as a new prognosis indicator in human squamous cell carcinomas promoting malignant transformation by both SNAI1-dependent and SNAI1-independent pathways. [Cancer Res 2008;68(12):4541–50]

Introduction

Metastasis is the most lethal consequence of tumor progression (1). Invasion is the first step during carcinoma metastasis involving changes in cell adhesion, polarity, remodeling of the extracellular matrix and the cytoskeleton (2). The changes observed during local invasion are associated with a process known as epithelial-mesenchymal transition (EMT), initially described as a critical event during embryogenesis and was thought to determine the individual role and tissue components (3). One of the hallmarks of EMT is the loss of E-cadherin expression (6). The mechanisms regulating the transcriptional repression of E-cadherin during tumor progression have been thoroughly investigated, especially since the description of Snai1 transcription factor as an E-cadherin repressor (7,8) and as key regulator of EMT processes during both development and tumor progression (6,9). We have described that lysyl oxidase–like 2 (LOXL2) interacts with Snai1 and promotes its stabilization by counteracting the action of GSK3β, leading to E-cadherin repression and EMT (10,11). LOXL2 is a member of the lysyl oxidase (LOX) gene family. Five LOX family genes have thus far been identified in mammalian genomes encoding the prototypic LOX and LOX-like proteins 1 to 4 (LOXL1, LOXL2, LOXL3, and LOXL4; refs. 12,13). All the members of the family show a highly conserved catalytic domain located at the COOH terminus, whereas the NH2 terminus region of the LOX isoforms is more divergent and is thought to determine the individual role and tissue distribution of each isoenzyme (14). The prototypic LOX (EC 1.4.3.13) plays a key role in the biogenesis of the connective tissue catalyzing cross-linkage formation in collagen and elastin components (15). LOX and LOXL1 have been shown to be required for proper elastic fiber homeostasis and cardiovascular system development (16,17). In addition to its biological role in normal connective tissue function, the LOX family of proteins has recently been implicated in tumorigenesis and metastasis (ref. 18; reviewed in ref. 19). Initial studies showed that LOXL2 overexpression promotes the invasiveness of tumor cells in vivo and in vitro (20,21) and its up-regulation has been reported in breast, colon, esophageal, pancreatic, prostatic, and head and neck squamous cell carcinoma (HNSCC) cell lines (4,10,20–24). However, few studies on the expression of LOX members in human tumor samples are presently available (19). Particularly, three studies have reported LOXL2 up-regulation in colon and esophageal tumors (25), head-and-neck, and oral squamous cell carcinomas (26,27), although another study reported the decreased expression of LOXL2 mRNA in HNSCC (28).

Our previous studies showed that silencing of either Loxl2 or Snai1 blocks tumor growth and induces differentiation (10,29,30); however, the specific contribution of each factor to malignant progression and their expression pattern in human tumors are largely unknown. Here, we have investigated the role of LOXL2 and SNAI1 in the regulation of keratinocyte behavior by analyzing the effect of Loxl2 and Sna1 interference in mouse malignant HaCa4 keratinocytes. Our results indicate that Loxl2 promotes malignant progression by both Snai1-dependent and Snai1-independent pathways.

To get further insights in the implication of LOXL2 and SNAI1 in tumor progression, we analyzed their expression in human and mice tumors. The expression of Loxl2 and Sna1 correlated with
malignant progression in the two-stage mouse skin carcinogenesis model. We have examined the expression of both proteins by immunohistochemistry in a cohort of 256 human laryngeal squamous cell carcinomas (LSCC). LSCC comprise the majority (96%) of human laryngeal malignancies (31). Comparative genomic studies have revealed differences in chromosomal pattern and carcinogenic progression between LSCC and other HN SCC (32). However, at present, the biological markers for molecular diagnostics of LSCC have a limited predictive value (reviewed in ref. 33). Among them, it is noteworthy to mention eIF4E overexpression, which predicts recurrence with discrete specificity (34), and loss of p16 expression, which is currently under evaluation (35). Our analysis of SNAI1 and LOXL2 in human LSCC showed that their expression is associated with local recurrence. Moreover, increased staining of LOXL2 with a heterogeneous pattern was also associated with decreased overall and disease-free survival. Our data show for the first time that LOXL2 can be considered as a new marker of poor prognosis in human LSCC. Importantly, the association between high LOXL2 mRNA expression and poor clinical outcome is also detected by data set analysis in lung squamous cell carcinomas (SCC) and lymph node–negative (N0) breast adenocarcinoma.

Materials and Methods

Cells and bidimensional cultures. Generation of HaCa4shEGFP, HaCa4shLoxl2, and HaCa4shSnai1 has been previously reported (10, 29). Briefly, HaCa4 cells were transfected with pSuperior vectors containing specific sequences to interfere with mLox2 (shLoxl2; ref. 10) or with mSna1 (shSnai1; ref. 29), or with short hairpin RNA against enhanced green fluorescent protein (shEGFP), as a control. Stable transfectants were selected with 1 μg/mL of puromycin for 2 to 3 weeks. At least four independent clones were isolated from each transfection (10, 29); one representative clone from each cell line is shown in the figures. Cells were grown in DMEM supplemented with 10% fetal bovine serum, 10 mMol/L of glutamine (Life Technologies), 100 μg/mL of ampicillin, and 32 μg/mL of gentamicin at 37°C in a humidified 5% CO2 atmosphere.

Patients. The current study was comprised of 256 laryngeal SCCs obtained from surgical patients from the Hospital La Paz, Madrid, Spain. Immunohistochemical studies were carried out on formalin-fixed paraffin-embedded tissue. The series included 249 males and 7 females with a mean age at diagnosis of 60.62 ± 10 years, 33 years old (range, 29–87; Supplementary Table S2). All cases were acquired from the archives of the Department of Pathology, Hospital La Paz with the approval of the head of the Department. All of the tumor samples were diagnosed between 1990 and 1996, and during follow-up for at least 10 years. To validate the external data set mRNA analysis, we analyzed two tumor retrospective samples series from lung SCC (n = 8) and lymph node breast adenocarcinoma (n = 8), respectively (36, 37). Both series were obtained from the Hospital 12 de Octubre, and the Hospital La Paz, Madrid, Spain. Lung SCC series included three well differentiated, two moderately differentiated, and three poorly differentiated tumors from patients ranging in age from 45 to 50 years old.

Tissue microarray construction. Representative areas from formalin-fixed, paraffin-embedded infiltrating carcinomas were carefully selected on HE-stained sections and two 1-mm diameter tissue cores were obtained from each specimen. The tissue cores were precisely arrayed into a new paraffin block using a tissue microarray workstation (Beecher Instruments). We constructed eight tissue microarrays containing approximately 32 tumor samples and positive and negative controls. Mouse skin carcinogenesis. The two-stage mouse skin carcinogenesis (single 7,12-dimethylben[a]anthracene application, followed by twice weekly 12-O-tetradecanoylphorbol-13-acetate applications for 16 weeks) was performed following standard protocols (38). Tumors were collected at different time periods after initiation and processed for reverse transcription-PCR (RT-PCR) and Western blotting as indicated below.

Microarray gene expression profiles. Microarray experiments were performed using Mouse Whole Genome V2 22 K array G4121B (Agilent Technologies). RNA was isolated using RNeasy Extraction Kit (QiAGen). RNA was labeled and array-hybridized using the Low RNA Linear Amplification Kit and the In Situ Hybridization Kit Plus (Agilent Technologies), respectively. After hybridization and washing, the slides were scanned in an Axon GenePix Scanner (Axon Instruments Inc.) and analyzed using Feature Extraction Software 6.1.1 (Agilent Technologies). Two different RNA samples obtained from each cell line were labeled with Cy5-DUTP. The RNA samples extracted from control cells were marked with Cy3-DUTP (Amersham). Two additional hybridizations were performed using the reciprocal fluorochrome labeling. Two independent clones from each cell line were analyzed, with similar results. The genes whose expression was up-regulated or down-regulated at least 2-fold in HaCa4shLoxl2 or HaCa4shSnai1 with respect to control cells were selected for analysis. A hierarchical clustering method was applied to group the genes and samples on the basis of the similarities in expression, and the unsupervised analyses were visualized using the SOTA and TreeView software assuming Euclidean distances between genes (39). Microarray raw data tables have been deposited in the Gene Expression Omnibus under the accession number GSE5658 (submitter: G. Moreno-Bueno).

Class prediction model analysis. For validation of the class prediction model, two independent data set of 51 lung SCC (36) and 286 lymph node negative (N0) breast adenocarcinoma (37) were analyzed. The microarray and clinical data were obtained from the GEO database, with the accession numbers GSE5123 and GSE2034, respectively. The log-rank test for survival data of each tumor type was obtained to classify samples as high and low expression on the basis of gene expression (LOXL2 and SNAI1) relative to the median across all samples. Statistical analysis of survival was performed with SPSS 14.0.

RT-PCR analysis and Western blotting. Total RNA from cell lines was extracted with Trizol (Invitrogen) and DNase treatment. RNA from proteins from tumors were obtained by disruption of the frozen samples with a poluton using Tripute reagent (Roche) or radioimmunoprecipitation assay buffer containing protease inhibitor cocktail (Roche). Two micrograms of RNA were employed for cDNA synthesis using oligo-dT and Moloney murine leukemia virus-recombinaseprimase (Promega). Primers for RT-PCR experiments are described in Supplementary Table S3. RT-PCR for E-cadherin, Snai1, Loxl2, and GAPDH were performed as previously described (10). Proteins from tumors and cell lines were analyzed by Western blot using the enhanced chemiluminescence detection system (GE Healthcare). The antibodies used are listed in Supplementary Table S3.

Organotypic cultures and transplantation assays. Organotypic cultures and transplantation assays on nude mice were performed and processed with two independent clones from each cell line as previously described (40). At least two organotypic cultures of each HaCa4-derived cell clone were studied. Eight mice were used for each HaCa4-derivative cell lines in transplantation experiments. After 1 to 2 weeks of starting the transplantation experiments, four transplants from each group were subjected to immunohistochemical analysis. Mice were housed and maintained under specific pathogen–free conditions and used in accordance with the guidelines approved by the Institutional Animal Care and Use Committee.

Immunofluorescence analysis. The optimal cutting temperature–embedded samples from either the organotypic cultures or in vivo transplants were fixed and subjected to immunofluorescence analysis as described (40). The primary antibodies used are described in Supplementary Table S3. The secondary antibodies included goat anti-rat, anti-mouse, or anti-rabbit coupled to Alexa 550 or Alexa 488 (Molecular Probes).

3 http://bioinfo.cnio.es/cgi-bin/tools/clustering/sotarray
Immunohistochemistry of tumors. LOXL2 detection epitope retrieval was performed in a steamer during 3 min in sodium citrate buffer 10 mmol/L (pH 6.0). SNAI1 analysis in tumors was performed as described (41). After endogenous peroxidase blocking, sections were blocked at room temperature for 30 min in PBS 3% normal goat serum, 3% bovine serum albumin, and 1% Triton X-100. Slices were then incubated overnight at 4°C with primary antibodies (diluted in PBS 1% normal goat serum, 1% bovine serum albumin, and 0.1% Triton X-100). Sections were further processed with appropriate secondary antibodies following the LSAB2/horseradish peroxidase Dako cytomation system protocol.

We considered LOXL2 heterogeneous pattern when an intense specific stain (cytoplasm and perinuclear) was observed in at least 20% of the tumor cells, when compared with the faint diffuse staining in the cytoplasm shown in the majority of the cells. In the case of SNAI1, the tumor stain was scored from 0 to 2 (0 negative, <5% of positive cells; 1 low and cytoplasm localization in —5% to 15% of the cells; 2 cytoplasm-nuclear stain in >15% of the cells). LOXL2 antibody specificity has been probed by immunohistochemistry in xenograft tumors derived from different cell lines (HaCa4 and Carb), which interfered with either an irrelevant sequence (shEGFP) or against Loxl2 (shLoxl2; ref. 10; Supplementary Fig. S1).

Statistical analysis. To test associations between categorical variables, we used the $\chi^2$ or Fisher’s exact test. $P < 0.05$ was considered statistically significant. All tests were two-tailed and 95% confidence intervals were adopted. These analyses were carried out using the SPSS 14.0 for statistical program (SPSS, Inc.). We plotted Kaplan-Meier estimates of the surviving (estimated separately for each stratum) and disease-free individuals. We compared survival curves using log-rank test.

Results

Silencing of Loxl2 or Sna1 reduces cell malignancy by different mechanisms. We have previously reported that silencing of Loxl2 or Sna1 has a dramatic effect on the tumorigenic behavior of HaCa4 cells (10, 29). To gain insights into the individual role of Loxl2 and Sna1 in this phenotype, we analyzed HaCa4 mouse SCC cells expressing short hairpin RNA that stably silence the expression of either Loxl2 or Sna1. HaCa4shLoxl2 and HaCa4shSna1 cells that expressed low levels of Loxl2 or Sna1, respectively (Fig. 1A), exhibited increased cell-cell contacts compared with
significant changes in the expression levels and organization of E-cadherin (Supplementary Fig. S2). On the other hand, HaCa4shSnai1 cells showed homogeneous cytoplasmic stain of control HaCa4-shEGFP cells. Importantly, Loxl2 staining of the same cultures showed almost complete absence of Loxl2 expression in the whole population of HaCa4-shLoxl2, in contrast with the localization in some cells. Importantly, Loxl2 staining of the same cells showed almost complete absence of Loxl2 expression in the whole population of HaCa4-shLoxl2, in contrast with the localization in some cells. Importantly, Loxl2 staining of the same cell population (Fig. 1A, right), with the typical membrane localization in ~50% of the cell population (Fig. 1B, middle); remarkably, these E-cadherin–positive cells seem to differentiate forming multilayers.

To examine the phenotypic differences of HaCa4shLoxl2 versus HaCa4shSnai1 cells in more detail, gene expression analysis of these cell lines could likely be mediated by β-catenin, as previously characterized in parental HaCa4 cells (42). To examine the phenotypic differences of HaCa4shLoxl2 versus HaCa4shSnai1 cells in more detail, gene expression analysis of control HaCa4 cells (Fig. 1A, right and B, bottom), indicating the specificity of E-cadherin re-expression in HaCa4shLoxl2 cells. The organization of β-catenin in these cell lines could likely be mediated by P-cadherin, as previously characterized in parental HaCa4 cells (42).

Table 1: Modified genes involved in cell differentiation (at least 2-fold) in HaCa4-shLoxl2 and HaCa4-shSnai1 compared with HaCa4-shEGFP control cells

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>shLoxl2</th>
<th>shSnai1</th>
<th>Description</th>
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<tr>
<td>Epidermal differentiation (n = 21)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Csf1</td>
<td>12.3</td>
<td>1.44</td>
<td>Cornifelin</td>
</tr>
<tr>
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<td>−20.75</td>
<td>−10.3</td>
<td>Endothelial differentiation, lysophosphatic acid G-protein–coupled receptor 7</td>
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<td>Figa</td>
<td>−18.75</td>
<td>−13.92</td>
<td>Folliculogenesis specific basic helix-loop-helix</td>
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<td>Heyl</td>
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<td>−12.71</td>
<td>Hairy/enhancer-of-split related with YRPW motif-like</td>
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<td>Klk10</td>
<td>23.82</td>
<td>2.25</td>
<td>Kallikrein-related peptidase 10</td>
</tr>
<tr>
<td>Klk1b16</td>
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<td>−2.98</td>
<td>Kallikrein-1–related peptidase b16</td>
</tr>
<tr>
<td>Klk7</td>
<td>47.42</td>
<td>1.48</td>
<td>Kallikrein-related peptidase 7 (chymotryptic, stratum corneum)</td>
</tr>
<tr>
<td>Kira8</td>
<td>−21.93</td>
<td>−5.74</td>
<td>Killer cell lectin-like receptor, subfamily A, member 8</td>
</tr>
<tr>
<td>Kppr</td>
<td>58.82</td>
<td>1.66</td>
<td>Keratinocyte expressed, proline-rich</td>
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<td>Krt23</td>
<td>11.16</td>
<td>62.45</td>
<td>Keratin 23</td>
</tr>
<tr>
<td>Krt8</td>
<td>2.09</td>
<td>19.08</td>
<td>Keratin 8</td>
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<td>−7.01</td>
<td>Neurogenic differentiation 6</td>
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<td>−5.55</td>
<td>Pleiotrophin</td>
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<td>1.63</td>
<td>Small proline-rich protein 2A</td>
</tr>
<tr>
<td>Sprv2d</td>
<td>18.84</td>
<td>−1.68</td>
<td>Small proline-rich protein 2D</td>
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<td>Sprv2f</td>
<td>19.66</td>
<td>−3.59</td>
<td>Small proline-rich protein 2F</td>
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<tr>
<td>Sprv3</td>
<td>11.63</td>
<td>2.58</td>
<td>Small proline-rich protein 3</td>
</tr>
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<td>Sprv1</td>
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<td>1.84</td>
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</tr>
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<td>Sprv2</td>
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<td>1.02</td>
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<td>EMT-related (n = 15)</td>
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<td>−1.67</td>
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<td>Epha5</td>
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<td>Eph receptor A5</td>
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<td>−7.4</td>
<td>Follistatin-like 1</td>
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<tr>
<td>Ifitd1</td>
<td>−16.5</td>
<td>−3.54</td>
<td>Intermediate filament tail domain containing 1</td>
</tr>
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<td>Iyd</td>
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<td>−5.35</td>
<td>Iodotyrosine deiodinase</td>
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<tr>
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<td>−12.57</td>
<td>−4.88</td>
<td>Protocadherin 11 X-linked</td>
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<tr>
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<td>−39.31</td>
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<tr>
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<td>SLIT and NTRK-like family, member 1</td>
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<td>−11.46</td>
<td>SLIT and NTRK-like family, member 6</td>
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<tr>
<td>Tspan1</td>
<td>−15.31</td>
<td>−7.53</td>
<td>Tetraspanin 1</td>
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<tr>
<td>Cdh1</td>
<td>12.77</td>
<td>1.8</td>
<td>Cadherin 1</td>
</tr>
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Control cells (HaCa4-shEGFP) interfered with an irrelevant sequence; Fig. 1B, top). HaCa4shLoxl2 cells re-expressed E-cadherin protein (Fig. 1A, right) with the typical membrane localization in ~50% of the cell population (Fig. 1B, middle); remarkably, these E-cadherin–positive cells seem to differentiate forming multilayers in bidimensional cultures. This pattern was more clearly observed by immunohistochemical staining of the HaCa4-shLoxl2 cultures (Supplementary Fig. S2), which also showed cytoplasmic E-cadherin localization in some cells. Importantly, Lox2 staining of the same cultures showed almost complete absence of Lox2 expression in the whole population of HaCa4-shLoxl2, in contrast with the homogeneous cytoplasmic staining of control HaCa4-shEGFP cells (Supplementary Fig. S2). On the other hand, HaCa4shSnai1 cells only express faint levels of E-cadherin transcripts (Fig. 1A, left), but no protein expression could be detected (Fig. 1A, right and B, middle), in agreement with our recent observations (10, 29). No significant changes in the expression levels and organization of β-catenin could be observed after Lox2 or Snai1 silencing in HaCa4 cells (Fig. 1A, right and B, bottom), indicating the specificity of E-cadherin re-expression in HaCa4shLoxl2 cells. The organization of β-catenin in these cell lines could likely be mediated by P-cadherin, as previously characterized in parental HaCa4 cells (42). To examine the phenotypic differences of HaCa4shLoxl2 versus HaCa4shSnai1 cells in more detail, gene expression analysis of both cell lines was performed (Fig. 1C; Table 1; Supplementary Table S1). A total of 220 genes displayed a >2-fold change in expression in either HaCa4shLoxl2 (L) or HaCa4shSnai1 (S) relative to control HaCa4shEGFP cells. Among them, 145 genes (~66%) were commonly regulated in both cell lines (36 up-regulated and 109 down-regulated; S/L columns, Fig. 1C). The remaining genes showed a different expression pattern depending of the cell type. Thus, the expression of 11 genes (5%; 1 up-regulated and 10 down-regulated) was specifically modified in HaCa4shSnai1 cells (S), whereas 57 genes (26%; 43 up-regulated and 14 down-regulated) were exclusively affected in HaCa4shLoxl2 cells (L). These results suggest that, although the majority of the genes (66%) are regulated...
by pathways involving both Snai1 and Loxl2, there are a significant number of genes (26%) modified by Loxl2 through Snai1-independent pathways. In addition, eight genes were differentially regulated in both cell lines (mixed color column, Fig. 1C). RT-PCR analyses validated the expression of selected genes (Fig. 1D). One notable finding was that besides E-cadherin, the expression of a great number of cell differentiation genes (16% of the total, 36 genes) is modified by either Loxl2 or Snai1 silencing (Table 1). Moreover, ~60% of them (n = 21) correspond to genes involved in epidermal differentiation. Remarkably, interference of Loxl2 has a much more severe effect than Snai1 silencing on the expression levels of differentiation genes (Table 1), particularly those associated with terminal epidermal differentiation and barrier function such as several members of the small proline-rich protein (Sprr) family (43). These results suggest a potential role for Loxl2 in the regulation of the epidermal differentiation program.

To further characterize the biological consequences of Loxl2 or Snai1 silencing, we analyzed the behavior of HaCa4-interfered cells in organotypic three-dimensional cultures (Fig. 2A). Under these growth conditions, control HaCa4-shEGFP and HaCa4-shSnai1 cells

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**Figure 2.** Comparative analysis of the three-dimensional organization and in vivo invasion properties of HaCa4 cells after stable silencing of Loxl2 or Snai1. A, organotypic three-dimensional cultures of HaCa4-shEGFP, HaCa4-shLoxl2, and HaCa4-shSnai1 after 1 week of culture. Histologic (H&E; left) and single or double immunofluorescence analysis for the markers indicated above each column (rest of panels) were performed; nuclei were stained with 4,6-diamidino-2-phenylindole (blue). B, HaCa4-shEGFP, HaCa4-shLoxl2, and HaCa4-shSnai1 were grown on collagen type I gel, transplanted onto the back of nude mice and allowed to grow for 1 week. Sections of each transplantation assay were analyzed by histology (H&E; left) and double immunofluorescence for the markers indicated above each column; nuclei were stained with 4,6-diamidino-2-phenylindole (blue). One representative example of each type of organotypic culture and transplantation assay is shown. Bars, 50 μm. K1, cytokeratin 1; K5, cytokeratin 5.
To better understand the potential implications of Loxl2 and Snai1 in tumors derived from the two-stage mouse skin carcinogenesis model, including benign papillomas (Pap) and SCC from well-differentiated (stages I and II) to poorly differentiated (stages III and IV) disease. The expression of Loxl2, Snai1, and the mesenchymal/extracellular marker SPARC (39) was investigated. Results indicated that Loxl2, Snai1, and SPARC are expressed in the most advanced tumor stages (Fig. 3), clearly suggesting a correlation between the expression of Loxl2 and Snai1 and tumor malignancy.

To investigate the role of Loxl2 and Snai1 in human tumors, we studied the expression of both proteins in human LSCC. A series of 256 LSCC with clinical follow-up for at least 10 years (Supplementary Table S2) was analyzed for Loxl2 and Snai1 expression by immunohistochemistry. The immunohistochemistry of Loxl2 in tumor tissue revealed two different patterns: a diffuse pattern with a faint Loxl2 stain and disperse cytoplasmic distribution (Fig. 4A, top left), and a heterogeneous pattern with an increased Loxl2 stain in the cytoplasm or perinuclear envelope of cells distributed in many tumor areas (Fig. 4A, top right, see arrows). Moreover, we detected Loxl2 at the tumor front in groups of cells apparently migrating in a collective manner (Fig. 4A, middle). In fact, in 86 out 256 tumors (33.6%), the increased heterogeneous expression pattern of Loxl2 was significantly correlated with moderately differentiated and poorly differentiated tumors (P = 0.016; Fig. 4B). In addition, a highly significant correlation between the heterogeneous pattern of Loxl2 expression and local recurrence of LSCC (P ≤ 0.001; Fig. 4B) was found. The relationship between Loxl2 expression and clinical survival was also investigated using a log-rank test which compares the survival of patients as a function of Loxl2 expression (diffuse versus heterogeneous pattern). A statistically significant difference was found between increased heterogeneous expression of Loxl2 and decreased overall survival (x² = 3.232; 1 df, P = 0.071; Fig. 5A, left) and disease-free survival (x² = 6.301; 1 df, P = 0.012; Fig. 5A, right). These results strongly support the hypothesis that Loxl2 can be considered as a new marker of poor prognosis in human LSCC. Additionally, to determine whether the analysis of Loxl2 expression could be applicable to other human tumors as a predictive marker, we examined the correlation between the Loxl2 mRNA expression level and overall survival in two independent gene expression data sets of 51 lung SCC (36) and
286 lymph node–negative (N0) breast adenocarcinomas (37) using the log-rank test. A statistically significant correlation was found between LOXL2 expression levels and decreased overall survival in lung SCC ($\chi^2$, 9.05; 1 df, $P = 0.003$; Fig. 5C) and lymph node–negative (N0) breast adenocarcinomas ($\chi^2$, 20.26; 1 df, $P \leq 0.001$; Fig. 5D).

Analysis of SNAI1 expression in the same cohort of LSCC tumors showed positive staining in $\sim$16% of tumors (40 of 251), but only 8 out 251 tumors (3.1%) exhibited high levels of SNAI1 (Supplementary Table S2), localized both in the nucleus and in the cytoplasm of tumor cells (Fig. 4A, bottom). Noteworthy, a significant correlation between SNAI1 expression and increased expression (heterogeneous pattern) of LOXL2 expression was observed ($P = 0.002$, Fig. 4B). In addition, the heterogeneous pattern of LOXL2 was observed in 75% of the tumors showing high levels of SNAI1 (Fig. 4B). Interestingly, SNAI1 immunostaining was markedly stronger at the invasion front in apparently actively migrating cells that, in addition, exhibited strong LOXL2 expression (Fig. 4A, bottom and middle, respectively), as previously reported in cervical SCC and colon carcinomas (41). Importantly, SNAI1 expression was also correlated with the local recurrence of LSCC ($P = 0.030$; Fig. 4B), supporting previous findings in breast carcinoma (46). However, SNAI1 expression was not significantly associated with either overall or disease-free survival in the present series of LSCC (Fig. 5B). Analysis of LOXL2 in the data set of lung SCC and lymph node–negative (N0) breast adenocarcinomas did not find any association between SNAI1 mRNA expression and clinical survival (data not shown). Nevertheless, we analyzed the expression of LOXL2 and SNAI1 proteins by Western blot in a small subset of tumors representing distinct differentiation grades of lung SCC and lymph node–negative (N0) breast adenocarcinomas. Results indicated that both SNAI1 and LOXL2 proteins are indeed increased in moderately or poorly differentiated tumors (Supplementary Fig. S3). These preliminary data, together with the analysis of SNAI1 in mouse skin and LSCC tumors, strongly support a model in which SNAI1 is stabilized at the protein level in tumor progression by the action of LOXL2, as we have previously shown in vitro (10). Altogether, these observations point to the existence of in vivo functional differences between SNAI1 and LOXL2 in relation to tumor progression. Although both molecules cooperate in invasion and local recurrence, LOXL2 plays additional, SNAI1-independent roles in other malignant events that contribute to poor clinical outcome.

**Discussion**

SNAI1 is an important player of malignancy by mediating key cellular events for tumor progression, like EMT, invasion, and cell survival (5, 6, 9). The mechanisms regulating SNAI1 expression and function have only recently been investigated; in particular, posttranslational modifications affecting SNAI1 protein stability and/or nuclear translocation have attracted much attention (reviewed in ref. 6). Among them, we recently reported that LOXL2, a member of the LOX family, interacts with and stabilizes...
Snai1 by a mechanism involving two Snai1 Lys residues and counteracting the action of GSK3β (10, 11). Increased LOXL2 or SNAI1 expression is correlated with the malignant phenotype in cells from mouse squamous and spindle cell carcinomas as well as several human carcinoma cells (7, 10, 29, 47). Moreover, the in vitro invasiveness and tumorigenic potential was dramatically blocked in malignant mouse HaCa4 keratinocytes interfered for Loxl2 or Snai1 (10, 29). Nevertheless, those studies do not discriminate between specific or redundant functions of SNAI1 and LOXL2 in tumor progression. To better understand the participation of both molecules, we have performed in-depth in vitro and in vivo studies of HaCa4 cells with silenced expression of Snai1 or Loxl2. Comparative gene expression analysis of those HaCa4-derived cells showed a cluster of genes that were commonly regulated by Loxl2 and Snai1, but a set of genes was specifically regulated in the Loxl2 knock-down cells. Interestingly, these genes were associated

Figure 5. Kaplan-Meier analysis of LOXL2 and SNAI1 expression in LSCC, lung SCC, and lymph node–negative (N0) breast adenocarcinomas. A, Kaplan-Meier plots in the cohort of 256 human LSCC showing that patients with positive (heterogeneous) LOXL2 stain (red) had statistically significant decreased overall survival (left) and disease-free survival (right) compared with patients with negative (diffuse) LOXL2 stain (blue). B, Kaplan-Meier plots of SNAI1 expression in human LSCC comparing negative (blue) and positive (red) SNAI1 stain with overall survival (left) and disease-free survival (right). C and D, the significant correlation of LOXL2 expression and overall survival is shown in two independent microarray data sets of 51 lung SCC (ref. 36; C) and 286 lymph node–negative (N0) breast adenocarcinomas (ref. 37; D); positive (red) and negative (blue) expression values of LOXL2 mRNA were obtained from average of expression ratio. P values were derived from log-rank tests.
with terminal epidermal differentiation and barrier function (43, 48), indicating a potential role for Loxl2 in the regulation of epidermal differentiation program. This was further evaluated by analyzing the behavior and homeostasis of the HaCa4-silenced cells in organotypic cultures. As expected, both Loxl2- and Snai1-silenced cells exhibited strongly reduced invasion capacity in organotypic cultures and in in vivo transplantation assays. Remarkably, only Loxl2-silenced cells showed expression and organization of both basal/suprabasal epithelial and differentiation markers in a pattern strikingly resembling the mouse skin structure in vitro and in vivo. These results suggest that Loxl2 influences the Snai1-dependent invasion properties and, perhaps, the Snai1-prosurvival action, but, in addition, Loxl2 has specific functions in the negative control of the epidermal differentiation program, apparently through the direct or indirect regulation of Snai1-independent target genes.

We also studied the expression of Loxl2 and Snai1 in tumor samples derived from mice skin carcinogenesis. This analysis showed a strong correlation between the expression of both proteins and tumor malignancy, and leads us to investigate the expression pattern of LOXL2 and Snai1 in human tumors. We selected a cohort of LSCC from 256 patients with clinical follow-up for at least 10 years and studied the distribution of LOXL2 and Snai1 by immunohistochemistry. As mentioned in the Introduction, at present, no reliable molecular markers for the prognosis of that specific type of SCC are available. Our results indicate that LOXL2 expression in LSCC is significantly associated with local recurrence and with decreased clinical and overall survival (see Figs. 4B and 5A), strongly supporting that LOXL2 can be considered as a new marker of poor prognosis in human LSCC. This remarkable outcome is supported by additional recent observations. First, gene profiling analysis of HNSCC showed that LOXL2 is expressed in a subset of tumors associated with a mesenchymal cell signature (26), and LOXL2 is considered as one of the 25-gene signatures for oral SCC (27). Second, the analysis of LOXL2 protein levels in colon carcinomas and esophageal tumors showed that LOXL2 protein is increased in the less-differentiated tumors (25). Third, when the LOXL2 mRNA expression level was used as a variable to predict disease-free survival in a data set of lung SCC (36) and lymph node–negative (N0) breast adenocarcinomas (37), a significant correlation between LOXL2 expression and a poor prognosis was detected (see Fig. 5C and D).

Regarding Snai1 expression, in the cohort of LSCC tumors, we observed a significant correlation between high levels of Snai1 expression with local recurrence and the heterogeneous pattern of LOXL2 distribution; however, we did not find any association of Snai1 expression with other clinicopathologic variables in either the LSCC series (see Fig. 5B) or in the data set of lung SCC and lymph node–negative (N0) breast adenocarcinomas analyzed at the mRNA level. Interestingly, the Snai1 immunostaining in LSCC was markedly stronger at the invasion front in apparently actively migrating cells, which is in agreement with previously reported results in other types of SCC (41). Moreover, preliminary data analyzing the expression of Snai1 and LOXL2 proteins in a small subset of lung SCC and lymph node–negative (N0) breast adenocarcinomas indicated that the expression of both Snai1 and LOXL2 increases in moderate and poorly differentiated tumors (Supplementary Fig. S3). These new data shed light on the potential stabilization of Snai1 by LOXL2 in tumor progression which deserves future analysis.

Altogether, these results confirm that LOXL2, but not Snai1 expression, predicts poor prognosis in LSCC but in addition they uncover that LOXL2 must function beyond, or apart from, Snai1 to promote tumor malignancy. This assumption is based on the observation that although the expression of both proteins correlates with local recurrence, only LOXL2 expression associates with poor prognosis. The specific participation of LOXL2 in regulating the reported prosurvival action of Snai1 (49), and thus, potentially local recurrence, is an important aspect that remains to be established. In addition, the elucidation of the Snai1-independent mechanisms through which LOXL2 influences tumor malignancy will be the subject of future studies. In particular, it should be relevant to analyze whether LOXL2 affects the differentiation program of other epithelial tissues, as shown here for epidermis. Whatever the precise molecular mechanisms underlying LOXL2 function, our present data show that LOXL2 can be considered as a new poor prognosis marker in human LSCC. Given the paucity of reliable molecular markers for this highly prevalent laryngeal malignancy, the identification of LOXL2 as a prognostic marker provided here can contribute to improve the clinical treatment of LSCC and point to its potential as a new therapeutic target in LSCC and, perhaps, in other human carcinomas.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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