

# The SNAIL family member SCRATCH1 is not expressed in human tumors

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**Abstract.** The SNAIL and SLUG transcription factors play important roles in embryogenesis owing to their anti-apoptotic properties and their ability to promote morphogenetic changes by inducing epithelial-mesenchymal transitions (EMT). These characteristics provide many of the proteins in these families with oncogenic and pro-metastatic capabilities when reactivated in cancers. The SCRATCH subgroup of the SNAIL superfamily, including SCRATCH1 and SCRATCH2, display distinct embryonic functions and diverge early in evolution. Despite the described overexpression of *SCRT1* (encoding for SCRATCH1) in a small subset of human lung cancers, there is little data supporting a role of SCRATCH proteins in tumorigenesis. To further explore this possibility, we assessed *SNAI1* (SNAIL), *SNAI2* (SLUG) and *SCRT1* (SCRATCH1) expression in a wide panel of human and murine tumors encompassing 151 primary tumors and 6 different cancer types, including melanomas and multiple different carcinomas. Whereas *SNAI1* and *SNAI2* are widely expressed in human and murine tumors, our results reveal that *SCRT1* transcripts are undetectable in nearly all of the examined tumors suggesting that SCRATCH1 plays a minor role, if any, in tumorigenesis. Our data therefore suggest that oncogenic properties are not shared by all SNAIL superfamily members but instead are specifically allotted to the SNAIL subgroup supporting the conclusions that SNAIL and SCRATCH subgroups are functionally divergent and strengthening the hypothesis that the oncogenic potential of SNAIL and SLUG proteins relies on the hijacking of their embryonic functions.

## Introduction

The epithelial-mesenchymal transition (EMT) is a trans-differentiation process that switches a polarized epithelial

phenotype into a highly motile fibroblastoid or mesenchymal phenotype. Required for morphogenetic movements during the embryonic development, EMT is also suggested to play an important role in promoting cancer cell dissemination (1,2). Additionally, we and others have shown that EMT was associated with the acquisition of stem-like properties (3,4) and with the inhibition of inherent primary failsafe programs (5), suggesting a major role during primary tumor growth. In light of its emerging role in tumor progression, signaling pathways regulating EMT induction have been deeply investigated, highlighting a prevalent role of various developmental gene regulators including the SNAIL transcription factor superfamily (6,7).

SNAIL superfamily members share a highly conserved carboxy-terminal DNA binding domain encompassing four to six zinc fingers, as well as an N-terminal repression domain, known as the SNAG domain (6). Through these two structures, SNAIL proteins specifically bind to E-box responsive elements and by recruiting the corepressor CtBP inhibit their target genes (8,9). The SNAIL superfamily encompasses five members, organized into two evolutionary and functionally distinct subgroups, namely the SNAIL subgroup, encompassing the SNAIL (*SNAI1*), SLUG (*SNAI2*) and SMUC (*SNAI3*) proteins, and the SCRATCH subgroup, encompassing the two SCRATCH1 (*SCRT1*) and SCRATCH2 (*SCRT2*) proteins (10,11). SNAIL and SLUG, the two most studied members of the family, are essential for neural crest and mesoderm formation in mouse and chick embryos, respectively (10-13). Downstream the CSF/c-kit signaling pathway, SLUG was also found to be a determinant for spermatogenesis, melanocyte development and hematopoietic progenitor cell survival (7). Down-regulation of *SNAI2* expression is thus associated with various pigmentation disorders such as the Waardenburg syndrome and piebaldism, as well as human congenital anemia (14). Inversely, *SNAI2* overexpression has been described in a large spectrum of human cancers including leukemia, esophageal carcinomas and mesotheliomas (14), wherein the *SNAI2* encompassing region is frequently amplified (14). *SNAI2* is expressed in human breast tumors (14), but normal mammary epithelial cells also display high levels of *SNAI2* (15), therefore rendering the role of SLUG in breast tumorigenesis less clear. *SNAI1* is also overexpressed in a wide panel of cancers including breast, colon and gastric cancers, hepatocellular carcinomas, melanomas and synovial sarcomas within which

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Table I. Sequences of primers and probes.

	NCBI RefSeq	Primer sequence	Universal Probe (Taqman) Library
<i>ACTB</i>	NM_001101.2	Forward: attggcaatgagcgggtc Reverse: ggatgccacaggactccat	#11
<i>ATP6AP1</i>	NM_001183.4	Forward: tgcagctctctacacttagatcc Reverse: ctgtgaaatcctcaatgctcag	#1
<i>CFL1</i>	NM_005507.2	Forward: gtgccctctccttttcgtt Reverse: ttgaacacctgatgacacat	#5
<i>GAPDH</i>	NM_002046.3	Forward: agccacatcgctcagacac Reverse: gcccaatacgaccaaacc	#60
<i>GUSB</i>	NM_000181.2	Forward: cgcctcgctatctgtattc Reverse: tccccacaggagtggtgtag	#57
<i>HPRT1</i>	NM_000194.1	Forward: tgaccttgattattttgcatacc Reverse: cgagcaagacgttcagtcct	#73
<i>PGK1</i>	NM_000291.2	Forward: ctgtggcttctggcatacct Reverse: cttgctgcttcaggacca	#42
<i>PPIB</i>	NM_000942.4	Forward: acttcaccaggggagatgg Reverse: agccgttggtgtctttgc	#20
<i>TUBA1C</i>	NM_032704.2	Forward: cccctcaagtcttagtcatgc Reverse: cattgccaatctggacacc	#58
<i>UBB</i>	NM_018955.2	Forward: aggatcctggatccgctaac Reverse: tcacattttcgatggtgtcact	#39
<i>SNAIL</i>	NM_005985	Forward: gctgcaggactctaaccaga Reverse: atctccggagggtgggatg	#11
<i>SNAIL2</i>	NM_003068	Forward: tggttgctcaaggacacat Reverse: gttgcagtgagggaagaa	#7
<i>SCRT1</i>	NM_031309.4	Forward: tcaaacttgacgcgttctcttcgg Reverse: agtcgctgaggtaccctttatcgt	None

it is invariably associated with dedifferentiation and invasiveness (16-23). Additionally, SNAIL was shown to be required for tumor growth and metastatic properties of human breast and mouse skin carcinoma cell lines (24,25) and associated with recurrence of breast tumors (26). Reactivation of each of *SNAIL* or *SNAIL2* genes has been associated with the acquisition of invasive properties due to their ability to induce an EMT (7,27). Interestingly, SNAIL stabilization by TNF- $\alpha$  through NF- $\kappa$ B activity was recently shown as a primary mechanism by which tumor-associated macrophages (TAMs) might promote tumor cell motility (28). Their anti-apoptotic properties might also contribute to their oncogenic potential. Indeed, SNAIL expression in MDCK epithelial cells induces an EMT associated with resistance to serum-deprivation or TNF- $\alpha$ -induced apoptosis (27). SLUG was also shown in hematopoietic precursor cells to function downstream p53 as a critical switch that prevents their apoptosis by antagonizing the transactivation of *PUMA* gene (*BBC3*) by p53 (29).

Despite their sequence homology (around 60% identity with SLUG and SNAIL C-terminal zinc-fingers), SCRATCH proteins functionally diverge from SNAIL and SLUG. In *Drosophila*, *Scrt* is exclusively expressed in dividing neuronal precursors and persists in post-mitotic neurons (30). Similarly, in the mouse, *Scrt1* is only expressed in neural tissues and in the adult nervous system (31). Expression of *Scrt2* during mouse development is even more restricted to cells that appear to be migrating radially to the neocortex and the hippocampus and in the cortical subventricular zone that gives rise to glial cells. Its expression progressively decreases, to be virtually undetectable in the adult brain (32). In light of these divergences and knowing that the SCRATCH proteins constitute an evolutionary distinct subgroup of the SNAIL superfamily (10,11), we sought to investigate whether tumor promoting properties are shared by all SNAIL superfamily members or rather restricted to the SNAIL subgroup. The detection of *SCRT1* transcripts in a small subset of lung

**SPANDIDOS** (31) and the anti-apoptotic properties of the **SCRATCH** ortholog Ces-1 (33) argue in favor of a role of **SCRATCH** proteins in tumorigenesis. To further explore this hypothesis, we assessed the expression of genes from each subgroup, namely *SCRT1*, *SNAI1* and *SNAI2* in a wide panel of human and murine primary tumors. Our results first confirmed the reactivation of *SNAI1* and *SNAI2* in multiple human cancers. In contrast, with the exception of a handful of breast tumors, we failed to detect *SCRT1* transcripts in any of the carcinomas and melanomas examined therefore excluding a preponderant role in human cancers. Further analyses of murine mammary tumors from the MMTV-*ERBB2/Neu* mouse model strengthened the conclusion that **SCRATCH1** is unlikely to play a role in tumorigenesis. Overall, these results suggest that oncogenic properties are specifically allotted to the **SNAIL** subgroup of the **SNAIL** superfamily.

## Materials and methods

**mRNA extraction and reverse transcription.** Total mRNA was extracted using TriReagent® (Sigma) and Phase Lock Gel® (Eppendorf) according to the instructions of the manufacturer. mRNA (1 µg) was reverse transcribed using the First-strand cDNA Synthesis® (Amersham Bioscience/GE Healthcare).

**Gene expression analysis in human primary tumors.** Taqman quantitative PCR analysis was carried out on a LightCycler® 2.0 System (Roche Applied Science) at least in triplicates. Housekeeping genes (*ACTB*, *ATP6AP1*, *CFL1*, *GAPDH*, *GUSB*, *HPRT1*, *PGK1*, *PIIB*, *TBP*, *TFRC*, *TUBA1C* and *UBB*) were used for normalization of target mRNA expression in each sample type. They were selected by systematic geNorm analysis as previously described (34). Real-time specific intron-spanning PCR assays were designed using the ProbeFinder software (Roche Applied Science). Transcription was compared with normal tissues provided by the Biologic Resource Center of the Centre Léon Bérard and/or commercial RNA (Clontech). An average between independent sources was used as a reference (indicated as Norm-Mean) in lung, kidney, colon and ESCC tumors. In breast tumors and melanomas, expression was normalized using human mammary epithelial cells (indicated as Norm) and three independent sources of normal human melanocytes (Melanocyte-mean) respectively as references.

*SCRT1* transcription was assessed by SYBR green quantitative PCR. Expression was compared with an average between independent sources in kidney and colon cancers. No *SCRT1* expression was detected in ESCC, lung tumors and melanomas as well as their normal counterparts. As no *SCRT1* transcript was expressed in normal mammary epithelial cells, relative expression of *SCRT1* in breast tumors is displayed in arbitrary units.

Sequences of primers and probes are listed in Table I.

**Gene expression analysis in MMTV-ErbB2/Neu transgenic mice-derived tumors.** SYBR green quantitative PCR was performed using *Rplp0* (encoding for 36B4) as an internal control gene. Relative expression is displayed in arbitrary

Table II. Sequences of specific murine primer pairs.

	NCBI RefSeq	Primer sequence
<i>RplpO</i>	NM_007475	Forward: gctgatgggcaagaacacca Reverse: ccggatgtgaggcagcagtt
<i>Snai1</i>	NM_011427	Forward: caccctcatctgggactctc Reverse: gctttggccactgtcctcat
<i>Snai2</i>	NM_011415	Forward: cagctccactccactctct Reverse: tgaaccactgtgatccttgg
<i>Scrt1</i>	NM_130893	Forward: agacctcgacagctcctacg Reverse: cccacgtagtcactgaggtga

units. Sequences of specific murine primer pairs are listed in Table II.

## Results

In order to evaluate the role of the **SNAIL** family transcription factor **SCRATCH1** in tumorigenesis, *SNAI1*, *SNAI2* and *SCRT1* expression was assessed by qRT-PCR in 132 human primary tumors encompassing 6 different cancer types and 19 murine primary mammary tumors. Accurate expression analysis was performed using the Taqman or SYBRgreen technology and a combination of 12 housekeeping genes selected by systematic geNorm analysis (34).

*SNAI1* and *SNAI2* are widely expressed in human primary tumors. Confirming previous work (35), *SNAI1* was found to be overexpressed (at least twice the expression of the corresponding normal tissue, dashed line) with high frequencies in breast tumors (overexpression: 97%, 0- to 72-fold, n=78; Fig. 1a) regardless of the subtype examined (*in situ* carcinoma, invasive ductal carcinomas or invasive lobular carcinomas), strengthening the hypothesis of its crucial role in breast tumorigenesis. *SNAI2* was not overexpressed in breast cancers compared to normal human mammary epithelial cells (overexpression: 0%, 0.1- to 1.8-fold, n=78; Fig. 1a). However, analysis of relative amount of *SNAI2* mRNA confirmed that both normal human mammary epithelial cells and breast tumors in fact display high *SNAI2* expression (Fig. 1a, inset graph) (15). In accordance with previous work, expression of *SNAI1* and *SNAI2* was increased in a significant proportion of esophageal squamous cell carcinomas (overexpression: 73%, 0.8- to 4.2-fold for *SNAI1*, and 80%, 0.9- to 5.4-fold for *SNAI2*, n=15, respectively; Fig. 2a) (36) and *SNAI1* was found to be overexpressed in melanomas (overexpression: 60%, 0- to 212-fold, n=10; Fig. 2c, left graph) (23,36). *SNAI2* transcripts are detectable at a similar high rate in both melanocytes and melanomas (Fig. 2c, right graph), wherein it was suggested to genetically program melanomas to metastasize (37). We additionally confirmed *SNAI1* and revealed the previously undescribed *SNAI2* overexpression in colon (overexpression: 44%, 0.5- to 8.2-fold for *SNAI1*, and 33%, 0.4- to 28.9-fold for *SNAI2*, n=9; Fig. 2d) and kidney cancers

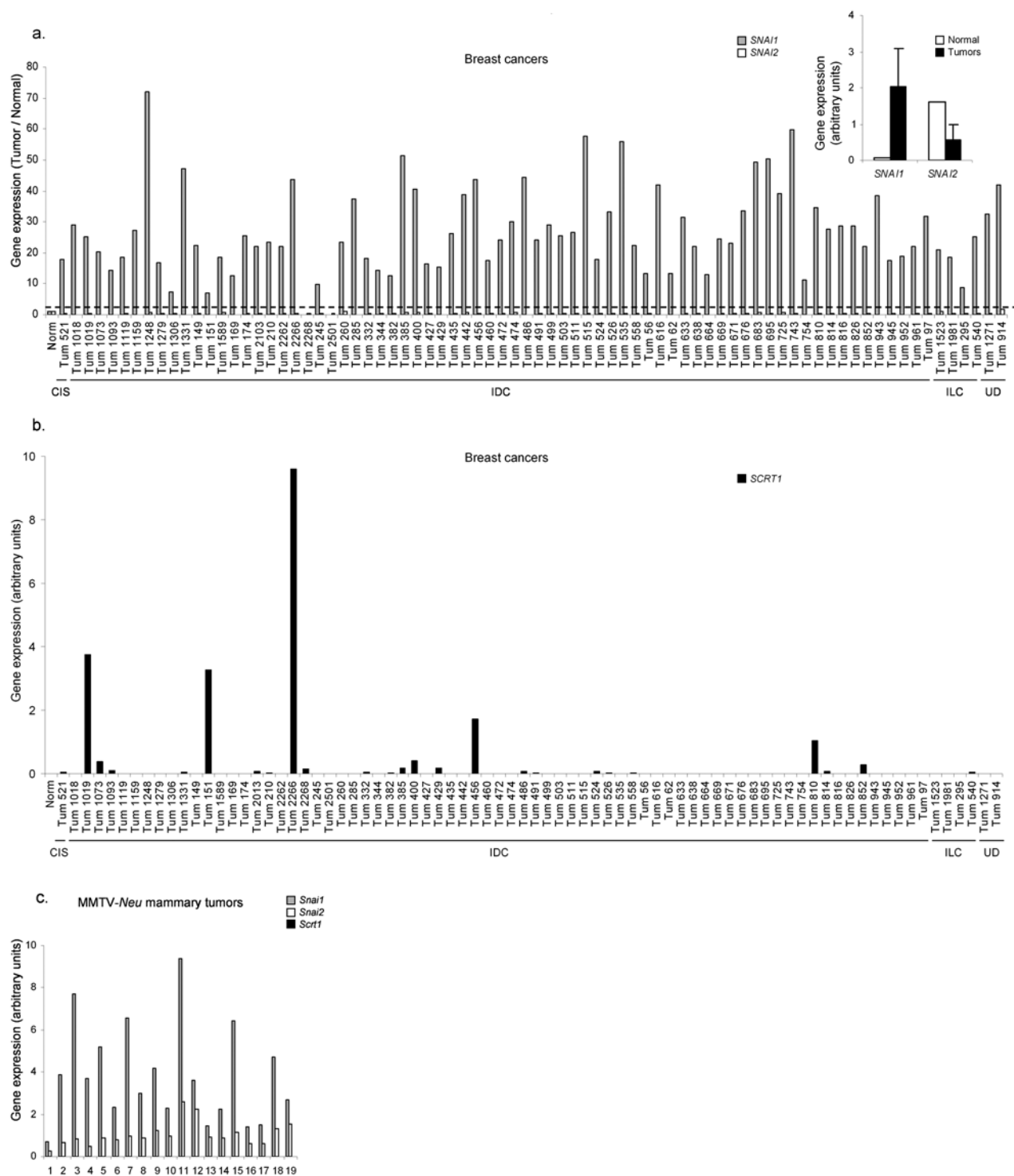


Figure 1. Expression analysis of SNAIL family genes in human and murine breast tumors. (a) *SNAI1* and *SNAI2* transcription was assessed by qRT-PCR in human breast tumors and normalized using normal human mammary epithelial cells. Relative amounts of transcripts are shown. CIS, carcinoma *in situ*; IDC, invasive ductal carcinomas; ILC, invasive lobular carcinomas; UD, undefined. Inset graph displays relative amounts of *SNAI1* and *SNAI2* mRNA to the normalizer genes in human mammary epithelial cells (Normal) and breast tumors (Tumors). (b) *SCRT1* transcription was assessed by qRT-PCR in human breast tumors and human mammary epithelial cells. Relative amounts of *SCRT1* mRNA to the normalizer genes are shown. CIS, carcinoma *in situ*; IDC, invasive ductal carcinomas; ILC, invasive lobular carcinomas; UD, undefined. Of note, as *SCRT1* could not be detected in normal epithelial cells, expression is thus displayed in arbitrary units. (c) Murine *Snai1*, *Snai2* and *Scrt1* transcription was assessed by qRT-PCR in MMTV-*ERBB2/Neu* transgenic mice-derived mammary tumors. Relative amounts of mRNA to the normalizer gene are shown.

(overexpression: 62%, 0.3- to 19.9-fold for *SNAI1*, and 38%, 0.1- to 41.6-fold, for *SNAI2*, n=13; Fig. 2e). Finally, both genes were found transcriptionally inactive in lung tumors (Fig. 2b).

*SCRT1* is not expressed in human carcinomas and melanomas. *SCRT1* mRNA was undetectable in ESCC, lung carcinomas, melanomas as well as in the corresponding normal tissues of origin (Fig. 2a-c) and was barely detectable in colon and



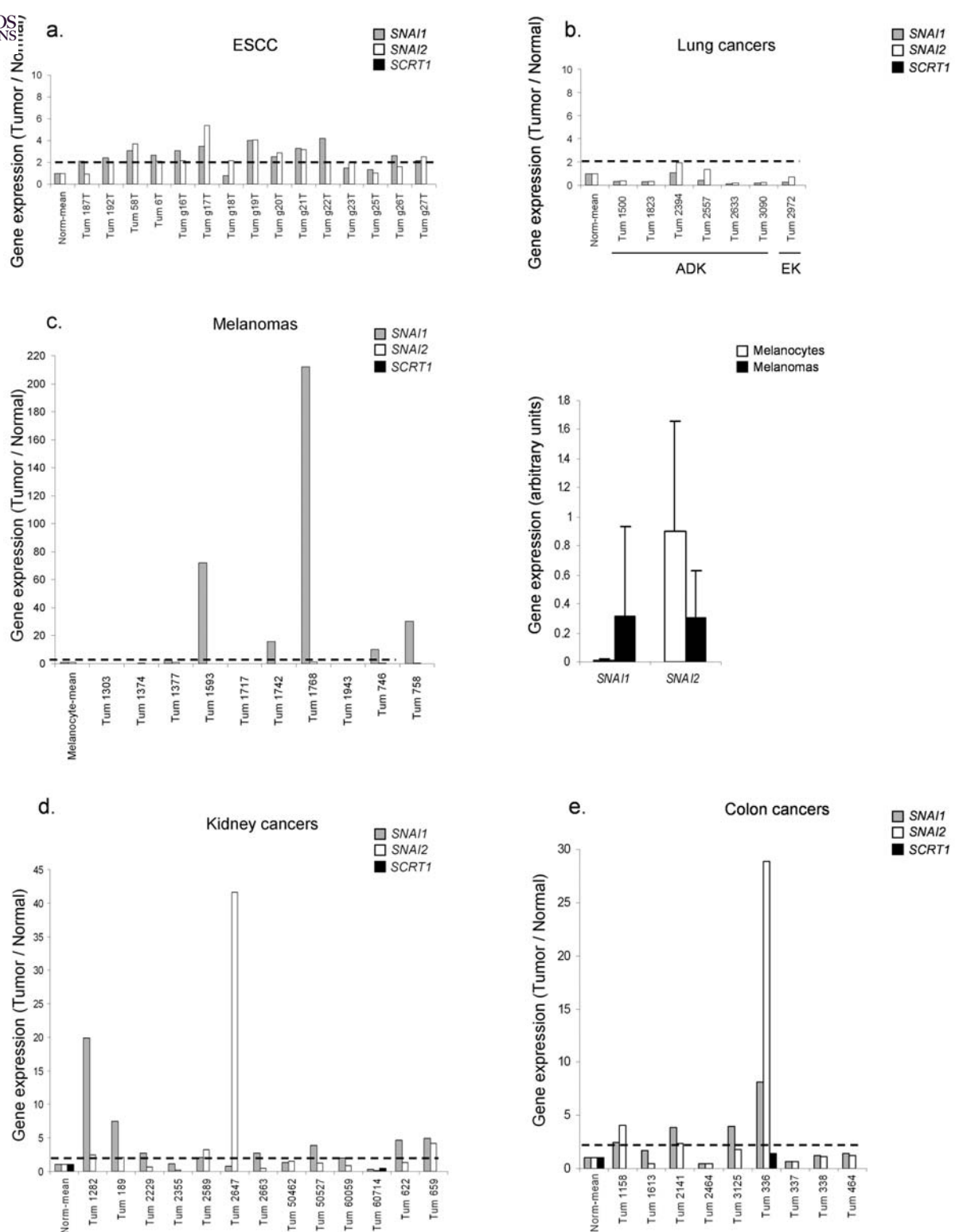


Figure 2. Expression analysis of Snail family genes in human tumors. (a-f) *SNAI1*, *SNAI2* and *SCRT1* transcription in (a) esophageal squamous cell carcinoma (ESCC), (b) lung cancers; ADK, adenocarcinomas; EK, epidermoid carcinomas, (c) melanomas, (d) kidney cancers and (e) colon cancers was assessed by qRT-PCR and normalized with that of healthy tissues or normal cell counterparts (Norm). Of note, *SCRT1* could not be amplified in normal esophageal squamous epithelium, normal lung and melanocytes and the corresponding tumors. Inset graph in (c) (right) displays relative amounts of *SNAI1* and *SNAI2* mRNA to the normalizer genes in melanocytes and melanomas.

kidney cancer specimens (Fig. 2d and e). In breast cancers, *SCRT1* mRNA was mainly undetectable although *SCRT1* mRNA could be amplified in 5 out of 78 tumor samples (Fig. 1b). Because of the limited and overall low expression

of *SCRT1* in human breast tumors, we therefore speculated that SCRATCH1 must have a minor role, if any, in breast carcinogenesis. To substantiate our conclusion, we next assessed *Snai1*, *Snai2* and *Scrt1* expression in 19 freshly

isolated murine mammary tumors derived from an MMTV-*ERBB2/Neu* mouse transgenic model (expressing the *ERBB2/Neu* proto-oncogene under the control of the MMTV promoter) (38). In accordance with our results in human breast tumors, *Snai1* and *Snai2* transcripts were found to be expressed in MMTV-*ERBB2/Neu* mouse derived tumors (Fig. 1c). However, no expression of *Scrt1* could be detected in these tumors, again supporting the idea that SCRATCH1 does not play a role in breast carcinogenesis.

## Discussion

The SNAIL and SLUG transcription factors are believed to promote cancer progression through their ability to promote EMT and to protect cells from apoptosis, properties reminiscent to their embryonic functions (6). Despite their evolutionary and functional divergence (10,11), several observations supported the hypothesis that oncogenic potential could also be allotted to the SCRATCH subgroup of the family. Indeed, *SCRT1* transcripts were detected in a small subset of lung carcinomas (31). Additionally, *C. elegans* SCRATCH ortholog Ces-1, likewise SNAIL and SLUG, was shown to display anti-apoptotic properties (33). To further explore this hypothesis, we investigated the expression of the Snail family related gene *SCRT1*, *SNAI1* and *SNAI2* in a wide panel of human tumors as well as in murine mammary tumors. Our results clearly established that, whereas *SNAI1* and *SNAI2* are widely overexpressed in many human cancers, *SCRT1* transcripts remained undetectable in most human cancers examined. Of note, we failed to detect *SCRT1* transcription in a set of lung adenocarcinomas and epidermoid carcinomas (Fig. 2a), strengthening the exclusivity of its previously described expression in small cell lung carcinomas with neuroendocrine (NE) features (31). As *SCRT1* is implicated in the maintenance of NE features, one can however assume that its expression in small cell lung carcinomas with NE features reflects the NE phenotype of these cells rather than a role in lung carcinogenesis.

It is noteworthy that *SCRT1* expression was detectable in a small subset of breast human cancers (5 out of 78; Fig. 1b) and in a limited number of human breast tumor cell lines (2 out of 15; data not shown). These data suggest that SCRATCH1 is unlikely to play a role in breast tumorigenesis. The rare expression observed in some breast tumors could rather reflect the presence of NE cells where SCRATCH1 is known to be functional (31); a hypothesis requiring further confirmation. However, using the Oncomine multiple array comparison software ([www.oncomine.org](http://www.oncomine.org)), we found that *SCRT1* mRNA is not enriched in tumors with NE features and does not take part of the neuroendocrine signature.

The lack of detectable *SCRT1* expression in the large panel of human tumors examined is likely to exclude a role of SCRATCH1 in carcinogenesis and melanomagenesis. As phylogenetic and embryonic expression analysis demonstrated that SCRATCH (SCRATCH1 and SCRATCH2) and SNAIL (SNAIL, SLUG and SMUC) proteins constitute evolutionary and functionally distinct subgroups (10), one could presume that only the hijacking of SNAIL subgroup functions may provide cancer cells with a selective advantage. However, as a neuronal specific network might be essential

for reactivating *SCRT1* expression, a specific role in promoting neuronal-derived tumorigenesis could not be excluded. Further investigations, including domain-swap chimera experiments, are warranted to definitively clarify the point.

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