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# Role of the epithelial-mesenchymal transition regulator Slug in primary human cancers

**Catarina Castro Alves[1], Fatima Carneiro[1], Heinz Hoefler[2,3], Karl-Friedrich Becker[2]**

*[1]Instituto de Patologia e Imunologia Molecular da Universidade do Porto (IPATIMUP) Rua Dr. Roberto Frias, S/N 4200-465 Porto, Portugal, [2]Institute of Pathology, Technical University of Munich, Trogerstrasse 18, D-81765 Munich, Germany, [3]Helmholtz Zentrum Muenchen - Deutsches Forschungszentrum für Gesundheit und Umwelt (GmbH), Ingolstaedter Landstraße 1, D-85764 Neuherberg, Germany*

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## 1. ABSTRACT

Epithelial-mesenchymal-transition (EMT) is a crucial process during morphogenesis of multi-cellular organisms. EMT not only is a normal developmental process but also plays a role in tumor invasion and metastasis. Indeed, molecules involved in EMT, such as the transcription factor and E-cadherin repressor Slug (SNAI2), have recently been demonstrated to be important for cancer cells to down-regulate epithelial markers and up-regulate mesenchymal markers in order to become motile and invasive. Here we summarize major studies focusing on Slug expression in human tumor samples. We review a total of 13 studies involving 1150 cases from 9 different types of tumors. It is becoming clear that this transcription factor plays a role in the progression of some tumor types, including breast and gastric cancer. Interestingly, Slug expression is not always associated with down-regulation of E-cadherin. The mode of action, the signaling pathways involved in its regulation, and the interplay with other EMT regulators need to be addressed in future studies in order to fully understand Slug's role in tumor progression.

## 2. INTRODUCTION

Epithelial-mesenchymal transition (EMT) is a fundamental process for morphogenesis in multicellular organisms. Mesenchymal cells are formed following the loss of epithelial cell polarity due to the disappearance of cell junctions, the reorganization of the cytoskeleton and the redistribution of the organelles (1). This change in phenotype is associated with an increase in motility and migration capabilities. For this reason, it has been proposed that similar EMT-like processes occur during tumor progression and invasion, in which cancer cells detach and migrate to organs away from the primary tumor, being these mechanisms responsible for conferring invasive and metastatic properties to cancer cells (2).

EMT can be governed by a variety of molecules, such as TGFβ and RTK/Ras signaling, autocrine factors and Wnt-, Notch-, Hedgehog-, and NF-κB-dependent pathways, that also play major roles during tumor invasion and metastasis (Reviewed in Ref. 3 and 4). One of the key features of EMT is the down-regulation of the expression

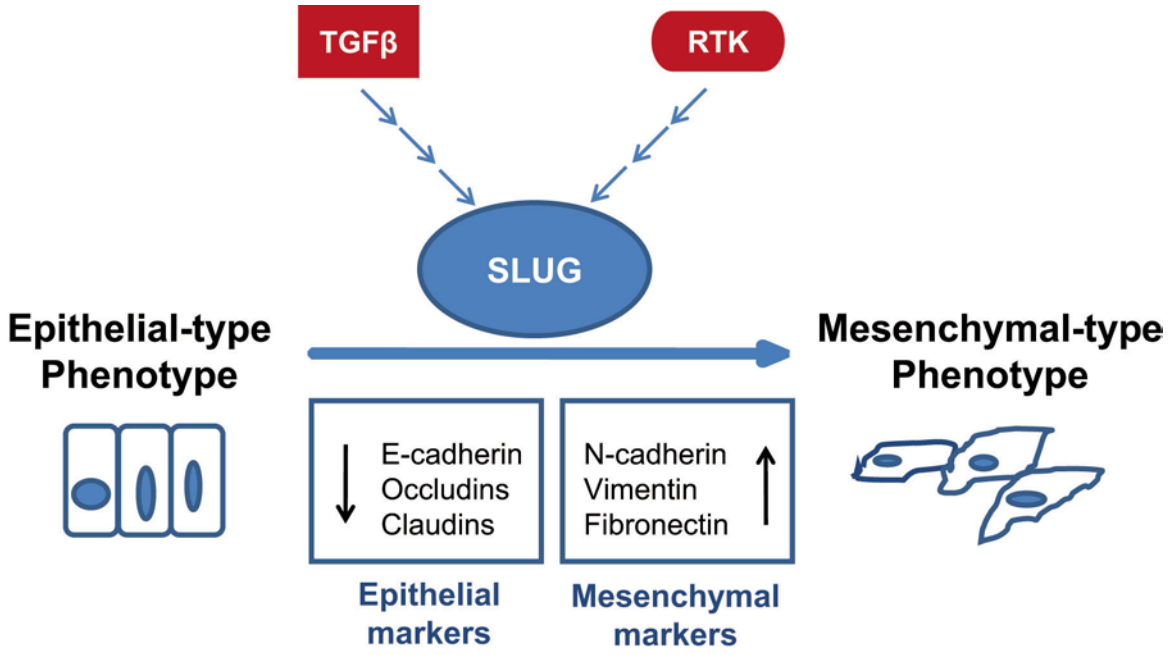


**Figure 1.** Slug's role in epithelial-mesenchymal transition. The diagram shows general signaling pathways regulating Slug and some of the molecules known to be affected by Slug activity. (TGFß, Transforming growth factor beta; RTK, Receptor tyrosine kinase).

of the cell adhesion molecule E-cadherin, a critical event in tumor invasion; E-cadherin expression is frequently decreased in carcinomas, and it can be inactivated by several mechanisms, like gene mutations, silencing by hypermethylation and transcriptional repression. *In vitro* studies have shown that several transcription factors can down-regulate E-cadherin gene expression in epithelial cells, inducing sometimes full EMT, leading to the expression of mesenchymal type proteins such as vimentin, N-cadherin and fibronectin, and down-regulation of other epithelial marker proteins such as tight junction proteins, desmosomes and cytokeratins (Figure 1). Among these transcription factors are Slug (5, 6), Snail (7), SIP1 (8) and others (9, 10, 11). Slug (SNAI2), a member of the Snail superfamily, was first identified in the neural crest and developing mesoderm of the chick embryos (12). Slug expression has been analyzed in various types of human cancers, and here we review and discuss the data obtained in these studies.

## 3. SLUG AND CANCER

### 3.1. Slug expression in various types of tumors

Up to date there are not many published studies reporting Slug expression in different types of cancers, and these are all very recent. Here we review and discuss these studies, as well as some *in vitro* data reporting findings that support the hypothesis of Slug having a role in tumor progression and invasion. The different tumor types analyzed were breast (3 studies) (13, 14, 15), esophageal squamous cell (2 studies) (16, 17), gastric carcinoma (1 study) (18), colorectal (1 study) (19), hepatocellular (1 study) (20), pancreatic cancer (1 study) (21), lung (1 study) (22), malignant mesothelioma (1 study) (23) and ovarian (2 studies) (13, 24). A total of 1150 cases of 9 different types of tumors were evaluated in the 13 studies here reviewed (Table 1). The main methods used to analyze Slug expression were RT-PCR, real-time RT-PCR and immunohistochemistry with commercially available anti-Slug polyclonal antibodies; *in situ* hybridization was also used to detect mRNA expression. The results obtained were quite heterogeneous, but so were the types of tissues analyzed and techniques, which together with the fact that the role of Slug and other EMT transcription factors in cancer is not yet fully understood, makes it difficult to compare and take conclusions out of the data here discussed. Nevertheless, we tried to summarize the major findings reported up to date, hoping that this will shed some light on the yet confusing, but promising study of the role that EMT regulators play in cancer.

#### 3.1.1. Breast Cancer

The first study where it was demonstrated that Slug expression is inversely correlated with E-cadherin expression in breast carcinoma cell lines was published in 2002 by Hajra and colleagues (25). Regarding Slug expression in breast cancer specimens, three studies were published to date. A study from T. Martin *et al.* (14) evaluated the expression of Slug in 114 breast primary tumors by immunohistochemistry (IHC) and Q-PCR (quantitative PCR). Slug expression was shown to be increased in tumors compared to normal background tissues. Slug expression also increased progressively with the Nottingham Prognostic Index (NPI) status and tumor grade but no correlation was seen with TNM status. Slug expression was higher in node-positive tumors and in

**Table 1.** Overview of the different studies of Slug expression in primary human tumors[1]

| Tumor type (number of cases) | Method of analysis | Major results | Reference |
|---|---|---|---|
| Breast carcinoma (n=114) | Q-PCR and IHC; frozen, formalin-fixed tissues | Slug overexpression associated with node-positive tumors and grade; identified as marker of poor prognosis. | (14) |
| Breast carcinoma (n=23 effusions) | RT-PCR; frozen, formalin-fixed | In comparison to Snail, Slug seems to be less relevant for E-cadherin regulation in breast cancer. | (13) |
| Breast carcinoma (n=128) | Real-time RT-PCR and IHC; frozen, formalin-fixed tissues | Slug overexpression associated with lymph-node metastasis. | (15) |
| Esophageal squamous cell carcinoma (ESSC) (n=203) | IHC; formalin-fixed tissues | Slug positive expression associated with depth of invasion, lymph node metastasis, stage and lymphatic and venous invasion.<br>Slug as marker of poor prognosis. | (16) |
| Esophageal squamous cell carcinoma (ESSC) (n=7, n=48) | cDNA microarrays<br>IHC; frozen, formalin-fixed tissues | Slug is identified as an overexpressed gene in ESCC. | (17) |
| Gastric Cancer (n=59) | QRT-PCR; formalin-fixed tissues | Slug overexpression and E-cadherin down-regulation associated with distant metastasis and TNM staging.<br>Slug expression correlated with Snail and SIP1 expression in diffuse and intestinal type tumors, respectively. | (18) |
| Colorectal carcinoma (n=138) | IHC; formalin-fixed tissues | Slug positive expression associated with Duke's stage and metastasis; marker of poor prognosis. | (19) |
| Hepatocellular carcinoma (n=18) | Real-time RT-PCR and IHC; frozen and formalin-fixed tissues | Slug overexpression associated with E-cadherin down-regulation and beta-catenin nuclear localization. | (20) |
| Pancreatic cancer (n=36) | IHC; formalin-fixed tissues | Slug is overexpressed in 50% of the cases.<br>Slug expression restricted to invasive margins of cancer tissue. | (21) |
| Lung adenocarcinoma (n=54) | RT-PCR; frozen tissues | Slug overexpression associated with postoperative recurrence and shorter survival. | (22) |
| Malignant mesothelioma (MM) (n=86 solid tumors, 24 effusions) | RT-PCR and IHC; frozen, formalin-fixed tissues | Slug expression correlated with MMP expression in effusions. | (23) |
| Ovarian carcinoma (n=78 effusions) | RT-PCR; fresh peritoneal and pleural effusions | Slug expression did not correlate with E-cadherin down-regulation in effusions. | (13) |
| Ovarian carcinoma (n=41 primary tumors, 15 metastasis, 78 effusions) | RT-PCR, IHC, Western blot and *in situ* hybridization; fresh peritoneal and pleural effusions, primary carcinomas and solid metastasis | Slug is differentially expressed according to tumor location. | (24) |

[1]A total of 1150 tumors cases from 13 studies have been summarized.

tumors from patients who had died from breast cancer or had metastatic disease, while the lowest expression was seen in patients who remained disease-free after tumor resection (14).

Another group studied Slug expression in malignant effusions from 23 patients diagnosed with breast cancer (13). Here, Snail seemed to predict a worse outcome in patients who had breast cancer metastatic to effusions, and a more pronounced suppression of E-cadherin expression was related to Snail and SIP1 expressions, and not to Slug. This seems to be the opposite of what was described in breast cancer cell lines (15, 25), where Snail expression did not correlate as well with loss of E-cadherin as Slug.

A third study came out in 2006 (15). Slug expression was analyzed by real-time RT-PCR and immunohistochemistry in a series of breast cancer cell lines and 128 primary tumors. In agreement to what was previously described (25), Slug correlated inversely with E-cadherin expression in the panel of cell lines analyzed, but, as reported by Martin *et al.*, (14) this was not the case in the primary tumors. The reason for this discrepancy between what is observed in cell lines and primary tumors remains to be elucidated. It is a known fact that cell lines do not always mimic what happens in *in vivo* tissues. Although Slug expression did not correlate with E-cadherin down-regulation in the study performed by Come *et al.* (15), the authors showed that it was higher in tumors when compared to normal breast tissue, particularly in those with lymph node metastasis.

According to these studies, Slug does not seem to have a critical role in the down-regulation of E-cadherin gene expression in breast carcinoma, although it seems to be correlated with lymph node metastasis and tumor grade.

### 3.1.2. Esophageal Squamous Cell Carcinoma

In esophageal squamous cell carcinoma (ESCC), one of the most aggressive carcinomas of the gastrointestinal tract, loss of E-cadherin expression was found to be associated with tumor invasiveness, metastasis and poor prognosis (26). Uchikado and colleagues, in a paper published in 2005, reported a series of 203 ESCC cases analyzed for Slug and E-cadherin expression, in an

attempt to see whether Slug could be related with the down-regulation of E-cadherin in this type of tumors (16). This study was performed by immunohistochemistry, using one of the anti-Slug polyclonal antibodies commercially available. Positive perinuclear and cytoplasmic staining was seen in 48% of the cases, and it was significantly correlated with reduced E-cadherin expression. Furthermore, Slug expression was seen to be associated with depth of tumor invasion, lymph node metastasis, stage, lymphatic invasion and venous invasion. An interesting observation of this study was that in a 5-year survival rate analysis, survival was significantly lower for the patients with positive Slug expression, as observed by Martin *et al.* in breast cancer (14), but only in patients harboring E-cadherin preserved tumors. This suggests that Slug can be important not only by repressing E-cadherin gene expression, but also by promoting tumor invasion by other mechanisms in tumors with intact E-cadherin expression.

Another line of evidence that Slug might play a role in ESCC comes from a paper published in 2006, where cDNA microarrays were used to study differential expression of several genes in 7 samples of ESCC (17). The authors identified 19 differentially expressed genes encoding zinc binding or modulating proteins associated with transcriptional regulation, ubiquitin-protein degradation and maintenance of zinc homeostasis. Among them was Slug, which was found to be up-regulated in tumors compared to normal control tissue. The results were validated by immunohistochemistry in 48 ESCCs. Positive Slug cytoplasmic staining was found in 71% of the cases, whereas no detectable levels of protein were found in normal tissue, although the authors do not mention how many nonmalignant samples were analyzed.

### 3.1.3. Gastric Carcinoma

Although its incidence tends to decrease in western world, stomach cancer is still the second most frequent cause of cancer related death (27).

Our group has recently analyzed a series of 59 gastric carcinomas, using real time RT-PCR from formalin-fixed tissues (18). We could show that Slug is up-regulated in tumors when compared to normal matched mucosa, and that this up-regulation is significantly associated with E-cadherin decreased expression particularly in the intestinal glandular subtype of tumors. We also showed that increased Slug expression together with E-cadherin down-regulation was significantly associated with the presence of distant metastasis and advanced TNM stages. Moreover, this was the first study analyzing associations between the expression of different EMT regulators in an attempt to better understand the specific role of each of these transcription factors known to regulate E-cadherin transcription and EMT, during gastric cancer progression and invasion. Interestingly, we found that Slug expression was associated with Snail expression in diffuse type gastric carcinomas, and with SIP1 expression in intestinal type tumors, which is in accordance with what some authors suggest, that the expression of the different EMT regulators could have different functions during tumor progression towards invasion and metastasis, possibly complementing each other's actions (28).

### 3.1.4. Colorectal Carcinoma

Colorectal carcinoma is one of the most frequent cancers worldwide, being the third most frequent in men and the fourth in women (29). Reduction of E-cadherin expression is seen in about 40% of colorectal cancer cases (30), but most of this loss is believed to be due to promoter hypermethylation (31).

The only study analyzing Slug expression in colorectal primary tumors suggests that positive Slug expression in colorectal carcinoma patients may be an independent parameter of poor prognosis (19). The authors first studied Slug mRNA expression in 6 colorectal cancer cell lines, and found it to be expressed in 5 out of 6 cell lines. Then they analyzed 138 colorectal cancers for Slug and E-cadherin expression, by immunohistochemistry, and found Slug positive expression in 37% of the cases, without any association with E-cadherin down-regulation (which was present in 58% of the patients), but significantly correlated with Dukes stage and the presence of distant metastasis. Although no direct association between E-cadherin down-regulation and Slug expression was detected, patients with positive expression of Slug and reduced E-cadherin expression showed the worst prognosis. The authors claimed that this absence of association could be due to the fact that in colorectal cancer, E-cadherin expression is very often down-regulated through promoter hypermethylation (31), rather than by gene structural alterations.

Furthermore, the overall survival of patients with Slug positive expression was significantly poorer and multivariate analysis indicated that Slug expression was an independent prognostic factor.

### 3.1.5. Hepatocellular Carcinoma

Hepatocellular carcinoma (HCC) is the fifth most frequent malignancy in the world, but current data indicate that its incidence is steadily increasing in the western countries (32). The prognosis of this type of tumor is usually poor, mainly because the number of patients suitable for surgical resection is limited (33). There is only one study, focusing on the role of TGF-beta1 and Laminin-5 (Ln-5) in EMT in HCC tissues and cell lines, where Slug expression was assessed in primary HCCs. Giannelli and colleagues studied Slug, Snail, E-cadherin, and beta-catenin in 18 HCCs by immunohistochemistry and real-time PCR, and showed that in HCC, but not in non-neoplastic liver of the same patients, Slug and Snail were up-regulated, E-cadherin was down-regulated and beta-catenin was translocated to the nuclei (20). *In vitro*, the authors showed that Slug and Snail expression could be induced by stimulating the cells with Ln-5 in HCC invasive cells, leading to dramatic morphological changes, E-cadherin delocalization and beta-catenin translocation to the nucleus.

### 3.1.6. Pancreatic cancer

A recent study addressed the expression and significance of Slug expression in this type of aggressive

tumor with a 5-year-survival rate <5% (21). It clearly shows that Slug expression is increased in pancreatic cancer when compared to normal surrounding parenchyma. However, in the 36 ductal adenocarcinomas analyzed, no obvious correlation was found between Slug and E-cadherin expression, nor stage or nodal *status*. In an attempt to clarify why Slug was up-regulated in this type of tumors, the authors used an orthotopic mouse model of human pancreatic cancer to analyze the expression of Slug and Snail. They found that Snail was expressed in ductal structures of the central part of the tumor, while Slug was mainly expressed in the invasive front, associated with E-cadherin reduction, supporting, together with other studies (18), that EMT regulators play distinct roles in tumor progression and invasion.

**3.1.7. Lung Adenocarcinoma**

Slug also appears to play a role in lung cancer, one of the most prevalent malignancies worldwide, considered to be the leading cause of cancer related death (29). A study analyzing mRNA of 54 surgically resected lung adenocarcinomas revealed that high expression of Slug was significantly associated with postoperative relapse and shorter patient survival (22). The authors had previously identified Slug as an invasion and metastasis-associated gene in a panel of lung cancer cell lines, using a cDNA microarray (34). Although no differences were found regarding gender, age, disease stage, tumor *status* and lymph node metastasis between patients expressing high and low levels of Slug, the median duration of postoperative recurrence was significantly shorter in the high expression group (median 11.0 months) in comparison with the low expression group (median 27.0 months), and the survival was significantly shorter (median survival 14.9 and 41.8 months in the high and low expression group, respectively). Furthermore, the study showed a direct correlation between Slug mRNA expression and invasiveness in a group of lung cancer cell lines established by the authors. At variance, Snail expression was similar in all the cell lines.

**3.1.8. Malignant Mesothelioma**

One study from 2006 (23) shows that expression of Slug in malignant mesothelioma effusions was associated with matrix matalloproteinase (MMPs) expression, but there was no association with E-, N-, or P-cadherin.

**3.1.9. Ovarian Carcinoma**

Ovarian carcinoma is the leading cause of death due to gynecological cancer in western countries. It metastasizes primarily to the serosal cavities, and is commonly associated with the accumulation of malignant peritoneal and pleural effusions. A study published in 2006 (24) showed that in primary tumors, Slug protein expression correlated inversely with E-cadherin mRNA and was shown to be localized in the nucleus, in opposite to what has been described by other studies mentioned in this review, in which Slug was shown to localize in the cytoplasmic and perinuclear region. Slug mRNA and protein were reported to be higher in primary tumors of the ovary and distant metastasis, when compared to effusions, where it was almost silenced, in agreement with the general up-regulation of E-cadherin in effusions when compared to primary tumors and distant metastasis in this type of tumors (35). A previous study from these authors showed no correlation between Slug and E-cadherin levels of expression in 78 malignant effusions, but no primary tumors were analyzed (13). The authors showed, in these two papers, that E-cadherin and its negative regulators Snail and Slug show site-dependent expression in ovarian carcinoma and hypothesize that loss of Slug expression could be one of the mechanisms regulating the re-expression of E-cadherin in effusions in ovarian cancer. They speculate that for some reason, re-expression of E-cadherin in the peritoneal or pleural microenvironment provides survival advantage for cancer cells. It is interesting to note that, according to these studies, Slug can play a role in ovarian cancer at the primary tumor level, where its expression is higher, down-regulating E-cadherin, and on another hand, its silenced expression at the effusions level could be responsible for the re-expression of E-cadherin at this site, as if mesenchymal-to-epithelial transition (MET) occurred. This line of evidence further supports the idea of EMT being a reversible, site-specific modulated process, and that MET could be important for metastasis survival.

**3.2 Relevant data from *in vitro* studies**

*In vitro* studies produced a lot of important data that support a putative role for Slug in tumor progression, particularly in invasion. After Bolos and colleagues (6) showed that Slug could bind to E-box elements at the promoter of the E-cadherin gene and repress endogenous E-cadherin expression, many authors started to look at Slug expression in tumors, since loss of E-cadherin is a frequent and important event in the progression of carcinomas, not totally explained by gene mutations or hypermethylation. Furthermore, there were also recent evidences that Snail, another member of the Snail family of transcription factors, could be a repressor of E-cadherin expression not only *in vitro* (7, 36), but also in primary tumors (37, 38).

Most *in vitro* studies were performed in non-human cell lines, but some were done in human cancer cell lines, especially in colon and breast cancer derived cells. Among the most important data, Slug was shown to correlate with E-cadherin loss in human breast cancer cell lines (25), to cause desmosome dissociation in a rat bladder carcinoma cell line (39), to negatively regulate cytokeratins 8 and 19 expression in human breast cancer cells (40), to down-regulate Claudin-1 expression in canine kidney cells (41), to regulate E-cadherin expression during Ras-mediated transformation of intestinal epithelial cells (42) and to repress occludin expression in a breast cancer cell line (43) as well as in a Raf1 transformed rat parotid gland model (44). In fact, there is increasing evidence that tight junction proteins may be important targets of Slug regulation, at least in stomach cancer. In the NCI-N87 human gastric carcinoma cell line, Slug transduction resulted in marked occludin down-regulation, and a dramatic change in the cell's phenotype (Castro Alves C, manuscript submitted) (Figure 2).

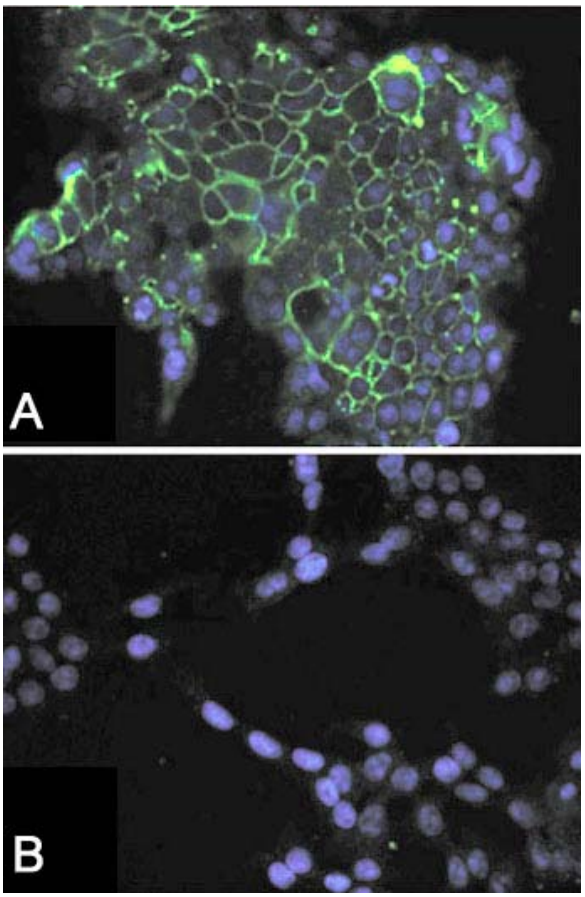


**Figure 2.** Occludin down-regulation detected by anti-Occludin (ZYMED) in Slug-transduced NCI-N87 cells, derived from a well differentiated gastric carcinoma. Wildtype (A) and Slug-transduced (B).

Furthemore, Gupta *et al.* (45) recently described that Slug expression is required for the formation of metastasis of transformed melanoma cells in a mouse model, claiming that the predisposition of this type of tumors to metastasize is due to the existence of lineage-specific factors associated to melanocyte differentiation such as Slug. Kajita and colleagues, in a paper published in 2004 reported that aberrant expression of Slug resulted in invasive growth and showed for the first time that Slug could also be involved in the acquisition of resistance to apoptosis elicited by DNA damage in epithelial cells (43). This is not the only report regarding the role of Slug in resistance to cell death. Slug was shown to be a target of the E2A-HLF oncoprotein in human pro-B leukemia, where it rescues hemotopoietic cells from DNA damage induced death (46, 47).

In conclusion, Slug was shown to interfere with the various types of epithelial cells junctions like adherens junctions (E-cadherin), desmosomes and tight junctions (claudin-1 and occludin), promoting invasive growth and resistance to apoptosis.

## 4. CONCLUSIONS AND PERSPECTIVES

Slug (SNAI2) is a vertebrate-specific Snail-related gene that was first identified in the neural crest and developing mesoderm in the chick embryos (12), where it is expressed in cells undergoing EMT. The first studies focusing on Slug in embryonic development showed that antisense oligonucleotides against Slug mRNA resulted in failure of EMT in chick embryos (12), and suggested that pathological activation of Slug (and other Snail family genes) could contribute to the onset of invasive and metastatic phenotype during the progression of cancers of epithelial origin, because the ability to break through an epithelial basement structure is reminiscent of the mechanism by which mesoderm and the neural crest originate (7, 12). The idea that EMT and its regulators could be implicated in processes such as invasion of tumor cells grew in the following years, after the evidence that Snail could repress E-cadherin expression and induce EMT in epithelial cells (7, 36) together with the known fact that E-cadherin loss of expression was an important step in tumor progression towards invasion and metastasis (48, 49, 50). In the meanwhile, also Slug was shown to repress E-cadherin expression in epithelial MDCK cells (6), and many *in vitro* studies suggested that Slug could be, indeed, important for cancer cells to change their phenotype into a more fibroblast-like phenotype with invasive capabilities, by losing epithelial markers and gaining mesenchymal markers (25, 39, 40, 41, 42, 43, 44).

Here we review the main studies focusing on Slug expression in primary tumors. It is not easy to compare studies using such a variety of sample types and methods, and providing some apparently contradictory data. Interpretation of the data is also somehow difficult because it is not clear yet how Slug itself is regulated. We know now that Snail's activity is influenced by its sub-cellular localization, which in turn is regulated by at least two kinases, GSK3beta and p21-activated kinase 1 (PAK1) (51, 52), but this has not been demonstrated for Slug yet.

The results we reviewed here suggest that Slug expression can be basal in some tissues but absent in others, since it is observed, both at RNA and protein level in non-neoplastic tissues from stomach and breast, although its expression was shown to be increased in tumors derived from both tissues, and absent in tissues like colon, ovary and esophagus. The reason for Slug presence in some tissues and absence from others is unknown, and in fact little is known so far regarding its pattern of expression, and, as mentioned before, how it is regulated.

It is not clear, from this set of data, whether Slug is in fact responsible for E-cadherin down-regulation in tumors. The results are quite heterogeneous regarding this matter, and not in accordance with most of the *in vitro* data, where the association is clear. In breast, for instance, there is a clear association in cell lines (15, 25) but not in primary tumors (15). However, one must consider that gene regulation is much more complex *in vivo* and that *in vitro* models are limited and do not always represent the *in vivo* situation.

What seems to be clear is the association of Slug expression with aggressiveness of the tumors, which is

demonstrated by the association with tumor grade, distant metastasis and patient shorter survival, although this does not always involve E-cadherin loss of expression. This suggests that E-cadherin is not the only target of Slug regulation *in vivo*, and possibly it is not even the most important one for tumor progression. An interesting example is described by Uchikado and colleagues (16), regarding ESSC. In the patients observed, Slug expression was found to be significantly associated with lower survival, but only in those patients harboring E-cadherin preserved tumors. This suggests that there are other targets of Slug action, direct and indirect, which result in increased aggressiveness of the tumors, independent of E-cadherin down-regulation. Furthermore, E-cadherin loss of expression may not be equally important for the progression of all types of carcinomas. The maintenance of some levels of cell-cell adhesion is not inconsistent with cell migration and invasiveness observed in some tumors, like in the case of breast invasive ductal carcinoma (IDC), where most of the carcinoma cells still express E-cadherin (15, 53). It is known from *in vitro* studies that Slug has other targets, although for many of them it is not known yet whether they are directly regulated by Slug, or a secondary effect of Slug overexpression. Tight junction proteins seem to be additional important targets of Slug regulation. Another important aspect that should be considered is the fact that E-cadherin gene promoter could not always be accessible to Slug binding due to promoter sequence alterations, which could explain why in some cases we see no association between Slug expression and E-cadherin down-regulation. Additionally, we do not know whether sub-cellular localization of Slug determines its activity and mRNA levels may not correspond to active protein.

Another interesting aspect is that Slug expression and its association with E-cadherin loss and aggressive characteristics of the tumors, seems to be independent of the tumor histotype, as seen in breast and gastric carcinomas (15, 18), which suggests that the mechanisms regulating Slug are somehow common among different histological types of the same tumors, so often presenting distinct molecular alterations and features. This feature could be very useful if Slug (and the other EMT regulators) are proven to be putative therapeutic targets for tumor invasion inhibition.

At the moment, still a lot of important factors remain to be elucidated. It needs to be demonstrated that molecules identified to be important for the regulation of Slug in cells or tissues from animal models have a similar function in human tissues. As more information is becoming available from *in vitro* and animal models, the molecules involved can be analyzed in human tumors. It is also important to clarify how the different EMT regulators (Slug, Snail, SIP1, Twist, and ZEB1) interact, since it is becoming clear that these transcription factors have different roles in invasion (18, 28). Analyzing several of these molecules both in human tissues and *in vitro* is probably the best way to understand in more detail the specific role of each one of them. Although some antibodies against Slug have been used in different studies, these may not work in every application and every tissue; thus, more Slug-specific antibodies should be generated in the future for general use.

In conclusion, based on the studies here reviewed, Slug seems to be important for tumor progression towards invasion and metastasis, although not always involving E-cadherin down-regulation. However, a lot of work is still needed regarding the mode of action and regulation of Slug and its interaction with other EMT regulators, in order to fully understand the observations of the studies mentioned in this review.

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**Send correspondence to:** Dr. Karl-Friedrich Becker,

Institut fuer Pathologie, Trogerstrasse 18, D-81675 Muenchen-Germany. Tel: 49-89-4140-4591, Fax: 49-89-4140-4915, E-mail: kf.becker@lrz.tum.de

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