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The role of developmental signaling pathways in non-small cell lung carcinoma

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On a global scale, lung cancer is the most widespread and deadly type of cancer and the non-small cell lung cancer histological subtype, contributes to a significant proportion of this mortality. It has been recently proposed that the main drivers of cancer progression and chemoresistance are cancer stem cells which can be identified through numerous biomarkers or through the overactivation of developmental signaling pathways which are essential for embryonic development but are normally suppressed in adulthood. The primary aim of this review was to compile experimental findings about mediators of three signaling pathways, namely Sonic Hedgehog, Notch and Wingless Integrated, in the prognosis and targeting of three non-small cell lung carcinoma histological types, namely adenocarcinoma, squamous cell carcinoma and large cell neuroendocrine lung carcinoma. Some mediators of all three signaling pathways can be used as biomarkers and overactivation is associated with shorter overall and disease-free survival of patients accompanied by metastasis, epithelial-to-mesenchymal transition and the acquisition of chemoresistance and radioresistance. Additionally, using antagonists to block overexpressed pathway mediators has yielded promising results *in vitro* with significant apoptotic and anti-tumor activity. Finally, numerous novel mediators of the three pathways have been identified and their pharmacological targeting has resulted in promising pre-clinical findings. The first in-human clinical trials of several drugs are currently being conducted. The current review supports further exploration of the three developmental signaling pathways in the prognosis and targeted treatment of non-small cell lung carcinoma with the aim of enhancing current treatment guidelines with the implementation of targeted therapies.

Keywords

Non-small cell lung carcinoma; developmental signaling pathways; Notch; Sonic hedgehog; Wingless Integrated

1. Introduction

With an estimated worldwide prevalence of 2.09 million cases in 2018, a mortality of 1.76 million deaths and with 246,440 new cases expected to arise in 2019 in the United States alone, lung cancer is the most frequently diagnosed cancer type and the one leading to the highest mortality [1, 2, 3]. Non-small cell lung carcinoma (NSCLC) is by far the most prevalent subtype of lung cancer accounting for 80% of all cases [4]. It is characterized by the acquisition of chemoresistance and high levels of recurrence even after surgical resection [4]. NSCLC encompasses several histological subtypes, the most common three being lung adenocarcinoma (AD), accounting for 40% of all NSCLC cases, squamous cell carcinoma (SCC) and large cell neuroendocrine carcinoma [4, 5]. Following diagnosis, patients usually present with an advanced form of NSCLC and only 15% survive after a 5-year period [6, 7].

Current treatment protocols for NSCLC include chemotherapy, targeted therapy and most recently immunotherapy combined with standard chemotherapeutic agents. A combination of the antibody pembrolizumab with carboplatin as well as combinations of pemetrexed and atezolizumab with carboplatin and cisplatin, serve as first-line combination treatment for advanced squamous and non-squamous NSCLC, respectively [8]. These treatment options have managed to prolong patient survival by 4 to 8 months [8, 9]. Patients with high levels of programmed death ligand-1 (PD-L1), defined as those whose receptor levels exceed 50%, can be treated with pembrolizumab monotherapy [8]. In addition to immunotherapy, radiotherapy and surgical resection are still part of clinical guidelines and both options have varying degrees of success [9].

The implications of better treatment for NSCLC in the medical field are wide-ranging and new approaches for tackling the disease are essential. A 2018 observational study in the US revealed that the mean monthly cost of targeted tyrosine kinase inhibitor (TKI) therapies for NSCLC treatment per patient was $20,106 [10]. In addition to this high economic burden, quality of life studies have shown that lung cancer patients experience numerous health problems on a psychological, physical and social level, the most common and debilitating ones being mental diseases, pain, physical disability and inability to perform expected social roles [11]. Hence, progress in the treatment of NSCLC is of pressing importance.

The implementation of immunotherapy in clinical guidelines and the observed treatment benefits illustrate a case where novel drugs significantly prolong the life expectancy of NSCLC patients. Targeted therapies for cancer are another novel strategy of pharmacological targeting through specific inhibition of certain signaling molecules which are predominantly expressed in cancer cells [12]. Some examples of targeted therapeutic agents currently used in clinical practice for NSCLC include drugs targeting mutated EGFRs, BRAF mutations, MET gene amplifications and ROS1 rearrangements, all observed in various subsets of patients [13]. Comparably, recent evidence suggests that the activation of developmental signaling pathways can play a significant role in cancer initiation and progression. Hence, the aim of this paper is to explore the association of three developmental pathways, namely the Hedgehog, Notch and Wingless/Integrated (Wnt) signaling pathways with NSCLC progression and treatment. Studies eligible for inclusion were those investigating biomarkers, different forms of pathway dysregulation, association with cancer hallmarks and pharmacological means of targeting three developmental pathways, Hedgehog, Notch and Wnt. Studies published within the period 2012-2019 were identified through the search engine PubMed using the keywords "Hedgehog", "Wnt", "Notch" and "NSCLC". Studies investigating the three most common NSCLC subtypes, namely lung adenocarcinoma (AC), squamous cell carcinoma (SCC) and large cell neuroendocrine lung carcinoma were part of the inclusion criteria.

2. The cancer stem cell hypothesis

A recently proposed explanation for the intractability of NSCLC is the existence of a cancer stem cell (CSC) niche within tissues which confers resistance to conventional therapies, promotes metastasis and drives relapse [14]. These cells constitute only a small subpopulation of all cancer cells found in the tumor tissue. They have an endless replicative potential, can easily metastasize to different organs, have higher tumorigenicity and exhibit higher expression of pluripotency genes [4, 5]. They are hypothesized to be the main drivers of tumor relapse after successful initial response to chemotherapy [4]. Pharmacological targeting of these cells can be challenging due to their heterogeneity and their resemblance to normal stem cells which are essential for proper maintenance and renewal of organs [5]. Several putative markers can confirm the presence of CSCs in tumors, including overexpression of certain receptors such as CD44, CD133, CD166, a side population (SP) phenotype observed in flow cytometry analysis, increased activity of the enzyme aldehyde dehydrogenase (ALDH) or upregulation of oncogenic pathways [4, 5]. However, the reliability of many of these markers has recently been disputed and they are not always indicative of CSC presence [4]. Developmental signaling pathways have also been found to play an essential role in the maintenance of a CSC phenotype [4, 5]. The three most commonly investigated ones are the Hedgehog, Notch and Wnt signaling pathways [5]. These pathways are necessary for the proper embryonic development in mammals and are normally suppressed in adulthood; however they can get reactivated in some individuals leading to uncontrolled growth and migration of cells [5]. A short overview of each pathway under normal conditions and results from recent research related to its dysregulation in NSCLC treatment is outlined in this thesis.

3. Hedgehog signaling pathway

3.1 Pathway description

The Hedgehog signaling pathway (HhP) is essential for the process of bronchial budding in lung formation during embryogenesis [15]. Three different divisions of the pathway have been characterized, including Sonic (Shh), Indian (Ihh) and Desert (Dhh) [16]. The Sonic subvariant of the pathway (shown in Fig. 1), which is the most frequently activated one in cancer and thus the main focus of the current review, involves several well-regulated steps [17]. The Sonic hedgehog (Shh) ligand is secreted from the endoplasmic reticulum and is post-translationally modified by the addition of fatty acids, namely a cholesteryl ester and a palmitoyl amide (the latter reaction is catalyzed by the enzyme Skinny Hedgehog (Hedgehog acetyltransferase)), added onto the N-terminus domain of the protein [18]. These post-translational modifications are required in order to increase signaling and to direct the binding of extracellular Shh to the membrane of surrounding cells [16, 18]. The release of the fully modified ligand is controlled by the protein Dispatched-1 which is located on the cell membrane [19]. Binding of Shh to the extracellular Patched receptor results in abrogation of the latter's repression of the nuclear receptor Smoothened (SMO). The activated SMO receptor can subsequently translocate to the primary cilium where it may activate the suppressor of fused homolog (SUFU) protein and modulate the expression of glioma-associated oncogenes (Gli) -1, -2 and -3 [20]. Gli-1 can alter cellular behavior as it is a transcription factor (TF) that can bind to respective Gli response elements on the DNA [20]. Gli-3 may function as a repressor of the activity of Gli-1, whereas Gli-2 functions as its inducer [17]. Recently emerging evidence from pre-clinical and clinical studies related to Sonic Hedgehog signaling will be presented as well.

3.2 Clinical evidence

Clinical evidence has shown frequent overactivation of numerous Sonic Hedgehog mediators, including receptors, ligands, signaling molecules and TFs [21]. The Shh ligand was associated with visceral pleural invasion in NSCLC and high SMO levels were associated with lymph node metastasis [22, 23]. Aberrant activation of three proteins, Shh, Gli-1 and Hedgehog interacting protein (HHIP; an inhibitor of the pathway), was demonstrated in surgical resections of AC and SCC and is possibly activated early on in cancer progression [24]. Gene expression of Dispatched-1 in tumor tissues was indicative of lower recurrence-free survival (RFS) and lower overall survival (OS) of NSCLC patients [19].

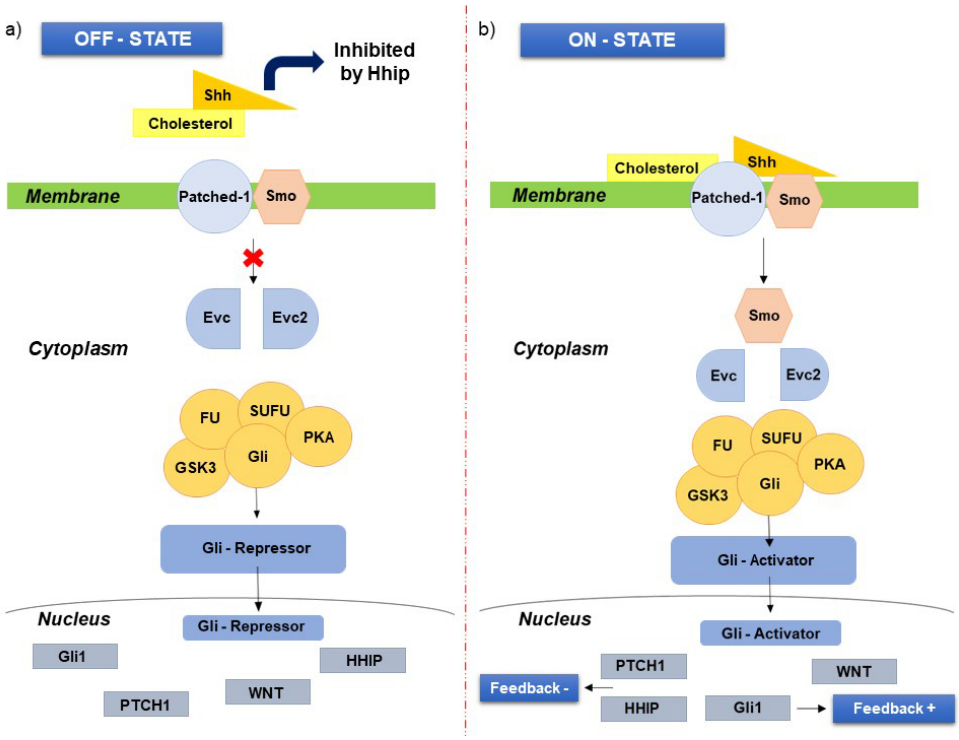

Figure 1. Shown are the mediators involved in Sonic Hedgehog signaling in the inactivated state of the pathway (a) and the activated state (b). Legend: Shh – Sonic Hedgehog ligand, HHIP – Hedgehog interacting protein, Smo – Smoothened, SUFU – suppressor of fused homolog, GSK3 – glycogen synthase kinase 3, Gli – glioma-associated oncogene.

Elevated Gli-1 and Gli-2 levels were shown to lead to epithelial-to-mesenchymal transition (EMT) by downregulating E-cadherin levels and conferring chemoresistance, respectively [25, 26, 27]. A possible explanation for Gli-1-mediated EMT induction is cross-talk with *Snail-1* genes and activation of the TGF-β pathway [26]. Presence of high Gli-2 was also associated with median patient OS of 8 months, compared to an 18-month OS for Gli-2 negative tumors [26]. Both mediators were additionally associated with the acquisition of chemoresistance to platinum-based chemotherapy in advanced-stage NSCLC tissues which were refractory to first-line chemotherapy agents [25]. In addition, Shh and Gli-1 engage in cross-talk with two lymphangiogenic factors - lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) and vascular endothelial growth factor-D (VEGF-D) which can facilitate lymph node metastasis and accelerate progression by stimulating angiogenesis near tumor tissues. Blocking Sonic Hedgehog signaling resulted in reduction of mRNA expression of these two molecules [22]. Despite all the aforementioned findings confirming the involvement of Gli-2 in OS and cancer progression, one study found no correlation of its expression with patient OS [17].

Cross-talk between Sonic Hedgehog signaling and other pathways has been investigated in several studies. The pathway relates the activity of NSCLC cells with lung fibroblasts, with the latter responding to the Shh ligand secreted by surrounding stromal cancer cells, with an increased secretion of metalloproteinases, as well as other mesenchymal and proangiogenic growth factors. The expression of pathway mediators was associated with distance of migration of these fibroblasts and it was more prominent in SCC compared to AC cell lines [28]. Phosphorylated STAT3 from the Janus Kinase (JAK) pathway can influence the pathway through thyroid transcription factor (TTF)-1 and is associated with early-stage and non-invasive cancers [24]. It has also been found that gene expression of the embryonic proteins Bmi1, FoxF1, Nanog and γ-catenin correlated with Gli-1 and lymph node metastasis [29].

Several novel pathway predictors of disease progression, clinical outcomes and CSC niche presence have been identified. A full-length, uncleaved Shh ligand was associated with the existence of a CSC phenotype and was indicative of worse survival outcomes for patients with stage I NSCLC with a time-to-progression (TTP) of 11.8 months [30]. Aldehyde dehydrogenase family 1 member A1 (ALDH1A1) which indicates canonical Sonic Hedgehog signaling was pinpointed as a putative marker of CSC presence, albeit it did not have biomarker potential in stage I and II NSCLC. Nevertheless, it showed correlated expression with HhP mediators in AC and SCC tissues [21].

3.3 Pharmacological targeting

In line with the abovementioned clinical observations, a growing body of experimental evidence supports the pharmacological targeting of pathway mediators. An extract of the Chinese herb *Scutellariabarbata D. Don* (SBE) was found to decrease the proliferation and induce chemosensitivity in various NSCLC cell lines by interfering with Shh signaling [31]. Using the SMO inhibitor GDC-0449, led to a concentration-dependent inhibition of AC cell proliferation and upon combination with cisplatin, the drugs demonstrated a synergistic effect in reducing the side population (SP), a flow cytometry proxy for a putative CSC niche [32]. An-

other SMO inhibitor, cyclopamine, successfully blocked cell division of the Gli-1 low expressing cell lines Hop62 and H322M but was not successful in inhibiting the growth of cell lines with high expression of Gli-1. Expectedly, small interfering (siRNA) blockade of Gli-1 managed to overcome this obstacle [33]. Another study found the same effect for cyclopamine at 3 μM, resulting in a decrease of Gli-1 protein expression, cell viability and cyclin D production [28]. Moreover, blocking SMO and Gli using Gli-I and vismodegib in two NSCLC cell lines decreased motility in wound-healing and 3D cell invasion assays and increased E-cadherin levels [26]. GANT61, a Gli-2 inhibitor, triggered apoptosis more effectively in comparison with other SMO inhibitors, leading to cellular death in almost 90% of primary tumor cell lines at a concentration of 5 μM [34].

Sonic Hedgehog inhibitors have also been used to improve the responsiveness of NSCLC cell lines to conventionally used chemo- and radiotherapy agents. In line with the observed correlation of chemoresistance and Sonic Hedgehog protein overexpression in cell lines, vismodegib led to restoration of sensitivity to cisplatin treatment and the two agents had synergistic anti-proliferative effects [25]. Sonic Hedgehog signaling was hypothesized to maintain a stem cell-like niche via Gli-1 and Gli-2 cross-talk with epidermal growth factor (EGF). Gli-1 was found to tightly regulate the expression of the embryonic transcription factor (TF) Sox2 and thus confer resistance to EGFR inhibitors in several NSCLC cell lines, a process which was reversed by an SMO inhibitor [17]. Similarly, a combination treatment of an SMO antagonist and radiation, significantly reduced tumor size in an additive manner in a mouse xenograft models [35].

One study investigated the efficacy of raising mouse antibodies against the C-terminus of Shh. The rationale consisted of targeting CSC tumor population as they express a full-length Shh ligand. Following specificity development, the antibody led to a decreased population of cells expressing Shh *in-vitro*, mainly through triggering apoptosis and slightly blocking Shh signaling. One possible reason proposed for this observation was the small size of the CSC population and its slow proliferation rate, as well as the potential uptake of the drug by surrounding cells [36]. IL-27 treatment of AC and SCC cell lines decreased the expression of numerous pluripotency genes, including *SHH* in the former and *NOTCH1* in the latter histological type. Both genes were putatively associated with the maintenance of stem cells [37].

3.4 Targeting novel pathway mediators

Recent research is focusing on additional proteins which can be exploited as potential pharmacological targets. Hedgehog acetyltransferase (HHAT) catalyzes the N-palmitoylation of Shh and blocking its function, halted pathway activity as indicated by low Gli-1 activation and concomitant cytotoxic and cytostatic effect on the AC cell lines A549 and Hop62 [19]. On the other hand, it was discovered that RUSKI-43 which blocks HHAT activity exhibited cytotoxic effects which were unrelated to the pathway [16]. RUSKI-201, another analogue which inhibits enzyme activity, led to specific inhibition of endogenous Shh signaling in the SCC cell line H520, by preventing the palmitoylation step of the Sonic ligand [16]. Hhat-EGFP, also involved in ligand palmitoylation was identified in a pancreatic cell line which was able to lead to decreased retention of Shh multimers, decreased pathway signaling and decreased invasion [18]. Blocking this enzyme in A549 NSCLC cells resulted in blockade of paracrine/juxtacrine Shh signaling to surrounding C3H10T1/2 cells co-cultured in the same assay [18]. Hematopoietic pre-B-cell leukemia TF (PBX)-interacting protein (HPIP) interacts with Shh proteins and could be targeted to reduce cell growth in A549 and H1299 cells [38]. Blocking the anti-apoptotic protein phosphatidylethanolamine-binding protein 4 (PEBP4) led to lower cell proliferation, prevented metastatic nodule formation and decreased the expression of Shh, SMO and Gli-1 [39].

WW45, a protein involved in the ubiquitination of Gli-1 prevents gene expression, reduces growth and metastasis [40]. The newly characterized activated kinase 1 (RACK1) receptor was found as an upstream effector in many signaling pathways. Expectedly, its upregulation led to tumor growth and metastasis and small interfering RNA (siRNA) blockage could reverse those effects, induce apoptosis and downregulate Gli-1 [41]. SCUBE2, a secreted and membrane-bound receptor, demonstrated the opposite effect and its activation was correlated with longer DFS and decreased cell proliferation, migration and levels of HhP mediators [42]. Set7, a methyltransferase of K436 and K595 residues on Gli-3, increased protein stability and improved DNA binding capacity of the TF to the *Gli1* promoter region. K436R and K595R mutants led to retarded tumor growth and decreased EMT in the A549 cell line [43].

3.5 Human clinical trials and prospective applications

The aforementioned studies seem to strongly support the use of Sonic Hedgehog inhibitors in human clinical trials. Nevertheless, there are several factors that need to be considered before proceeding to human trials. Expectedly, since the Sonic Hedgehog pathway involves an intricate cascade that is related to many other mediators and factors, including non-canonical ones, its targeted inhibition can be challenging [25]. As proposed by Wang et al., (2016), drugs that work further downstream in the pathway are expected to inhibit both canonical and non-canonical signaling and should be considered for future use [26]. Additionally, resistance to vismodegib, a SMO antagonist approved by the Food and Drug Administration (FDA) for the treatment of basal cell carcinoma, despite occurring at low rates, has been documented in patients with medulloblastoma and basal cell carcinoma [44, 26]. Hence, newer drugs like itraconazole have been used as they can circumvent the acquisition of vismodegib resistance by exploiting alternative binding sites on the SMO protein [26]. Two ongoing studies are currently investigating the effects of itraconazole in a phase 0 CT and a phase II CT where it is administered as an adjunct to platinum-based chemotherapy for NSCLC patients [45, 46].

4. Notch signaling pathway

4.1 Pathway description

The Notch signaling pathway is involved in determining cell differentiation during the process of branching morphogenesis in the developing embryo and has a role in regulating functional lung physiology during adulthood [47]. The canonical pathway starts with juxtacrine signaling whereby a Notch receptor binds to an adjacent cell membrane-bound ligand of the Jagged or Delta-like family of proteins (shown in Fig. 2) [48]. Upon binding, an initial cleavage of the receptor occurs via the activity of the enzyme

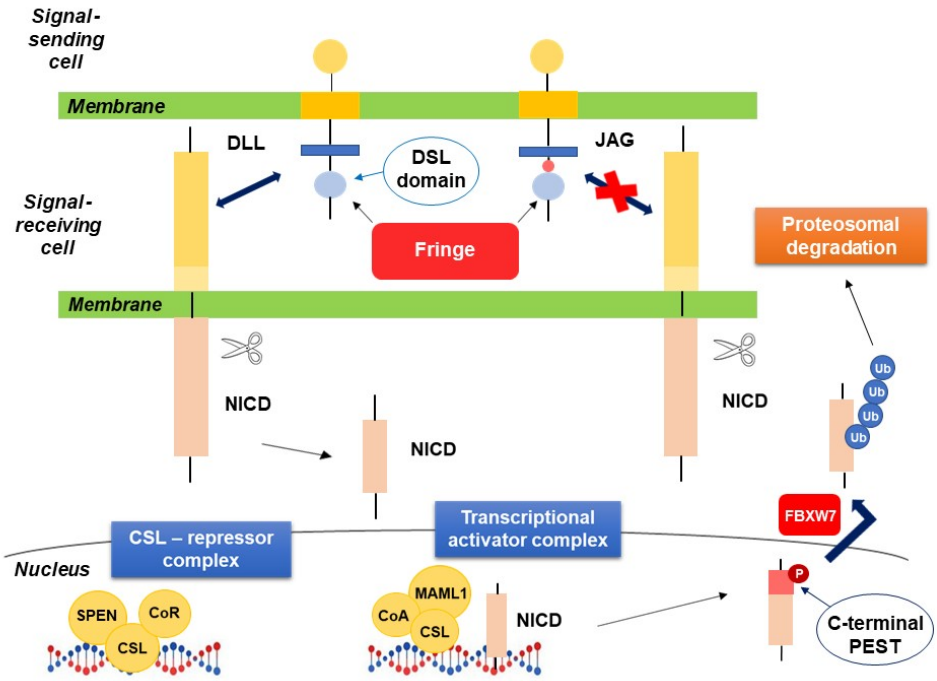

Figure 2. Shown are the mediators involved in canonical Notch signaling in the inactivated state of the pathway. Legend: DLL – Delta – like ligand, JAG – Jagged, NICD – Notch intracellular domain.

A-disintegrin and metalloprotease (ADAM) and subsequently, the presenilin-γ-secretase complex is responsible for the final cleavage of the Notch receptor at an S3 site [49]. This results in the intracellular detachment of a Notch intracellular domain (NICD) which translocates to the nucleus. Once translocated, NICD forms a transcription complex with other TFs and activates genes of the *Hes* and *Hey* families as well as VEGF, NFkB, BcL family members, *c-myc*, Ras and cyclin D1 [47, 48, 50]. In total, there are four different variants of the Notch receptor: Notch-1, Notch-2, Notch-3 and Notch-4, all of which have different functions in NSCLC [51]. Furthermore, there are two Jagged ligand variants, JAG1 and JAG2, as well as three Delta-like ligand (DLLs) variants: DLL1, DLL3 and DLL4 [52, 53]. The following section outlines recent clinical evidence for the association of different Notch pathway mediators with NSCLC.

4.2 Clinical evidence

Various Notch signaling molecules have been analyzed in NSCLC tissues and they tended to have higher predictive values for AC compared to other NSCLC subtypes in 2437 observed samples [53]. The first Notch receptor variant, Notch-1, has an ambiguous role in NSCLC since its expression is predictive of different outcomes based on the analyzed cancer subtype; while it has a protective role in AC and is associated with longer OS, it does not have a protective role in SCC [53, 54]. In AC, Notch-1 correlated with vascular pathways and immune markers while in SCC, it was associated with increased cell proliferation and mitotic genes [55]. Its co-expression with *c-met* correlated with staging and OS in resected tumors. The association was higher in AC than in SCC, suggesting the two mediators can be used in combination as biomarkers in this histological type [56]. In one study, the presence of Notch-1 and the intracellularly cleaved protein N1-ICD-V1754 were found in 50% of clinical cases analyzed, mostly of SCC origin; they were negatively correlated with clinical stage and nodal status and showed no correlation with tumor grade and size [57]. Notch-1 has also been implicated in the acquisition of resistance to gefitinib and in EMT induction in tumors; antagonizing the receptor with either short hairpin (shRNA) or a γ-secretase inhibitor (GSI) reversed the observed effects [58]. Furthermore, prolonged cadmium exposure upregulated Notch-1 levels and conferred cisplatin resistance upon the AC cell line H1975 [59]. Along this vein, the role of Notch-2 in NSCLC progression is ambiguous based on recent evidence. It was found to be overexpressed in females, current smokers and patients with stage IIIA AC. It was significantly associated with higher tumor recurrence and metastatic involvement in the subset of patients that was analyzed [60]. On the other hand, two other studies found that Notch-2 that suppresses apoptosis, can promote the differentiation of Club-like cells, which are the resident stem cell-like cells in lung tissues and was predictive of longer OS [53, 61]

Studies have shown that Notch-3 is tumor-promoting and indicative of shorter DFS and OS, higher tumor stage, lymph node metastasis and poorer tumor differentiation status [52, 53, 62, 63, 64]. It has been implicated in resistance acquisition to cisplatin and poor response to platinum-based chemotherapy. Its inhibition using siRNA led to resensitization to platinum-based drugs, induced apoptosis and decreased growth and invasion [63, 64]. Some of the genes that Notch-3 regulates include *Notch1*, *Hes1*, *Jagged1*, *Vimentin* and *Snail*, the last two of which correspond to the decreased levels of EMT observed after targeting [62]. It was found to have a non-redundant role in putative tumor-promoting

Table 1. Association of Notch ligands with OS

Ligand	Outcome
JAG1	Higher OS [62], lower OS [67]
JAG2	Lower OS [60]
DLL1	Higher OS [62]
DLL3	No predictive value [60]
DLL4	Higher OS [60]

cells (TPCs) from NSCLC cell lines and its inhibition led to a two-fold reduction in growth and five- to six-fold reduction in sphere-formation [65]. Moreover, its expression was corelated with two CSC markers, namely CD44 and ALDH1A1 [63]. Contrary to the evidence presented thus far, one study reported that Notch-3 has a non-canonical pro-apoptotic function in endothelial cells of the tumor stroma as well as in vascular cells, thus leading to an inhibitory effect on angiogenesis [49]. Jagged-1 can act as a suppressor of this activity and its interaction with Notch-3 could be exploited by pharmacological means to target angiogenesis [49]. Another study found no association between Notch-3 expression and OS or histological subtype [60]. While one study found no predictive role of Notch-4 in NSCLC, other studies found that its expression led to vasculogenic mimicry and was predictive of lymph node metastasis and TNM stage [52, 53, 66]. The associations of elevated Notch ligands with OS outcomes in NSCLC patients are presented in Table 1.

4.3 Pharmacological targeting

Preclinical studies have used various pharmacological agents to target different Notch pathway mediators with the aim of suppressing tumor growth. Notch-1 has been targeted in several such studies leading to promising results. A tocotrienol mixture reduced its levels in a dose-dependent manner, led to apoptosis and inhibited cell growth and invasiveness [51]. Similarly, the analogue delta-tocotrienol decreased the expression of Notch-1 and its downstream genes, led to G0-G1 cell arrest and decreased their invasive capability three-fold compared to controls [69]. Contrary to these findings, a combination of daurinoline, a plant alkaloid, and paclitaxel, a microtubule-stabilizing chemotherapeutic, upregulated Notch-1 levels and subsequently reversed EMT and invasive phenotypes [70]. Further studies are warranted to establish the exact effects that arise as a consequence of Notch-1 blockade in various NSCLC cell lines.

Similar results were found for the pharmacological targeting of Notch-3 and its cleaved form, NICD3. Using Notch-3 siRNA or GSI in two cell lines led to their sensitization to paclitaxel by reducing the IC_{50} value and induced apoptosis via the intrinsic pathway [71]. A combination of gemcitabine, a deoxycytidine analogue and 10 or 20 μM of N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT), a Notch signaling blocker, decreased Notch-3 levels, induced Bcl-2 mediated apoptosis and decreased the colony number of cancer cells [72]. Comparably, N4ICD levels were decreased using a combination of terfenadine, an anti-histamine drug targeting the histamine H_1 receptor along with the anthracycline epirubicin. The combinational effect of the drugs decreased EMT and proliferation markers, while simultaneously increasing the levels of N1ICD and N3ICD [73].

The Notch signaling pathway has been implicated in the acquisition of resistance of NSCLC cancer cells to radiotherapy as prolonged exposure has resulted in overexpression of several pathway mediators [47]. Experiments on mouse xenografts found that artificially induced high-expressing Notch cells were significantly more radioresistant compared to normal cells and the hypoxic fraction increased after radiation [47]. Accordingly, several studies have investigated whether targeting the pathway can render resistant cells responsive to radiotherapy. A combination of the flavonoids rhamnetin and cirsiliol led to the induction of an inhibitory miR-34a which decreased the levels of Notch-1 and eventually rendered NSCLC cell lines more susceptible to radiation-induced apoptosis [50]. Employing a combination of a GSI and hypoxia-inducible factor-1 (HIF-1) inhibitor had a synergistic effect and reduced post-irradiation NICD3 activation [48].

Combinations of Notch inhibitors with standard-treatment chemotherapeutic agents have also been employed in several studies. A combination of BMS-906024, a GSI, with cisplatin, etoposide or crizotinib, a TKI targeting ALK and ROS1, revealed that the last drug demonstrated the highest suppressive effect at a concentration of 0.8 μM in two NSCLC cell lines [74]. Additionally, 50-100 nM of BMS-906024 in combination with either paclitaxel or cisplatin showed a synergistic effect in 25 AC cell lines; the most effective cytotoxic response was seen in KRAS/BRAF-wild type cells that demonstrated low N1ICD expression [75]. A combination of a GSI and ABT-737, a Bcl-2 inhibitor, revealed a synergistic mode of action of the two drugs via suppression of pro-survival Bcl-2 proteins and upregulation of anti-survival proteins in a Bim-dependent manner [76].

4.4 Novel pathway mediators

In addition to the canonically activated pathway mediators, recent studies have identified numerous proteins and signaling molecules which can modulate Notch signaling and several studies have specifically focused on their relation to Notch-1. Nuclear factor E2 related factor 2 (Nrf2) was upregulated along with Notch-1 after radiation. Expectedly, its knockdown in cell lines inhibited post-radiation migration and invasion capacity and could be combined pharmacologically with Notch inhibitors [77]. Targeting Nrf2 using siRNA in combination with radiation, led to an enhancement of reactive oxygen species (ROS) production by tumor cells and abolished Notch-1 expression [78]. Blocking leptin with siRNA resulted in a decrease of Notch-1 and pJAK/STAT mediators [79]. Interferon regulatory factor 4 (IRF4) was correlated with Notch-1 and -2 expression [80].

Other factors have also been associated with a modulation of Notch signaling and their targeting has led to a decrease in cancer hallmarks. High expression of the gene *KIAA0247*, encoding for a membrane protein, could act as a potential prognostic marker for slower cancer progression [81]. It could lead to decreased Jagged1 and NICD levels and subsequently reduced cell proliferation, metastasis and EMT-associated proteins [81]. Overexpression of Notch activation complex kinase (NACK) correlated with lymph node metastasis and low degree of differentiation, as well as low OS and DFS. Targeting its miRNA led to reduced migration, increased apoptosis, and decreased expression of the TFs Hes1 and HeyL [82]. Overexpression of the lncRNA-X-inactive specific transcript (XIST) could inhibit proliferation and TGFβ1-

Table 2. The association of Notch pathway mediators with NSCLC outcomes

Signaling mediator	Role in Notch signaling	Outcome of targeting
Histone deacetylase 6 (HDAC6) [84]	Deacetylation of HSP90; link for TGF-β1-induced activation of Notch1	Prevented HEY-1, HES-1 upregulation
Forkhead box J2 (FOXJ2) [85]	Inhibits TGF-β1-mediated EMT; lower expression in cancer	Increased Notch1 and NICD1 levels
Zinc-finger RNA (ZFR) binding protein [86]	Increases Notch1 levels	Reduced cell migration potential
lncRNA-Low Expression in Tumor (LET) [87]	Reduces NICD1 levels	Downregulation led to higher TNM stage, low OS
Rnd3/RhoE (Rho GTPase) [88]	Downregulated; associated with higher NICD expression	Forced regulation led to an inhibition of Notch-regulated proliferation
miR-129-5p [89]	Inhibits DLL1 homologue in A549 and H460 cells	Diminished stemness markers, reduced resistance
miR-223 [90]	Upregulated; leads to overactivation of Notch	Reduced CD44+ subpopulation of CSCs, improved response to erlotinib
Rumi, protein-O-glucosyltransferase [91]	Two to three-fold higher expression in NSCLC	Knockdown led to decreased Hes1, Hey1, cell proliferation and migration

induced EMT by binding to miR-137 and thus reversed the effects of Notch downregulation [83]. Several other novel pathway mediators along with their role in Notch pathway signaling and the results of their pharmacological targeting are presented in Table 2.

4.5 Clinical trials

There are several ongoing clinical trials which aim to evaluate the use of Notch inhibitors in the treatment of NSCLC patients. A phase I clinical trial of RO4929097, a GSI, in combination with erlotinib in patients with stage IV NSCLC, has been recently conducted [92]. A phase IB of demcizumab, an antibody targeting DLL4, in combination with pemetrexed and carboplatin has also been conducted. The most common adverse side effects observed in treated patients were pulmonary hypertension and congestive heart failure [93]. Nevertheless, the treatment regimen showed antitumor activity in 50% of the patients and it successfully downregulated *NOTCH1* and *NOTCH2* gene expression levels. The tolerable doses established for further testing were four cycles of 5mg/kg given once every three weeks in combination with standard-dose carboplatin and pemetrexted, followed by continuous chemotherapy treatment for responding patients [93]. Several other clinical trials for demcizumab are also currently being conducted in various combinations [94, 95].

5. Wingless/Integrated (Wnt) signaling pathway

5.1 Pathway description

The Wingless Integrated (Wnt) pathway is involved in cell fate determination and differentiation of various cell types during lung growth [96]. It can be subdivided into the canonical and non-canonical variant of the pathway, the former one depending on the protein β-catenin to exert target gene expression. The canonical pathway (shown in Fig. 3) consists of binding of the ligand Wnt to its receptor Frizzled (Fz) and the co-receptor lipoprotein receptor-related protein 6 (LRP6), subsequent activation of Dishevelled (Dvl) and final inhibition of the Axin complex [97]. Since the Axin complex contains two catalytic subunits, namely casein kinase 1 (CK1) and glycogen synthase kinase 3 (GSK3), their inhibition prevents the stepwise phosphorylation of β-catenin [97]. Once these two subunits are inactivated, unphosphorylated β-catenin levels rise in the cytosol and can thereafter translocate to the nucleus where they form transcription complexes with TF members of the TCF/LEF family or the initiator of transcription, p300 [97, 96]. This can eventually lead to an increase in activation of genes encoding for metalloproteinases, *c-myc*, *c-jun* and cyclin D among others [96]. Numerous antagonists of the pathway exist and the most important families and their corresponding members are listed in Table 3. The following sections present recently emerging evidence for the role that the canonical Wnt pathway plays in NSCLC progression and treatment.

5.2 Clinical evidence

Clinical evidence has shown that overexpression of the canonical Wnt signaling pathway in patients with NSCLC is associated with worse prognosis and shorter OS [102]. One common mechanism of attaining upregulated expression is through the methylation of CpG island regions on the promoters of certain genes. This was observed in a cohort of 111 NSCLC samples where methylation marks on Wnt-associated genes, namely *SFRP1, SFRP2, PRKCB* and *WIF1*, were indicative of NSCLC diagnosis [98]. Other genetic factors analyzed include germline single nucleotide polymorphisms (SNPs) analyzed in stage I and II NSCLC patients who had undergone surgery or received chemotherapy. A polymorphism close to the gene *WNT16* was significantly associated with recurrence and a polymorphism near *FZD4* was associated with recurrence and patient survival [103]. Similar analyses of stage III and IV patients treated with platinum-based chemotherapy revealed that SNPs in *AXIN2*, *CXX4* and *WIF1* genes were associated with decreased OS in the cohort of patients with an increased number of such polymorphisms [100].

Other studies have investigated the prognostic value of several ligands and signaling molecules of the Wnt pathway. Elevated Wnt2 serum levels were found to be highly expressed in AC and SCC tissues compared to normal samples and they had an independent prognostic role for OS and RFS in the former NSCLC subtype [104]. In one study, Wnt5a expression was found in 61.95% of NSCLC cases analyzed; it was mostly correlated with SCC, male sex and shorter OS and it was also associated with vascular

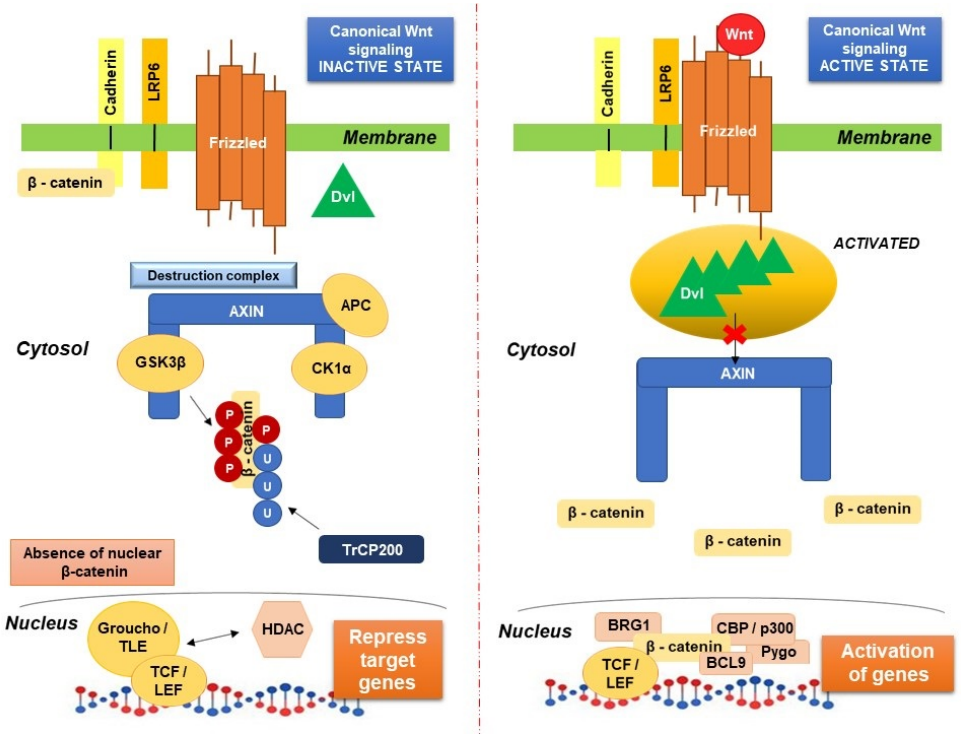

Figure 3. Shown are the mediators involved in canonical Wnt signaling in the inactivated state of the pathway (left) and the activated state (right). Legend: Frizzled – Fz, LRP6 – lipoprotein receptor 6, Dishevelled – DVL, GSK3 – glycogen synthase kinase, CK1 – casein kinase 1.

mimicry and microvessel density [105]. Wnt-5 can contribute to angiogenesis by increasing microvessel formation via downregulation of peroxisome proliferator-activated receptor-gamma (PPAR-γ) and upregulation of VEGF-A expression [106]. Additionally, the ligand can contribute to EMT by downregulating β-catenin levels and other attachment proteins in the AC cell line H1975 [107]. Its joint expression with RTK-like orphan receptor (ROR2) was predictive of TNM staging and poor patient prognosis [108]. Furthermore, increased levels of Wnt-5 and -11 in post-surgical tissues of SCC and AC patients were correlated with decreased levels of E-cadherin and increased levels of N-cadherin respectively, both markers being predictive of cellular migration [109]. Overexpression of β-catenin was associated with acquisition of resistance to gefitinib and the emergence of EGFR mutations [102].

5.3 Pharmacological targeting

Targeting different mediators of the Wnt pathway in experimental studies has shown promising results. One common mode of action of numerous newly identified pharmacological agents includes upregulation of Wnt pathway suppressors. Treatment with triptolide restored the expression of WIF-1 and four other inhibitory proteins by decreasing histone methylation levels which in turn decreased nuclear β-catenin levels [110]. Comparably, bisdemethoxycurcumin (BDMC) prevented TGF-β1-induced EMT transformation and migration of cells from the invasive NSCLC cell line 95D by upregulating WIF-1 expression [99]. TMU-35435 which functions as a histone deacetylase (HDAC) inhibitor successfully induced G_2/M stage arrest of four different NSCLC cell lines while leaving non-cancerous lung cells intact. Its mode of action was through upregulation of several genes encoding for Wnt inhibitors, possibly by catalyzing the presence of activating acetylation histone marks [111]. The antitumor effects of norcantharidin consisted of decreased methylation of WIF-1 gene promoters and increased expression of SFRP1. These molecular changes consequently decreased β-catenin translocation from the cytoplasm to the nucleus [112].

Another mode of action of pharmacological agents implemented in studies includes diminishing the resistance acquired to commonly employed chemotherapeutic agents. Using siRNA to block Wnt3, sensitized cells to 2μM cisplatin and led to significant levels of apoptosis [113]. Chloroquine resensitized cells to paclitaxel by generating ROS that led to AKT phosphorylation and subsequent inhibition of GSK-3β activity and Wnt signaling. This in turn led to anti-proliferative effects on cancer cells via inhibition of autophagy and diminishing of efflux pumps and other CSC markers, whereas lung fibroblasts remained unaffected [97]. Increased resistance to taxanes was noted due to prominent DNA methylation patterns in the intergenic region of SFRP1 in AC cell lines [114]. Overexpression of SFRP1 or β-catenin blockade using the antagonist FH535 restored sensitivity to taxanes by phosphorylating GSK3β. Clinical observations support these experimental findings since patients with SFRP1 positive tumors are more responsive to taxane therapy [114]. Cell lines resistant to erlotinib were found to have a two to four-fold increase in *c-met* signaling and two to eight-fold increase in Wnt signaling. Using 8 μM of three inhibitors, including XAV939 for Wnt signaling, resulted in a 95% growth inhibition of a drug resistant SCC cell line [115].

5.4 Novel mediators

Novel mediators that have emerged in the Wnt pathway and that have been previously shortly touched upon include methyltransferases and other epigenetic markers. DNA methyltransferase

Table 3. Antagonist families in the Wnt signaling pathway and their corresponding members

Antagonist family	Members
Secreted frizzled related protein (SFRP) [98]	SFRP1, SFRP2, SFRP3, SFRP4, SFRP5
Wnt inhibitory factors (WIF) [98, 99]	WIF-1
Dickkopf (Dkk) [100]	
Dvl antagonists [100]	Idax, human homolog of Dapper (HDPR1)
Miscellaneous	Cerberus, [100] Disabled-2, [100] Wnt-7a, [101] Frizzled-9 [101]

1 (DNMT1) can prevent the expression of Wnt-7a and Frizzled-9 which have tumor suppressive effects. The expression of the enzyme can be upregulated by carcinogens found in cigarette smoke and its effects can be counteracted by demethylating agents [101]. Furthermore, its inhibition via siRNA resulted in lower βcatenin levels in the cytoplasm and decreased EMT markers in the highly invasive 95D NSCLC cell line [116]. Another enzyme, histone methyltransferase G9a, which catalyzes H3K9 dimethylation, is closely related to DNMT1 activity by repressing gene expression and increasing the levels of Wnt3a. Its selective inhibition led to an upregulation of numerous tumor suppressor genes, including those encoding for APC, DKK and WIF1, the last two of which are involved in the pathway and the downregulation of the Wnt gene target *c-myc* [117]. Similarly, SETDB1, another H3K9 methylase, was overexpressed in tissues from NSCLC patients and it was observed to alter the expression of numerous genes involved in the degradation of βcatenin in three NSCLC cell lines. Its shRNA inhibition resulted in antitumor effects and increased cytoplasmic levels of βcatenin [118]. The effects of two DNA mehtyltransferases, namely, DNMT3A and DNMT3B which methylate the gene promoter of *Wif1,* could be reversed by treatment with miR-29s [119, 120].

Several miRNAs have emerged as promising novel regulators of cancer growth and progression in NSCLC. The overexpression of miR-181c was significantly higher in cells resistant to cisplatin and its blockade with anti-miR-181c led to a decrease in the cisplatin IC_{50} value by upregulating WIF-1 levels [121]. Similarly, miR-128-3p was associated with chemoresistance to first-line chemotherapeutic agents and metastasis via an overactivation of the Wnt and the TGF-β signaling pathways. Early on in the treatment with platinum-based chemotherapy, cells expressing the miR could migrate easily despite being treatment-naïve [122]. Cells overexpressing this miRNA further had an increased number of stemness markers, including CTR2 drug transporters. Silencing of the mRNA led to the silencing of three genes encoding for pathway antagonists, namely WIF1, SFRP2, and Axin1, and this in turn could prevent the migration of β-catenin to the nucleus [122]. Other miRNAs that have been identified in other studies include miR-367, which indirectly increased Wnt-1 signaling, miRNA-384 which decreased the overall signaling activity of the pathway and miRNA-376 which suppressed the agonistic activity of the liver receptor homolog-1 (LRH-1) [123, 124, 125].

6. Conclusion

The primary aim of the current review was to present the accumulating evidence of the involvement of the three major developmental signaling pathways in the initiation and progression of NSCLC. Three key findings emerged from this study. Firstly, overactivation of the three most common developmental signaling pathways (Shh, Notch and Wnt) is associated with shorter OS of patients and faster progression to more advanced stages of cancer via upregulation of processes like EMT, angiogenesis and metastasis. Key mediators include Gli-1, Gli-2 and fully palmitoylated Sonic Hedgehog ligand involved in the Shh pathway, Notch-1 and Notch-3 as tumor promoters in the Notch pathway and Wnt-2, Wnt-5 and WIF1 in the Wnt pathway. Secondly, all pathways have been experimentally targeted using numerous FDA-approved inhibitors or siRNA in experimental studies and such targeting has led to anti-proliferative and anti-invasive effects in NSCLC cell lines. Shh inhibitors can be synergistically combined with both chemo- and radiotherapy agents to sensitize resistant cells to conventional treatments. Likewise, Notch blockade was observed to have the same effects while Wnt inhibitors have only been explored in combination with chemotherapy. This supports the idea that antagonizing proteins of the developmental signaling pathways could provide a benefit for a subset of patients in which they are overexpressed. Lastly, numerous novel mediators with biomarker potential and therapeutic targetability in all three pathways, have been identified in recent studies and may represent a novel way of controlling pathway overactivation. Current ongoing clinical trials for two of the pathways are expected to provide insight into the possibility of extrapolating the success of NSCLC treatment with targeted therapies in pre-clinical settings to the first in-human studies.

7. Discussion

The current paper represents the first comprehensive review of the involvement of the three main developmental signaling pathways in NSCLC. As such, it can serve as a signpost for future research in the field of tackling NSCLC using targeted pharmacological therapies. Nonetheless, this study is subject to several limitations. Firstly, it does not explore the interactions among the three pathways in depth. Since numerous studies have discovered that crosstalk between these developmental pathways as well as crosstalk of these pathways with numerous others occurs in NSCLC, they should be incorporated in future systematic reviews. Additionally, due to the strict inclusion criteria followed, many research studies that were not published in the period between 2012 and 2019 were excluded, thus increasing the possibility of overlooking important findings. Moreover, since the Wnt signaling pathway is currently a widely explored topic in NSCLC, this study only presented selected evidence for its overexpression and involvement in this cancer subtype with the main focus on the underlying epigenetic regulators and mechanisms. Future studies that focus exclusively on one pathway can incorporate all pub-

lished findings and present an entire overview of their involvement in this cancer type. Lastly, many of the included studies used different tumor cell lines and drug concentrations and doses were often not reported which could further limit the extent to which some of the findings can be extrapolated. It is also worth mentioning that only a small number of studies investigated the role developmental signaling pathways play in large cell neuroendocrine carcinoma.

Future studies could consider several different aspects that have been explored in this review. Firstly, distinct NSCLC subtypes, should be observed and tackled differently; for instance, considerable differences exist between the response of SCC and AC cell lines to targeted therapies due to differential activation of the Notch pathway or the higher microvessel density present in AC [106]. Notably, as mentioned in a previous section of this thesis, Notch receptors have been found to have a higher predictive value for AC and this finding should be taken into account in future studies. On the other hand, while most studies found evidence for the important role that upregulated pathway mediators play in NSCLC prognosis and progression, many studies could not find such correlations. This indicates that more meticulously designed experiments need to be conducted to determine their role. As mentioned before, cross-talk between these developmental pathways should also be explored in-depth. For instance, Wnt3a was found to cross-react with Notch-3 to drive cellular migration and loss of adjunctions in the surrounding tissues[126, 127]. And such findings hint at the possibility of discovering numerous other interconnections. Furthermore, the correlation of the discussed pathways with other mediators involved in angiogenesis, EMT or metastasis can be exploited in multimodal treatment combinations. Such approaches can be used in individualized patient treatments (i.e. precision medicine) and are especially promising when dealing with acquisition of chemo- and radioresistance. *In vivo* systems should be employed more often to test such combinations and to establish drug tolerability when looking for the best equivalents to be implemented in human trials.

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Conflict of interest

The authors declare that they have no conflict of interest.

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