Notch: from fly wings to human hematological tumors

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SUMMARY

Notch history begins in 1919 with Thomas Hunt Morgan studies on fruit fly mutants. From then, this gene aroused lively interest in the scientific community since it is involved in a wide variety of processes, including morphogenesis, tissue homeostasis, and stem cell maintenance. Deregulation of Notch signaling characterizes several human tumors. Hematopoietic system is affected by mutations of Notch receptors, Notch ligands, and proteins controlling their stability. Approximately 60% T acute lymphoblastic leukemia (T-ALL) patients carry activating Notch1 mutations prompting blasts growth. In addition, multiple myeloma is characterized by Notch signaling hyper-activation due to an abnormal expression of the Jagged2 ligand; this affects not only myeloma cells, but also their interaction with bone marrow microenvironment, influencing tumor burden and bone disease. These findings make Notch a rational target of a therapeutic approach. Inhibitors of the Notch activating enzyme, γ-Secretase, have been successfully used in vitro and in vivo and are currently under clinical trials for T-ALL and breast cancer. Yet a wide use of these inhibitors is prevented by frequently occurring drug resistance. To elucidate the mechanism underlying this phenomenon, a number of pathways have been identified mediating Notch biological effects: AKT and c-Myc are frequently deregulated in leukemic patients and account for resistance to γ-Secretase inhibitors by acting downstream Notch receptor. Therefore, the interaction of Notch with other cancer-associated proteins should be clarified to predict the biological outcome of a Notch targeted therapy and possibly, to exploit combined treatments against the key deregulated elements in Notch-associated cancers.

Key words: *Receptors, Notch; Multiple Myeloma; Precursor Cell Lymphoblastic Leukemia-Lymphoma; Proto-Oncogene Proteins c-myc; Intracellular Signaling Peptides and Proteins; Enzyme Inhibitors; Amyloid Precursor Protein Secretases; Proto-Oncogene Proteins c-akt; PTEN Phosphohydrolase*

The history of Notch

The history of the Notch gene starts in the early 1900, just when the word *genetics* was coined by William Bateson (1905) and the concept of Mendelian inheritance was being rediscovered. In those years, the future Nobel prize winner Thomas Hunt Morgan began his study of mutations in the fruit fly *Drosophila melanogaster*. Morgan experiments discovered the basis of the modern science of genetics, by demonstrating that genes, intended as mechanical basis of heredity, were carried on chromosomes. During this study, Morgan first noticed a strain of *Drosophila* with a toothed (or notched) wing margin: this strain was called Notch (1).

Since 1958, the Notch locus was characterized genetically and phenotypically due to an array of mutations; heterozygous mutations yielded a dominant phenotype characterized by notched wings, thickened wing veins, and minor bristle abnormalities (2); recessive mutations could either be lethal, resulting in embryonic lethality and nervous system hypertrophy, or affect wing or eye morphology (3).

Only in 1983, Artavanis-Tsakonas and colleagues undertook the molecular analysis and sequencing of this gene by exploiting an inversion involving the Notch locus; in this way, they isolated chromosomal segments from the Notch region on chromosome 3C7 of Drosophila salivary gland (4).

Initial studies on Notch biological effects concerned its neurogenic role in Drosophila development (5).

In early 90's, it was demonstrated that Notch signal was well conserved in higher organisms including vertebrates and that it controlled the proliferation and differentiation of stem cells (6). In the same years, for the first time, Ellisen et al. (7) associated a translocation involving human Notch1 isoform with cancer: a small percentage of patients with T acute lymphoblastic leukemia carried the translocation t(7;9)(q34; q34.3). This genetic rearrangement resulted in the fusion of the 3' portion of Notch1 on chromosome 9 to the Jb joining region of TCR-β on chromosome 7 with the consequent overexpression of a truncated constitutive form of Notch1.

From then on, the interest of the scientific community produced a huge amount of information concerning the Notch signaling. Notch pathway resulted to be involved in a wide variety of processes, including normal morphogenesis, adult tissue homeostasis, and stem cell maintenance (8-10). Loss of function of components of this pathway causes inherited genetic diseases such as Alagille syndrome, spondylocostal dysostosis (SCD), and cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL; 11). On the opposite, upregulation of Notch activity has been associated with cancer in several cellular contexts with the notable exception of epidermal keratinocytes in which Notch plays a role as tumor-suppressor gene.

Structure and activation mechanism

The Notch family of genes is composed of four Notch receptor isoforms, Notch-1 to -4, and five Notch ligands, Jagged-1 and -2 sharing homologies with the Drosophila Serrate and Dll-1, -3, -4 homologous to Drosophila ligand Delta. Although the mammalian Notch receptors are structurally very similar, they have distinct functions and they are expressed at different stages of development and in different cells. All Notch receptors are synthesized as a single transmembrane polypeptide in the endoplasmic reticulum and subsequently are transported to the cell surface through the trans-Golgi network. During the transfer to the cell surface Notch undergoes post-translational modifications such as glicosylation and is cleaved by a furin-like protease producing a heterodimer.

The structure of the two Notch subunits is composed by different domains. The extracellular portion has a negative regulatory role and contains several tandem repeats related to epidermal growth factor (EGF). The EGF motifs are implicated in ligand binding.

Three copies of LIN12/Notch cysteine rich repeats located immediately downstream the EGF domain act in receptor activation, preventing signaling in the absence of ligand. Deletions or mutations in Notch repeats produce a constitutively active receptor.

The heterodimerization domain (HD), cleaved during the separation of the extracellular and the intracytoplasmatic portion, is responsible for the stable association of the two subunits through a non-covalent Ca^{++}-dependent bond (12).

The intracellular domain of Notch contains: the RAM domain, located downstream the transmembrane region, interacts with CSL transcription factors, Six ankyrin repeats, folded into a helix-loop-helix structure with a β-hairpin/loop region, which mediate further protein-protein interactions, and the C-terminal domain that carries two characteristic features: a polyglutamine region and a proline, glutamic acid, serine and threonine rich region, termed PEST, which contains the signal sequence for ubiquitination necessary to address the protein to proteosomal degradation (13).

As shown in Figure 1 Notch signaling occurs when the ligands, expressed on neighboring cell, bind and interact with Notch, inducing its activation as a result of two consecutive cleavages. The first is mediated by ADAM/TACE metalloproteases, whereas the second occurs intramembranously and is mediated by γ-Secretase (14). This releases the intracellular domain of Notch (ICN) from the membrane and allows it to translocate into the nucleus. Nuclear Notch interacts with the transcriptional factor CSL (from CBF1 in mammals, Su(H) in flies, and LAG-1 in C.elegans) and activates downstream target genes involved in proliferation including c-myc, p21, p27, CycD1 (8,9,15,16), in cell migration as chemokine receptors, CCR4, CCR8 and CXCR6 (17) and CCR6 (18), cytokines or their receptors including IL-6 (19) and IL-8 (20).

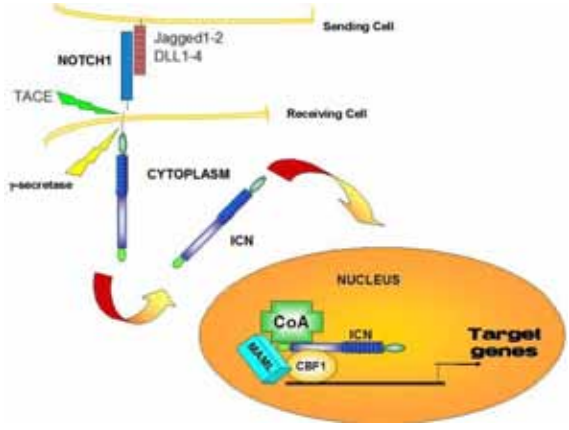

Figure 1. Mechanisms of Notch signaling
Signal-sending cell expresses jagged or Dll ligands that trigger Notch heterodimer dissociation. This event results in two sequential proteolytic cleavages, the first by TACE metalloproteinase, and the second by γ-secretase. The latter frees Notch C-terminus from the plasma membrane, allowing it to translocate to the nucleus. Here, intracellular Notch (ICN) binds to CBF-1 transcription factor and recruits co-activators (CoA), among which Mastermind-like proteins (MAML). Such ICN-dependent transcriptional complex induces the expression of target genes (i.e. c-Myc, Hes1, pre-TCRα)

Notch receptors in malignancies of the hematopoietic system

Notch proteins act as developmental morphogens. They are widely expressed in all self-renewing tissues and they can regulate cell proliferation and survival, or activation of differentiation mechanisms in a context-dependent manner. Since Notch is critical in many fundamental processes, it is not surprising that aberrant gain or loss of Notch function could be directly associated with multiple human disorders, as developmental diseases or cancer (21). Among hematological malignancies, Notch gain-of-function mutations or abnormal activation has been clearly linked to T-cell acute lymphoblastic leukemia (T-ALL) (22), and recently to multiple myeloma (23-24). Moreover, a role for Notch receptors has been proposed, but not univocally shown in acute myeloid leukemias (AML) (25-26) and lymphomas (27-28). This work will review Notch role in T-ALL and MM, focusing on the interplay with other signaling pathways.

Notch1 in T-ALL

In human cancers, as reported, Notch1 was firstly identified in T-ALL as a partner in the t(7;9)(q34;q34.3) translocation, occurring in less than 1% of T-ALL cases (7).

To further elucidate Notch1 role in oncogenesis, the group led by David Baltimore (Nobel Laureate in Medicine in 1975) obtained the first direct evidence that the truncated Notch1 plays a causative role in the development of T-ALL (29). They used retroviral constructs to express Notch1 gene truncated in three different positions, encoding proteins that resembled polypeptides found in cells bearing the t(7;9). BALB/ cByJ bone marrow cells were retrovirally transfected with these Notch1 vectors, then transplanted into lethally irradiated syngeneic mice. After a variable period of latency, roughly 50% of mice showed sudden cachexia, abnormal white blood cell counts, and leukemic blasts in the peripheral blood. All tumors was composed of immature T cells blocked at different maturation levels and, noteworthy, high level expression of the proteins encoded by the transgenic Notch1 cDNAs in tumors occurred with a frequency of 100%.

As genetic studies went on, Notch1 gene was found to be mutated in nearly the 60% of human T-ALL (22,30). Two major mutation hot-spot domains were identified: HD (heterodimerization domain, between exons 26 and 27) and PEST (in exon 34). HD mutations are single aminoacid substitutions or in-frame deletions/insertions and weaken the association of the heterodimer, allowing ligand- independent activation of Notch (31). PEST mutations are out-of-frame insertions or deletions producing premature stop codons: the resulting truncated proteins lack the proteasome-targeting sequence and display increased half-life. Recently, a new class of Notch1 activating mutations were identified and functionally characterized in human T-ALL. They are called JME mutations and consist of expansions of the extracellular juxtamembrane region receptor caused by internal tandem duplications of exon 28 and adjacent intron (32). Notably, all known Notch1 mutations still require γ-secretase cleavage to trigger intracellular signals.

Exploiting Notch receptor as a pharmacological target in T-ALL

The high occurrence of Notch hyper-activation in T-ALL suggests the use of anti-Notch drugs as a promising therapeutic strategy in these tumors. As previously described, Notch1 activation requires two proteolytic cleavages

to release the ICN that in turns translocates to the nucleus where it binds the CBF1 transcription factor and MAML family coactivators, triggering the expression of target genes. Pharmacological interventions against Notch signaling could target any of these steps, but blocking the final γ-Secretase-mediated cleavage appears the most promising strategy.

γ-Secretase is an intramembranous aspartyl protease initially identified as responsible for the cleavage of the amyloid precursor protein and the abnormal production of neurotoxic amyloid-β peptide in Alzheimer's disease (AD) (33). Thus, the first γ-Secretase inhibitors (GSIs) were developed as a new therapeutic approach for the treatment of Alzheimer's disease. Since most of the GSIs also affect Notch cleavage, their use as anti-tumor compounds was suggested. However, the first data about GSI anti-cancer activity were not obtained in leukaemias, but in solid tumors. Curry et al. (34) demonstrated that GSI-I triggers apoptosis in primary and immortalized Kaposi Sarcoma cells overexpressing activated Notch-1, -2, and -4; Hallahan et al. (35) demonstrated that Notch pathway inhibition with GSI-IX resulted in a reduction of cell viability of human medulloblastoma cell lines and primary tumor cultures.

GSI efficacy in acute leukemia firstly came from the study of Weng et al. (22): they found that a number of human T-ALL cell lines showed a G_0/G_1 cell-cycle arrest following GSI- XXI administration for 4- 8 days. However, since these first studies it was clear that only a few cell lines were responsive to GSI treatment, as only 5 out of 30 tested T-ALL cell lines responded to the treatment independently from Notch1 mutations.

These initial observations were supported by several other works. In the study by De Keersmaecker et al. (36), T-cell acute lymphoblastic leukemia cell lines were treated with GSIs or combinations of GSIs with standard chemotherapies or glucocorticoids. Results indicated that GSI induced growth arrest in 5 out of 8 tested lines. The study of Kogoshi et al. (37) confirmed these data focusing on apoptotic response: they showed that, in GSI- sensitive cells, the treatment triggered an increase in apoptosis rate. These studies indicate that GSIs are effective in only 10% of T-ALL cell lines and that cell response to GSIs depended on Notch1 mutational status.

Since lack of response to GSIs prevents their use for therapeutic purpose, efforts have been made to elucidate the mechanisms of such resistance.

Studies in murine models of Notch-induced T-cell leukemia and T-cell precursors differentiation have identified several signaling intermediates including pre-T-cell receptor, Lck, protein kinase C, phosphatidylinositol 3-kinase (PI3K), Akt/protein kinase B, extracellular signal-regulated kinase (ERK) and nuclear factor B (NFk-B), as possible downstream regulators of Notch (38-41). Among these, two pathways seem to play a major role in T-ALL by transducing Notch proliferative signals: c-myc and PI3K/AKT.

In 2006, Weng and coworkers (42) identified c-Myc as a direct Notch1 transcriptional target. Moreover, they demonstrated that c-Myc inhibition interferes with the pro-growth effects of oncogenic Notch1, while enforced expression of c-Myc rescues Notch1-dependent T-ALL cell lines from Notch withdrawal. Later on, the group of Paul J. Utz (43) compared the effect of GSI-XXI treatment on the phosphorylation status of 82 signaling proteins in sensitive and resistant cells through a reverse phase protein microarray profiling. They showed that GSI-sensitive cell lines mainly display changes in the phosphorylation of protein belonging to the mTOR pathway prior to the onset of G_0/G_1-arrest. mTOR, in combination with other proteins, forms the complexes mTORC1 or mTORC2. It is a serine/threonine kinase, which in response to growth factors and nutrients, regulates cell growth and cell cycle progression by the phosphorylation of downstream effectors and through translation control (44). Since variations of mTOR pathway occurred independently from Akt inhibition and the enforced expression of c-Myc had reversed GSI-induced down-regulation of mTOR, the authors proposed a model in which Notch stimulation of mTOR requires c-Myc activity and occurs independently from Akt.

The importance of c-Myc pathway as intermediate in Notch oncogenic activity makes this pathway a possible target of activating mutations that would act downstream and independently from Notch. Indeed, such mutations exist, affect c-Myc stability and, as expected, their presence in T-ALL cells induces resistance to GSI treatment. In fact, O'Neil and colleagues showed that T-ALL resistant cell lines harbored FBW7 gene inactivating mutations or homozygous deletion (13). This gene encodes an ubiquitin ligase required for both c-Myc and ICN degradation, thus acting as a tumor-suppressor. The presence of FBW7 mutations in GSI-resistant cell lines suggested that GSI resistance raised from stabilization of both ICN and its principle downstream target c-Myc. FBW7 mutations are clinically relevant since they have been identified in about 30% T-ALL patients (45).

c-Myc stability can also be affected in T-ALL through epigenetic modification: we recently found that the PPP2R3A gene, encoding the regulatory subunit B of protein phosphatase 2 alpha (PP2A) was silenced by hyper-methylation in 69% T-ALL patients (46). PP2A plays a role in post-translation modification of c-Myc by dephosphorylating Ser62 which makes c-Myc a suitable substrate to FBW7 activity (47). In addition, PP2A affects AKT signaling, the other Notch-related pathway, through Thr308 dephosphorylation: this modification is associated with high-risk acute myelogenous leukemia (48).

The most relevant information concerning the role of AKT pathway in T-ALL resistance comes from Palomero and co-workers (49); they showed that forced expression of a constitutively active form of AKT in the GSI-sensitive cells rescued them from the growth-inhibitory effects of GSI. Moreover, the analyzed GSI-resistant cells lacked the expression of the tumor-suppressor PTEN (an inhibitor of AKT activation) owing to frameshift mutations, and, accordingly, exhibited an abnormal activation of the PI3K/Akt pathway compared to the GSI-sensitive, PTEN-positive cell lines. The authors argued that aberrant activation of the PI3K-AKT signaling, due to PTEN mutations, induced resistance to NOTCH1 inhibition. In accordance, shRNA anti-PTEN made sensitive cells resistant to GSI. These data have a clinical relevance since the authors found loss of PTEN protein in 17% T-ALL patients.

Overall, these studies indicate that the function of c-Myc and AKT in the control of human T-ALL cell proliferation is an important and still unsettled issue and that a possible effective Notch-based therapy for T-ALL will have to take into account their possible deregulation in each single T-ALL patient. Most likely, an effective therapy should be tailored as a combined therapy targeting those pathways specifically deregulated in each subgroup of patients.

Notch role in multiple myeloma: the importance of the microenvironment

Multiple myeloma (MM) is a hematological malignancy resulting from a clonal proliferation of plasma cells in the bone marrow. Hematopoietic lineages development and differentiation are largely under Notch control: under physiologic conditions, hematopoietic stem cells express Notch receptors, whereas bone marrow (BM) stromal cells express Notch ligands. This microenviron-

ment provides signals for stem cell survival and differentiation, which are opportunely exploited by MM cells.

The complex interactions with BM stromal cells are at the basis of features characterizing this malignancy and they are responsible for MM cell proliferation, chemoresistance and bone disease. This explains why, although there have been major advances in the treatment of MM in recent years, it still remains largely incurable.

Recently Notch activation has been described in MM. MM cells can autonomously activate Notch signaling through homotypic interactions since they express both Notch receptors, Notch-1, -2, and -3, and their deregulated ligands (Figure 2). Indeed overexpression of Notch ligand Jagged 2 was observed in MM cells due to promoter hypomethylation (23) or overexpression of Jagged2-specific ubiquitin-ligase skeleotrophin (50).

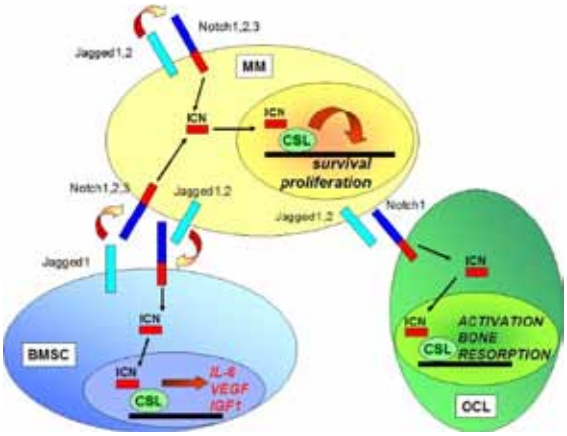

Figure 2. The Notch signaling in multiple myeloma
Multiple myeloma cells trigger Notch activation through Jagged ligands on themselves or on neighboring bone marrow stromal cells (BMSC) and osteoclasts (OCL). In turns, Jagged 1 expressed by BMSC can activate Notch on MM cells. Notch signal outcome is different depending upon the cell type: in MM cells, it induces proliferation and expression of anti-apoptotic factors, in BMSC, it supports the production of myeloma-promoting factors as IL-6, VEGF, and IGF-1, while in OCLs it results in activation and immoderate differentiation

Although Notch ligands can be detected on MM cells, they are abundantly expressed by BM stromal cells and BM macrophages (51): consequently, also these stromal cells can activate Notch signaling in MM cells through heterotypic interactions (Figure 2).

The outcomes of Notch signaling activation in MM cells are promotion of proliferation, inhibition of apoptosis, and decreased sensitivity of MM cells to chemotherapeutics. At molecular level, stimulation through Jagged2 results in secretion of IL-6, vascular endothelial growth factor (VEGF), and insulin-like growth factor (IGF), three relevant inducers of MM cell survival and growth (23).

Notch signaling plays a role also in the communication from MM to stromal cells (Figure 2); Jagged2-overexpressing MM cells activate Notch signaling in Notch-bearing pre-osteoclasts and osteoclasts, inducing differentiation, activation, and secretion of pro-inflammatory mediators that increase tumor burden and osteolytic bone lesions (26,52).

Several soluble or membrane bound factors relevant in MM, resulted to be under Notch signaling control, albeit in different cellular context:

i) CXCR4, controlled by Notch1 in bone marrow-derived dendritic cells and endothelial cells (53,54), promotes different outcomes as MM cells recruitment in BM (55), due to directional migration toward BM stromal cells secreting the ligand chemokine SDF-1, and transendothelial migration, promoted by cell adhesion to the endothelium (56); also, SDF-1 increases the proliferation of both MM cell lines and primary MM cells, while protecting them against dexamethasone-induced apoptosis (57).

ii) Notch1 was shown to upregulate CCR6 expression in human Langerhans cells development from blood monocytes (18). In MM malignancy, the axis MIP3α/CCR6 contributes to *in vitro* osteoclasts differentiation and bone lesions development in MM patients as shown by the detection of significantly higher MIP3α levels in MM patients with bone involvement versus those without osteolytic lesions. MM cells produce MIP3α rarely, nevertheless they are able to promote its secretion, and the expression of its receptor CCR6 by BM osteoprogenitor cells (58).

iii) IL-8, whose expression is positively regulated by Notch1 in bone inflammatory disease (20), is a potent activator of osteoclastic differentiation and bone resorption (59) and a growth and chemotactic factor for MM cell lines and patient plasma cells (60).

iv) Matrix metalloproteinases (MMPs) are often implicated in tumor invasion, metastasis, and tumor-stroma interaction. Notch was reported to play a role in the regulation of MMPs in pancreatic cancer cells (61) and in inflammatory settings as osteoarthritic disease (20). Bone marrow stromal cells of MM patients frequently display matrix metalloproteinases MMP-1 and MMP-2 overproduction. (62). Also myeloma cells have been reported to produce MMP-9 and MMP-2, involved in degradation of collagen IV, the major constituent of the basement membrane (63). The relevance of MMPs expression in MM has been demonstrated through the 5T2 MM mouse model, closely resembling human MM: the application of metalloproteinase inhibitors resulted in a significant reduction in the tumor burden, a significant decrease in angiogenesis, and a partially protective effect in the development of the osteolytic bone disease (64).

Compared to the studies of Notch role in T-ALL, the findings about Notch activity in myeloma are far more recent and less abundant. Nonetheless, a number of in vitro works evaluated the effect of GSI on MM tumor growth and osteoclastogenic activity. The recently synthesized GSI-15 (RH02015SC, Maybridge, Acros Organics, Belgium) reduced the proliferation and induced apoptosis in MM cells cultured alone or co-cultured with OCL. Noteworthy, GSI completely abolished the increase in OCL activity induced by MM cells (52). Nefedova and colleagues (65) reported an analogous effect of Notch signaling on MM cell lines showing that forced retroviral expression of only Notch-1, but not Notch-2, protected tumor cells from melphalan- and mitoxantrone-induced apoptosis. Nevertheless, in disagreement with the above reported proliferative effect of Notch, in this case Notch-induced resistance to apoptosis resulted to be associated with up-regulation of p21WAF/Cip and growth inhibition of cells. Recently the same group displayed GSI anti-tumor effect using xenograft and SCID-hu model of MM; furthermore, they presented *in vivo* data indicating that GSI prevents BM-mediated drug resistance and sensitizes MM cells to chemotherapeutic drugs doxorubicin and mephalan.

In conclusion, although T-ALL and MM display different pictures at molecular and cellular level, the occurrence of Notch signaling deregulation in these malignancies makes this pathway a rational and promising target for anti-cancer therapy. Nevertheless, several clarifications are still necessary concerning the interaction of Notch with other cancer-associated proteins

and the possible biological outcomes. This would allow predicting the efficacy of a therapy addressed to Notch inhibition and possibly to plan combined treatments targeting the key deregulated elements in different patients subsets.

Conflict of interest

We declare no conflicts of interest.

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