CHAPTER II

LITERATURE REVIEWS

2.1 *Notch* and Notch signaling

Notch genes encode heterodimeric transmembrane receptors that regulate differentiation, proliferation and apoptosis in vertebrates (Artavanis-Tsakonas *et al.*, 1999). A mammalian family of Notch receptors consists of four Notch proteins (Notch1-4) and five ligands, i.e. Jagged1, Jagged2, Delta-like1 (Dll1), Dll3 and Dll4. The extracellular domain of Notch receptors contains multiple epidermal-growth-factor (EGF)-like repeats and a unique cysteine-rich Notch/Lin12 domain (LN). An intracellular domain contains the RAM domain and six ankyrin repeats (ANK, also known as CDC10 repeats), two nuclear-localization signals (NLSs), followed by the transactivation domain (TAD) and a PEST sequence. The mammalian Notch ligands contain an amino-terminal structure called DSL (Delta, Serrate and LAG-2), which is common to all family members, followed by EGF-like repeats. Jagged1 and Jagged2 have a cysteine-rich domain (CR) following the EGF-like repeats (Figure 2.1) (Radtke and Raj, 2003).

Upon receptor-ligand interaction, the Notch receptors are subjected to proteolytic processing. The first cleavage is mediated by TACE (tumour-necrosis factor-α-convering enzyme/ metalloproteinase), followed by a second cleavage mediated by the γ-secretase. As a result, the intracellular domain of Notch (ICN) is released from the cell membrane and enters the nucleus, where it binds to a transcription factor CSL (CBF-1 in mammals, suppressor of hairless in *Drosophila* and LAG-1 in *Caenorhabditis elegans*). ICN displaces co-repressors (CoR) and recruits co-activators (CoA) to CSL, leading to transcriptional activation of downstream target genes (Radtke and Raj, 2003). Mastermind-like (MAML) proteins family are required for stabilization of Notch/CSL/CoA complex (Figure 2.2).

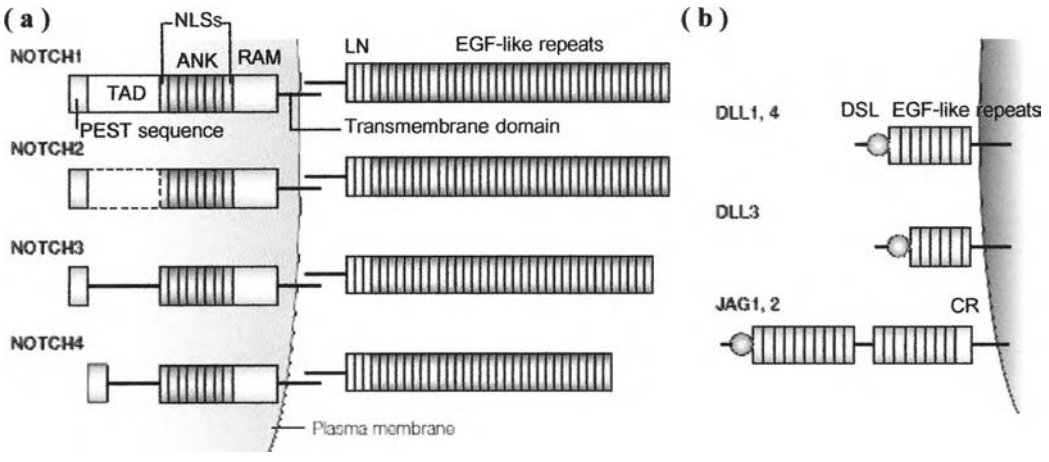

Figure 2.1 Structure of Notch receptors (a) and Notch ligands (b). The extracellular domain of Notch receptors contains multiple EGF-like repeats and LN. The intracellular domain contains the RAM domain and six ANK, two NLSs, followed by TAD and a PEST sequence. Notch ligands contain DSL, EGF-like repeats and CR (found in Jagged 1 and 2). (modified from (Radtke and Raj, 2003))

2.2 Mastermind-like (MAML)

The human MAMLs, a family of transcriptional activator proteins, consists of three members (MAML1-3). Each MAML contains an N-terminal basic domain and two acidic domains (I and II) in their middle region and the C-terminus. The basic domain show highest sequence identity, which contains all ICNs binding site. Two acidic domains consist of TAD1 (aa 75-300) and TAD2 (aa 303-1016). TAD1 contains a p300/CBP binding site and recruits this co-activator molecule to the Notch transcriptional complex, while TAD2 is a glutamine-rich region that is required for transcription *in vivo* (Figure 2.3) (Wu and Griffin, 2004).

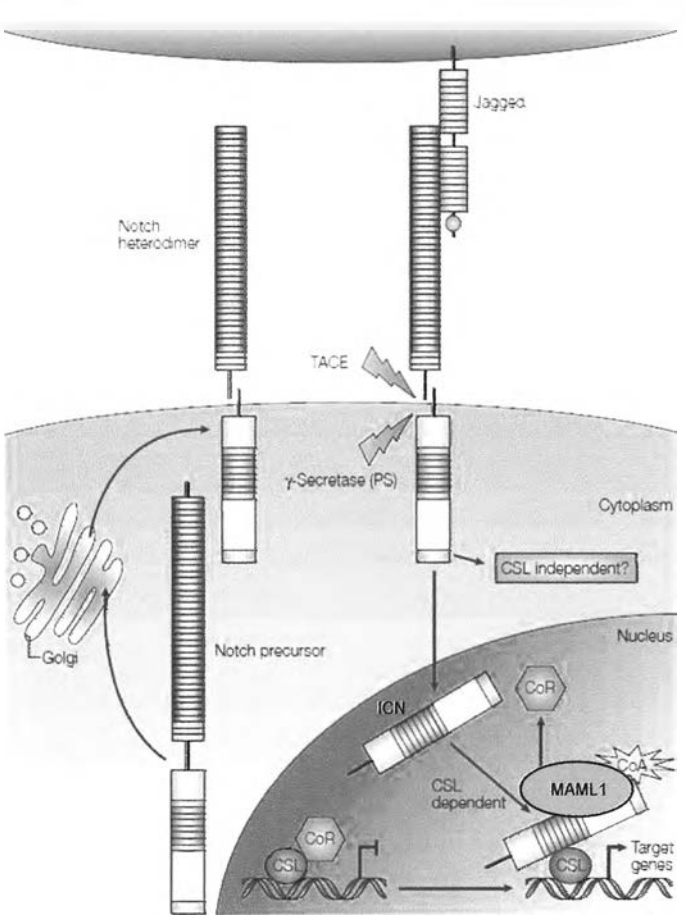

Figure 2.2 Notch signaling. Interaction of Notch receptors with their ligands induces two sequential proteolytic cleavages. The first cleavage within the extracellular domain is mediated by TACE. The second cleavage occurs within the transmembrane domain and is mediated by the γ-secretase. The liberated ICN enters the nucleus and binds to the transcription factor CSL. This interaction leads to transcriptional activation of Notch target genes by displacement of CoR and simultaneous recruitment of CoA. (modified from (Radtke and Raj, 2003))

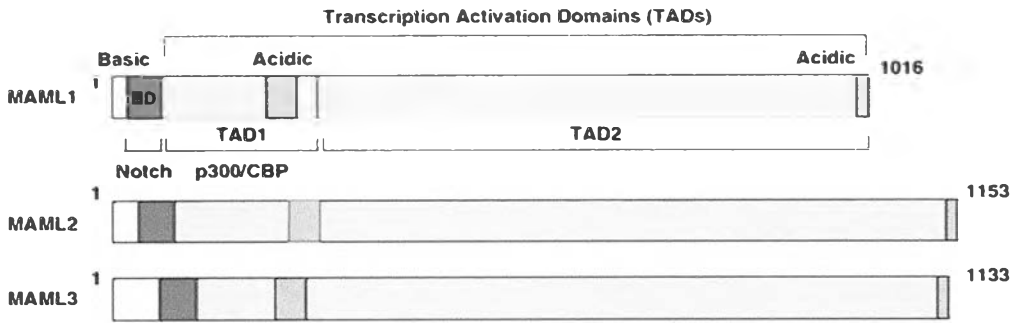

Figure 2.3 Structure of MAML proteins. The human MAML family consists of three members: MAML1, MAML2 and MAML3. They contain an N-terminal basic domain (BD) and two acidic domains (TADs). BD is responsible for interactions with the ankyrin domain of the Notch receptors. There are two TADs: TAD1 contains the CBP/p300-binding site and TAD2, the activities of which are required for Notch signaling *in vivo*. (McElhinny *et al.*, 2008)

MAMLs form a ternary complex with CSL-ICN through their N-terminal conserved basic domains, leading to transcriptional activation of downstream target genes. They interact with CSL only in the presence of ICN, forming a stable DNA-binding ternary complex. MAMLs activate transcription of Notch target genes when Notch receptors are stimulated by ligands, or when constitutively active forms of Notch ICN are expressed. One of the best characterized Notch targets is the HES (hairy/enhancer of split) family of transcription factors (Bailey and Posakony, 1995) and c-myc (Satoh *et al.*, 2004, Weng *et al.*, 2006).

Originally, MAML1 was defined as a transcriptional co-activator for Notch signaling activation through interactions with Notch and CSL. Recently, however, its coactivator activities for other transcription factors were also revealed, including MEF2C (Shen *et al.*, 2006), p53 (Zhao *et al.*, 2007), β-catenin (Alves-Guerra *et al.*, 2007) and NF-κB (Jin *et al.*, 2010). These studies indicated that a co-activator function of MAML1, independent of its function as a co-activator of Notch signaling pathway, exists.

2.2.1 Dominant negative mastermind-like1 (DN-MAML1)

There was several reports studied the roles of MAML family members in modulation of Notch signaling. One effective tool is a dominant negative MAML1 mutant, DN-MAML1. It consists of 62 amino acid peptide of basic domain in the N-terminus of MAML1 but lacks two TADs, therefore the ability to form complex with Notch and CSL remains in DN-MAML but it is deficient in transcriptional activation (Figure 2.4). Since MAML binds to all Notch receptors in a similar manner, DN-MAML1 is pan Notch inhibitor and inhibits transcriptional activation from all Notch receptors (Weng *et al.*, 2003).

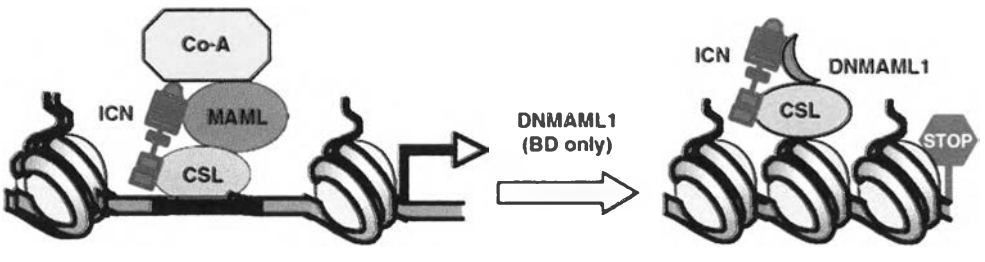

Figure 2.4 Inhibition of Notch-mediated transcription by expression of a dominant negative MAML1 mutant, DN-MAML1. DN-MAML contains the BD but lacks the entire TADs, therefore the ability to form complex with Notch and CSL remains but it is deficient in transcriptional activity. (McElhinny *et al.*, 2008)

DN-MAML has been useful in investigating the role of Notch signaling in multiple cellular processes. Weng *et al.* found that the suppressive effects of Notch signaling by DN-MAML1 induced the growth arrest of SUP-T1, a human T-ALL cell line (Weng *et al.*, 2003). The expression of DN-MAML1 in bone marrow cells interfered with Notch signaling, resulting in inhibition of both the early T-cell differentiation and marginal zone B cell generation (Maillard *et al.*, 2004). These data suggested that the role of MAMLs were essential in the T/B lineage decision, as Notch signaling is known to govern this process (Maillard *et al.*, 2004).

2.3 The role of Notch in tumorigenesis

Notch signaling plays a key role in the differentiation of various types of cells. Some studies suggested that Notch can function as a tumor suppressor gene on the other hand, Notch may act as an oncogene or tumor promoter gene.

Aberrant Notch signaling is linked to cancers. In human, the oncogenic role of Notch was first identified in T-cell neoplasia. Aberrant *Notch1* has been reported as a causative factor in the development of T-cell acute lymphoblastic leukemia and lymphoma (T-ALL) (Ellisen *et al.*, 1991). Human breast cancer cell lines and tumor tissue samples exhibited accumulation of NICD (Stylianou *et al.*, 2006). Overexpression of Notch1 and Jagged1 are observed in B- and T-cell–derived tumor cells of Hodgkin and anaplastic large cell lymphoma (Jundt *et al.*, 2002). These studies suggested that Notch1 was activated by Jagged1 and this activation may be a causative factor in the development of Hodgkin lymphoma (Jundt *et al.*, 2002).

A tumor suppressive role for Notch signaling has been observed in B-cell acute lymphoblastic leukemia cells. Mammalian truncated form of all Notch receptors (ICN1-4) induced growth arrest and apoptosis in B-cell malignancies and Hes1 was identified as an important mediator of Notch signaling in inducing B-cell death (Zweidler-McKay *et al.*, 2005). Interestingly, elevated levels of Notch2 protein is shown in human breast cancer which correlated with increased patient survival, whereas elevated levels of Notch1 was associated with reduced patient survival (Parr *et al.*, 2004). This study suggested that Notch1 may be oncogenic, whereas Notch2 may be tumor suppressive.

2.3.1 Notch signaling in cervical cancer

Infection by high-risk human papillomavirus (HPV) (such as HPV type 16 , HPV-18, and HPV-31) and continued expression of the viral *E6* and *E7* genes are causally linked to the progression of human cervical cancers, a major subset of neoplasia in women worldwide (zur

Hausen, 1996). Several research papers indicated that Notch signaling is associated with HPV cervical cancer but the role of Notch signaling in cervical cancer remains controversial.

2.3.1.1 Oncogenic Notch signaling in cervical cancer

For the oncogenetic functions, Weijzen *et al.* shown that in Ras transformed human cell lines, Notch1 is a downstream effector of oncogenic Ras, overexpression and deregulation of Notch signaling are necessary to maintenance of the transformed phenotype in Ras-transformed human cell lines (Weijzen *et al.*, 2002). Chakrabarti *et al.* found that activated MAPK signaling cooperates with deregulated Notch signaling to recreate features of HPV-driven invasive cervical carcinomas. In addition, E6 amino acid 83 variants enhance MAPK signaling through a Ras-dependent. These variants inhibit oncogenic Ras-mediated transformation that are potentially permissive for cervical tumor progression (Chakrabarti *et al.*, 2004). Nair *et al.* showed that in HaCaT-activated Notch1 (AcN1) cells, AcN1 inhibited p53-induced apoptosis and is dependent on a PI3K-PKB/Akt-dependent pathway as mediator of apoptosis resistance (Nair *et al.*, 2003b). In contrast, Alimirah *et al.* suggested that p53-mediated upregulation of Notch1 expression in human cancer cell lines contributes to cell fate determination after genotoxic stress (Alimirah *et al.*, 2007). Veeraraghavalu *et al.* suggested that Manic Fringe (MFng), a negative regulator of Jagged1-Notch1 signaling, is linked to activation of the Notch pathway in the progression of HPV-associated cervical neoplasia. They found high levels of Jagged1 expression and this is correlated with absence of Manic Fringe in the cervical tumor-derived cell line CaSki (Veeraraghavalu *et al.*, 2004). Furthermore, they compared the expression patterns and the role of two Notch ligands, Jagged1 and Delta1 in the progression of human cervical cancer. They found that Jagged1 is preferentially upregulated in human cervical tumors, sustains transformation by HPV 16 E6-E7 oncogenes, generates resistance to anoikis. Induction of PI3K by Notch signaling is independent on CSL and is linked to the function of Deltex1 (Veeraraghavalu *et al.*, 2005).

The other cell signaling pathway that has been shown to be disregulated in human cervical cancers is NF-κB (Prusty *et al.*, 2005). NF-κB complexes are either homo or

heterodimers, of which NF-κB p50 and p65 are the major active form. They are sequestered in the cytoplasm by their inhibitory proteins IκBs (IκB-α, IκB-β, IκB-ε), of which the function of IκB-α is well characterized. Phosphorylation of IκB-α at serines 32 and 36 by the IκB kinase (IKK) complex results in its ubiquitination and subsequent proteasome mediated degradation, leading to translocation of NF-κB to the nucleus where it can turn on transcriptiontion of target genes. Increased NF-κB signaling can result in blocking of apoptosis, increasing proliferation or inducing epithelial-mesenchymal transition through induction of various target genes. Some of the downstream target genes of NF-κB are c-Myc, VEGF, IL-6, IL-8, Bcl-2, Bcl-XL, CyclinD1 and IκB-α. Ramdass *et al.* studied both Notch and NF-κB pathways in human cervical cancer CaSki. They found that these two signaling components coexpressed and colocalized intracellularly. NF-κB is proposed to be downstream of Notch signaling (Figure 2.5) (Ramdass *et al.*, 2007). Yu *et al.* used small interfering RNA to block Notch1 and PS1, a core component of γ-secretase. They demonstrated that RNA interference–mediated silencing of Notch1 or PS-1 blocked the Notch signaling and inhibited the growth of tumor cells both *in vitro* (HeLa) and *in vivo* (Yu *et al.*, 2007a).

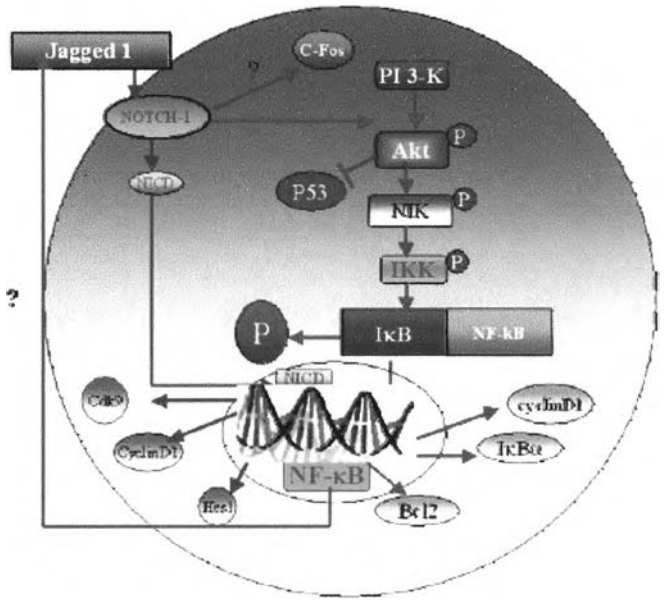

Figure 2.5 Possible cross talk between Notch, NF-κB and PI3K-AKT pathways. The activated NF-κB pathway might be helping tumor progression by increasing proliferation or by blocking apoptosis through the induction of target genes like *cyclinD1* and *Bcl2*. Schematic diagram showing the possible cross-talk of these pathways that support neoplastic transformation. (Ramdass *et al.*, 2007)

2.3.1.2 Tumor suppressive Notch signaling in cervical cancer

For the tumor suppressive functions, Talora *et al.* showed that high levels of Notch1 can be tumor suppressive and Notch1 exerts specific protective effects against HPV-induced transformation through suppression of *E6/E7* expression. This is mediated through up-regulation of Fra-1, decreasing c-Fos expression and subsequent suppression of activator protein (AP)-1 activity. Down-modulation of Notch1 expression may have an important role in late stages of HPV-induced carcinogenesis (Talora *et al.*, 2002). In addition, they showed that in cervical cancer cell lines, activated Notch1 suppresses activity of the helix–loop–helix transcription factor E47, via ERK1/2 activation, resulting in inhibition of cell cycle

progression. They suggested that activation of Notch signaling in cervical cancer cells results in cell cycle arrest (Figure 2.6) (Talora *et al.*, 2005).

Yao *et al.* found that activated Notch1 results in growth inhibition of HeLa by inducing G2-M cell cycle arrest and apoptosis and the growth inhibition was correlated with inhibition of NF-κB p50 activation, these results suggested that NF-κB inhibition may be involved in antitumor mechanisms of Notch1 activation (Yao *et al.*, 2007). Wang *et al.* determined whether increased Notch1 signaling inhibits or stimulates the process of transformation. They found that overexpression of active Notch1 (ICN) significantly inhibited the *in vitro* growth of cervical carcinoma cells through suppression of AP-1 activity (Wang *et al.*, 2007). Thus, Notch1 signaling plays a key role and exerts dual effects, functioning in context-specific manner.

2.4 MAML family members linked to cancer

The MAML proteins are essential regulatory proteins in Notch signaling. They are linked to cancer such as mucoepidermoid carcinoma, T cell leukemia and cervical cancer.

2.4.1 MAML2 and mucoepidermoid carcinoma

Mucoepidermoid carcinoma (MEC) (Adamec *et al.*, 2006) arises from major and minor salivary glands throughout the upper aerodigestive tract. There are two main histological components of the tumor: mucous cells that contain intracellular mucin and epidermoid (squamous type) epithelial cells. MEC shows an unusual pattern of mixed cell differentiation. Wu *et al.* cloned the chromosomal breakpoints of the t (11;19) (q14–21;p12–13) translocation and identified the genes involved in this translocation, MAML2 and MECT1. They found that MECT1-MAML2 alters Notch signaling and possibly contributes to tumorigenesis in the MEC (Tonon *et al.*, 2003).

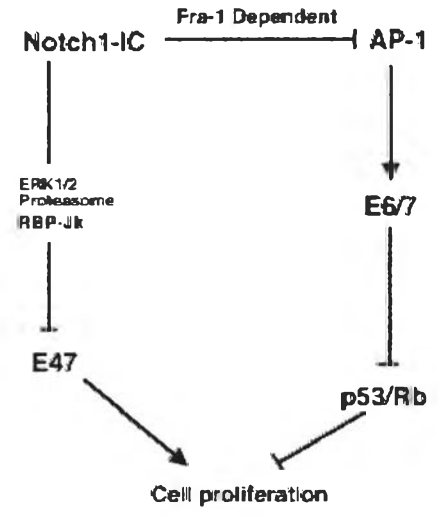

Figure 2.6 A model of Notch1-mediated growth arrest in cervical cancer cells. Notch1 signaling inhibits AP-1 activity, through a Fra-1-dependent mechanism, which results in reduction of HPV E6/E7 expression. The loss of the E6 and E7 proteins, which otherwise target p53/Rb tumor suppressor proteins for accelerated proteasome-mediated degradation, reactivates these dormant tumor suppressor pathways. Additionally, and independently from the latter pathway, Notch1 targets E47 protein for degradation, thereby depriving cancer cells of a key mediator of progression through the cell cycle. The repression of E47 also resulted in a positive feedback loop that reinforced the Notch1-mediated growth inhibitory signal. These two pathways appear to proceed in parallel to each other and each of them is sufficient to inhibit cell proliferation. However, both pathways may be activated simultaneously. (Talora *et al.*, 2005)

2.4.2 Modulating Notch signaling via the MAML family suppresses growth of Notch-dependent leukemia cells

Weng *et al.* found that a MAML1 dominant negative peptide that contains MAML1 (13–74 aa) fused to GFP, which was able to form a DNA-binding complex with RAM-ANK and CSL, strongly inhibits CSL-dependent transcription and the effects of this dominant

negative mutant caused a significant growth suppression of Notch1-transformed lymphoid cell lines expressing membrane-tethered Notch1, or ICN1, or TAN1 from t (7;9) translocation, but not the growth of other non-Notch1 transformed cell lines (Weng *et al.*, 2003).

2.4.3 MAML proteins and cervical cancer

Wu *et al.* cloned MAML1 as a binding protein for high-risk HPV type 16 E6 in a yeast two-hybrid screening (Wu *et al.*, 2000). Their preliminary studies indicated that transforming variants of E6 interact with human mastermind-like proteins (unpublished data). They proposed that these interactions possibly interfere with the functions of MAML proteins.

2.5 Gamma secretase inhibitor (γ-secretase inhibitor: GSI)

γ-secretase is a protease complex located on the membrane. It comprised of a catalytic subunit presenilin (PS), an accessory subunit presenilin enhancer (pen-2), nicastrin and anterior pharynx-defective phenotype-1 (aph-1) (Figure 2.7) (Kimberly *et al.*, 2003). PS is described as the catalytic subunit containing separate binding and catalytic sites (Tian *et al.*, 2003).

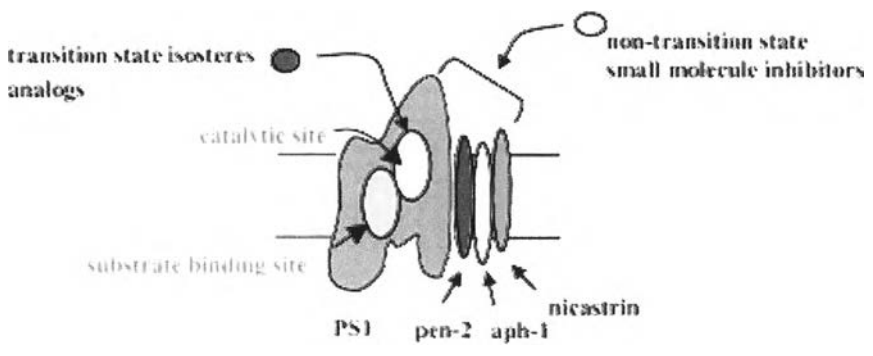

Figure 2.7 Structure of γ-secretase. It is a protein complex containing at least presenilin,pen-2, nicastrin and aph-1 (Tian *et al.*, 2003).

Cerebral accumulation of the amyloid-β peptide is considered a central event in the pathogenesis of Alzheimer's disease (Selkoe, 1994). The peptide is derived by proteolytic cleavage events from the amyloid precursor protein (APP) by two enzymes, termed β- and γ-secretase, cleaving at N-terminus and C-terminus of the peptide, respectively (Selkoe *et al.*, 1996). One of γ-secretase inhibitor, *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester or DAPT (Figure 2.8), reduced the level of brain Aβ in PDAPP transgenic mice (Dovey *et al.*, 2001). This finding provided useful tool for regulating mechanism of γ-secretase.

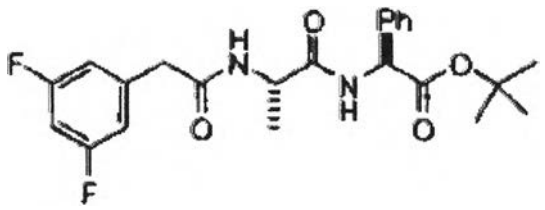

Figure 2.8 Structure of *N*-[*N*-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine *t*-butyl ester (DAPT) (Kornilova *et al.*, 2003).

γ-secretase is required for proteolytic activation of Notch receptor upon ligand binding. The intracellular domain of Notch (ICN) is released from the cell membrane and enters the nucleus, where it regulates gene transcription. A number of other transmembrane proteins, such as ErbB4 (Ni *et al.*, 2001, Lee *et al.*, 2002), E-cadherin (Marambaud *et al.*, 2002) and CD44 (Lammich *et al.*, 2002), are also γ-secretase substrates.

Ramdass *et al.* reported that the κB activity, pAKT and pIKK levels were decreased by DAPT while they significantly increased with overexpression of activated Notch1 (AcN1) in CaSki (Ramdass *et al.*, 2007). These studies indicated that Notch1 can modulate NF-κB activity through PI3K-PKB/AKT pathway. In Jurkat and HepG2, Suwanjunee *et al.* showed that DAPT treatment for 4 days decreased *Hes1* expression, cell proliferation and increased

Notch1 expression. DAPT treatment of both cell lines did not result in apoptosis, but caused cell cycle arrest (Suwanjunee *et al.*, 2008).

Although, several research papers indicated Notch signaling is associated with HPV cervical cancer. However, the role of Notch signaling in HPV cervical cancer remains controversial. The purpose of this research is to study the effects of suppressing of Notch signaling in HPV-positive cervical cancer cells, using pharmacological inhibitor and genetic (DN-MAML1) approaches.