Tumor Suppressor Role of Notch1 and Raf-1 Signaling in Medullary Thyroid Cancer Cells

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Abstract: There is a growing body of literature suggesting that signaling based therapy might be a potential approach for medullary thyroid cancer (MTC). In this review we focus on the tumor suppressor role of Notch1 and Raf-1 signaling in MTC. Interestingly these two pathways are minimally active or absent in these tumors and activation of Notch1 and Raf-1 significantly reduces tumor growth *in vitro*. Therefore, identification of compounds that induce these pathways could be a potential strategy to treat patients with MTC.

Keywords: medullary thyroid cancer, Notch1, Raf-1, achaete scute complex-like 1, Notch1 activating compound, ZM336372.

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumor (NET) originating from the parafollicular C cells of the thyroid. Although it represents only 3–5% of thyroid cancers, it is the cause of 13% of deaths secondary to thyroid malignancy (Chen et al. 2005). Once diagnosed the 10-year survival rate is 30–50%. Approximately 80% of patients diagnosed with MTC will have sporadic MTC. These patients classically present in their 50s or 60s with a solitary thyroid nodule.

Approximately 20% of patients with MTC will have an inherited form the disease. MEN2A, MEN2B and familial medullary thyroid cancer (FMTC) are transmitted in an autosomal dominant fashion and are all associated with mutations of the RET proto-oncogene. Because 6–7% of patients with presumptive sporadic MTC carry a germ line RET mutation (Eng et al. 1995; Marsh et al. 1996), all patients with apparently sporadic MTC should undergo genetic screening. The outcome of patients with an inherited form of MTC varies with genotype and early prophylactic thyroidectomies are recommended for family members with a RET mutation. The age at which the prophylactic thyroidectomy should be performed varies with mutation, and in some instances, i.e. MEN2B or RET codon 883 or 918 mutation, it should occur within the first 6 months of life (Chen et al. 2005; Massoll and Mazzaferri, 2004).

Similar to other NETs, MTC produces hormones. The classic tumor marker and most commonly secreted hormone, is calcitonin. Secretion of calcitonin, calcitonin gene-related peptide, and rarely ACTH can be associated with symptoms such as diarrhea, facial flushing, bronchospasm, and if ACTH is secreted, signs of Cushing's syndrome (Chen et al. 2005; Kunnimalaiyaan and Chen, 2006; Saad et al. 1984). With the exception of poorly differentiated MTC, calcitonin levels preoperatively can be used to predict both tumor burden and post-operative calcitonin normalization (Cohen et al. 2000). In addition, post-operative calcitonin doubling time can be used to predict prognosis. Doubling time greater than 2 years is associated with 100% 10-year survival, whereas doubling time under 6 months is associated with 8% 10-year survival and doubling time between 6 months and 2 years is associated with 37% 10-year survival (Barbet et al. 2005).

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In the majority of patients with de novo MTC, metastases are present at the time of diagnosis. Half of all patients will have cervical lymph node involvement, 15% will have compression of airway or laryngeal nerve involvement, and 5% will have distant metastases (Saad et al. 1984). At time of diagnosis, the minimal surgical procedure should be total thyroidectomy and central neck dissection. Preoperative staging may indicate a need for further neck dissection. Despite fairly aggressive initial surgery, most patients with MTC will have residual or recurrent disease. If recurrent tumor cannot be resected surgically, treatment options are limited. Chemotherapy and radioactive iodine are not beneficial and external beam radiotherapy can be associated with significant morbidity. Given the high likelihood of persistent/recurrent disease post-operatively and the lack of benefit of current therapeutic options, new treatments are needed.

Signaling based Therapy

Several signaling pathways, such as the phosphatidyl-inositol 3-kinase (PI3K)/Akt, mitogen activated protein kinases (MAPKs), and Notch1/Hairy Enhancer of Split-1 (HES-1)/achaete-scute complex like-1 (ASCL1) signaling pathway, have been shown to play important roles in regulating the growth of NETs (Chen et al. 2005; Kunnimalaiyaan et al. 2005a; Kunnimalaiyaan et al. 2005b; Kunnimalaiyaan et al. 2006a; Kunnimalaiyaan and Chen, 2006; Lal and Chen, 2006; Nakakura et al. 2005; Sippel et al. 2003b; Sippel et al. 2003a; Sippel and Chen, 2006). Thus, a potential therapeutic target could be manipulation of these various cellular signaling pathways. In this review we focus on two such signaling pathways as a potential target as a therapy for medullary thyroid cancer.

The Role of Notch1 as a Tumor Suppressor

Notch1 pathway is a highly conserved pathway throughout the animal kingdom that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts (Kadesch, 2004; Maillard and Pear, 2003; Yoon and Gaiano, 2005). A growing body of literature suggests that Notch1 signaling is very complex in nature. Recently, Notch1 has also been shown to play an essential role in the neuroendocrine (NE) differentiation of cells in the lung and gastrointestinal tract (Apelqvist et al. 1999; Hald et al. 2003; Jensen et al. 2000; Murtaugh et al. 2003). Both the Notch1 receptor and its ligands (Delta1 and Jagged1, for example) are transmembrane proteins with large extracellular domains. Binding of any one of the Notch ligands promotes two proteolytic cleavage events in the Notch receptor resulting in the release of the active Notch1 intracellular domain (NICD) (Allenspach et al. 2002; Artavanis-Tsakonas et al. 1999; Bray, 2006). The released NICD then translocates to the nucleus, binds with the DNA-binding protein complex CSL (CBF1, Su (H), and LAG-1) and resulting in activation of various target genes such as HES-1 (Artavanis-Tsakonas et al. 1999).

Notch1 signaling is very minimal or absent in prostate cancer, and NETs such as small cell lung cancer (SCLC), carcinoid, and medullary thyroid cancer (MTC) (Kunnimalaiyaan et al. 2005a; Kunnimalaiyaan et al. 2005b; Nakakura et al. 2005; Radtke and Raj, 2003). Therefore, the lack of Notch1 signaling in NETs led us to examine the potential role to influence cellular differentiation, proliferation and/or survival in these cancers.

Abolition of ASCL1 in transgenic knockout mice led to the failed development of pulmonary NE cells, a lack of thyroid C-cells, a 50% reduction in adrenal chromaffin cells, and death at birth (Ito T et al. 2000; Lanigan et al. 1998) suggesting that ASCL1 is required for the development of NE cells in the body including C-cells (see review (Chen et al. 2005)). We and others have characterized the expression of ASCL1 in several human cancer cell lines and tumors and found that ASCL1 is, as predicted, highly expressed in MTC, SCLC, carcinoids, and pheochromocytoma, where as ASCL1 is absent in pancreatic cancer tissues and cell lines (see review (Chen et al. 2005)). Therefore, inhibition of ASCL1 expression may be an important way to suppress tumor growth. The pathways such as Notch1 that regulate ASCL1 expression have been well characterized. Interestingly, recent studies have shown that Notch1 signaling is very minimal or non-existent in NETs (Kunnimalaiyaan et al. 2005a; Kunnimalaiyaan et al. 2005b; Kunnimalaiyaan et al. 2006b; Nakakura et al. 2005; Sriuranpong et al. 2001). Furthermore, we have reported the lack of active Notch1 protein but high levels of expression of CgA and ASCL1in several human MTC tumor tissues and MTC-TT cell line (Kunnimalaiyaan et al. 2006b). These results further support that negative regulation of ASCL1 by Notch1 pathway. As expected activation of

Notch1 pathway in MTC and SCLC cells led to a significant reduction in ASCL1 protein and growth suppression (Kunnimalaiyaan et al. 2006b; Ravi et al. 1998). Activation of doxycycline inducible Notch1 in TT cells by varying concentration of doxycycline led to dose dependent increase in Notch1 protein and HES-1 protein. As expected the level of ASCL1 is reduced with increase in Notch1 (Kunnimalaiyaan et al. 2006b). Further, we observed that activation of the Notch1 significantly reduced the growth of TT cells and the reduction in growth is dependent on the level of Notch1 protein (Kunnimalaiyaan et al. 2006b). We also found that Notch1 regulates calcitonin level in a dose dependent manner. Furthermore, the levels of reduction in growth and hormone production depend on the amount of Notch1 protein present in the cell (Kunnimalaiyaan et al. 2006b). These observations clearly support the hypothesis that Notch1 functions as a tumor suppressor in MTC tumors and cell lines.

Activation of Raf-1 Inhibits Growth in MTC Cells

Ras regulates multiple signaling pathways of which the best understood is the Ras/Raf/ mitogen-activated extracellular protein kinase (MEK)/ extracellular signal-regulated kinase (ERK) pathway. R*as* and *raf* are proto-oncogenes and expression of these genes activates signaling pathways, which in turn control cellular growth. Therefore, the ras/raf signaling pathway has been recognized as an important process in cancer biology (see reviews (Chen et al. 2005; Dhillon and Kolch, 2002; Dhillon and Kolch, 2004; Kolch, 2000; Sridhar et al. 2005)). Despite several findings and new insights on this signaling pathway, the role of Raf in cancer cells remains controversial yet interesting. Recently, we have shown that activation of raf-1 pathway in MTC cells by expression of estradiol inducible estrogen receptor fused with catalytic domain of raf-1 fusion protein led to complete suppression of ASCL1 mRNA and protein (Carson-Walter et al. 1998; Chen et al. 1996; Sippel et al. 2003a). Decrease in the level of ASCL1 protein correlated with reduction in calcitonin and CgA. Furthermore, raf-1 activation in MTC cells led to a significant growth suppression (Park et al. 2003).

Earlier we have reported the importance of ASCL1in MTC tumor proliferation (Chen H et al. 1997; Chen et al. 1997; Chen et al. 2005; Park et al. 2003; Sippel et al. 2003a). Recently, we and others have reported that Raf-1 activation in MTC- TT cell line results in growth suppression as well as reduction in NE hormones (such as calcitonin and serotonin), ASCL1, and levels of the RET proto-oncogene (Carson-Walter et al. 1998; Chen et al. 1996; Park et al. 2003; Sippel et al. 2003a). Further, it has been shown that growth inhibition by raf-1 activation in MTC-TT cell line induces an autocrine-paracrine protein, leukemia inhibitory factor (LIF), and this alone could mediate differentiation and cell growth inhibition (Park et al. 2003). Furthermore, we have recently shown that activation of raf-1 pathway in these cells lead to inactivation of GSK-3β by phosphorylation at ser9. We also showed that inactivation of the GSK-3β alone resulted in differentiation and cell growth inhibition (Kunnimalaiyaan et al. 2007). These findings are interesting because raf-1 activation not only activates its own raf-1/MEK/ERK pathway but also cross talk with other pathways, which in turn could possibly regulate growth.

Therapeutic Approaches for the Future

These results suggest that Raf-1 or Notch1 activation may be a possible therapeutic target in MTC. Other than gene therapy, applications to deliver activated *raf-1* or *notch1* to tumor cells are limited. Therefore, identification of pharmacological agent(s) that activate these pathways in MTC cells should be undertaken. In this regard, development of high throughput platform assays to screen for activators of these pathways would be highly beneficial. However, thus far many research groups are working on the identification of compounds, which inhibit these pathways for other tumors such as pancreatic cancer and breast cancer.

Based on the results that Raf-1 activation in MTC cells led to growth inhibition, we explored the possibility of pharmacologically activating compound for raf-1 activator in MTC cells. Though the compound ZM336372 was originally identified as a small molecule inhibitor of Raf-1 (Hall-Jackson C.A et al. 1999) recently we have shown that it activates raf-1 pathway in NET (Kunnimalaiyaan and Chen 2006; Van Gompel et al. 2005). When we treated NET cell lines such as pancreatic carcinoid and pulmonary carcinoid cancer cell line with ZM336372, there was significant reduction

in growth and hormone production (Van Gompel et al. 2005). Furthermore, we have shown that phosphorylation of Raf-1 and ERK1/2 by ZM336372. Recently we have observed that treatment of MTC cells with ZM336372 resulted in growth inhibition suggesting that activation of raf-1 pathway is required for the anti-tumor proliferation effect (Kunnimalaiyaan et al., manuscript in submission). Taken together the results of over expression of ectopic raf-1 and activation of endogenous raf-1 by ZM336372 in NETs, suggests that raf-1 could be a potential target for novel therapeutic anticancer strategies.

In summary, given the important role of Notch1 and raf-1 in the regulation of growth of MTC, we hope that activating compounds for these signaling pathways will have novel and potent therapeutic value for the treatment of MTC patients. However, a fundamental question is whether the concentrations of ZM336372 or other similar compounds required to activate Notch1 or raf-1 are achievable in the humans. Therefore, further studies examining the therapeutic and palliative potential of this drug alone or in combination with other drugs for patients with metastatic MTC are warranted.

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