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Molecular targets in medullary thyroid carcinoma

Review Article

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Summary

A high percentage of thyroid cancer has been associated with a number of oncogenic genetic variations. The RET proto-oncogene *(REarranged during Transfection*; RET*)* is thought to play an important role in the etiology of thyroid tumours and is clearly implicated in Medullary thyroid carcinoma. RET variations which confer oncogenic gain of function, appear to result in the development of cancer due to uncontrolled cellular proliferation as well as failure to undergo normal differentiation and the loss of apoptotic functions. Understanding the biological role of the RET proto-oncogene in thyroid carcinoma is an important area of biomedical research which leads not only to better understanding of the pathophysiological changes involved in oncogenesis but also determines the optimal management and identifies molecular targets allowing the development of novel therapeutic approaches. This review looks at the structure and function of the RET proto-oncogene, its understood method of activation and important molecular targets identified to date. It also studies agents used for novel molecular targeting in treatment of thyroid carcinomas.

I. Introduction

Thyroid cancer has been associated with a number of oncogenic genetic abnormalities which are fairly unique due to the fairly high percentage relating to genetic rearrangements and chromosomal translocations (eg RET proto-oncogene, RET/PTC, NTRK, ras mutation, Braf mutation, met genes and Pax 8/PPARgamma etc). In addition, tumor suppressor gene malfunction (eg RB1 and p53) appear to be associated with anaplastic thyroid tumors (Moretti et al, 2000) and other genes (eg miR) are thought to further modulate the oncogenic response (eg to RET/ PTC (Jazdzewski et al, 2008).

Study of these genetic variations is important to not only understand the fundamental mechanisms of oncogenesis and the development of molecular target therapies but to also identify those at risk with resultant prophylactic protocols.

Recent research has shown that receptor tyrosine kinases (RTK) are vital oncogenic agents as RTK activation promotes cellular angiogenesis and increases cellular proliferation, invasion and metastases. The RET proto-oncogene *(REarranged during Transfection*; RET*)* is regarded as a proto-type RTK and is thought to play an important role in the etiology of thyroid tumours because of the numbers of rearrangements frequently present in thyroid tumour cells (Porter and Vaillancourt, 1998) and its clearly defined role in Medullary thyroid carcinoma (Machens and Dralle, 2007a). RET variations which confer oncogenic gain of function, appear to result in the development of cancer due to uncontrolled cellular proliferation as well as failure to undergo normal differentiation and the loss of apoptotic functions.

The RET proto-oncogene plays a pivotal role in at least 4 clinical syndromes (Donis-Keller et al, 1993; Mulligan et al, 1993). Multiple Endocrine Neoplasia (MEN) Type 2 A and B and Familial Medullary thyroid carcinoma (FMTC) (Mulligan et al, 1993; Carlson et al, 1994; Eng et al, 1995) and Hirschsprung's disease (HSCR) (Luo et al, 1993; Edery et al, 1994; Takahashi et al, 1985), in a unique "switch on"/ "switch off" manner (van Heyningen V, 1994).

II. The structure of the RET proto-oncogene

Since the initial association of the RET proto-oncogene (10q 11.2) with MEN2 syndromes in 1993

(Donis-Keller et al, 1993; Mulligan et al, 1993), exploration of its oncogenic role has resulted in the rapid advancement of knowledge as to its structure and possible mechanisms of action.

The RET (Rearranged during Transfection) proto-oncogene consists 21 exons which lie over a 55Kb area at 10q11.2 (Ceccherini et al, 1993). It remains one of most important proto-oncogenes within the human genome, encoding a RTK which is a critical signaling component required for normal nervous system and kidney development, as well as spermatogenesis (Takahashi et al, 1985). RET is mainly expressed in cells of neural origin and is involved in cellular proliferation, migration and differentiation of cells (Pachnis et al, 1993).

Like other RTKs, RET is made up of at least 3 functional areas (viz: extracellular, trans-membrane and intracellular regions) each appearing to have specific functions (Ceccherini et al, 1993). In terms of RET activationin the extracellular domain, the cysteine molecules appear to be key areas. A possible explanation is that genetic variation impairs the correct sequencing of the RET protein thus influencing its function (Asai et al, 1995). In addition to the usual mechanism, extracellular domain mutations (eg C620S) may affect polarity due to the unfolding of RET, which has also been shown to also result in TK activation (Carlomagno et al, 2006).

The function of transmembrane domain mutations are unclear but in recent studies into the transmembrane S649L RET mutation appears to be associated with late-onset non-aggressive disease. (Colombo-Benkmann et al, 2008). Activation of RET RTK via the intracellular or TK portion of the gene activation is easier to understand. On the one hand, malfunction at critical areas probably affects molecule-molecule signaling connections with other RET-related molecules (eg GDNF) leading to defective downstream signaling. RET receptor dimerization as well as RTK activation in the absence of the normal ligand co-factors may thus result (Carlomagno et al, 1997). On the other hand, other possible oncogenic cellular mechanisms include the autophosphorylation of RET (eg. MEN2B) as well as the modifying the sub cellular distribution of the active kinase (Santoro et al, 2002).

The uncommon co-segregation of HSCR and MEN2 in the same patient is a fascinating finding as it involves both gain and loss of function in the same patient (Verdy et al, 1982; Mulligan et al, 1994; Borst et al, 1995; Blank et al, 1996; Caron et al, 1996; Peretz et al, 1997; Borrego et al, 1998; Decker and Peacock, 1998; Romeo et al, 1998; Sijmons et al, 1998; Inoue et al, 1999; Pasini et al, 2002; Dvorakova et al, 2005; Moore and Zaahl, 2008). The most commonly reported mutations associated with MEN2-HSCR are C620R(65;162) and occasionally C620S and rarely C620W (Decker et al, 1998). This has led to the concept of the so-called "Janus gene" mutation which like the Roman god of doorways can face in both directions. The association is not limited to codon 620 cysteine substitutions, however. Although it must be bourne in mind that the prevalence may differ between Paediatric surgical centers specializing in HSCR and endocrine surgical centers specializing in MTC (5% at best), a 32 - 35% association has been reported with codon 618 (C 618R2x >C 618S-) missense mutations (Decker and Peacock, 1998) as well as a 15-20% association with codon 609 (C 609Y mostly) and a 4% association with 611 mutations. Genetic variation in these areas possibly resulting in the malfunction of the same or similar vital signaling pathways. The reasons why this occurs as well as the factors modifying its phenotypic expression remain areas of ongoing research.

In addition to the potential tyrosine kinase activation via fusion genes, there are yet other genetic variations identified in RET malfunction (Knowles et al, 2006). The first group are those regions in the extracellular portion of the gene which control protein folding (mutations which result in a non-folded RET protein at the endoplasmic reticulum). Those occurring within the 1st cadherin-like domain in HSCR (Kjaer and Ibanez, 2003) are thought to influence its pathogenesis (Griseri, 2005). Those affecting one of the 6 cysteine radicals (viz: 609, 611, 618, 620, 630 and 634 positions) affect gene function and influence RET activity resulting in MEN2A (Carlomagno et al, 1996; Iwashita et al, 1996). The second group of RET gene variations are those within the terminal tyrosine kinase (KD) intracellular region of the gene (eg exon 16 M918/T mutations in MEN 2B (Carlomagno et al, 1996; Iwashita et al, 1996). A third area is emerging in those gene variations which affect the docking sites for downstream signaling pathways(Geneste et al, 1999). There is a further group of MEN-related genetic variations, associated with non-cysteine areas of gene which raises interesting questions as to the oncogenic mechanism. This latter group appeared to occur more frequently than expected in our own series(Moore et al, 2007) in keeping with other genetic pools (Pinna et al, 2007).

III. RET-proto-oncogene activation

Oncogenic RET mutations mostly occur de novo in sporadic MTC and their effect on protein synthesis and function clearly involves a number of downstream molecular signaling pathways (Carlomagno et al, 1996). Hereditary MTC can with rare exceptions be traced back in family trees (Machens and Dralle, 2008).

Genetic variations impair the RET tyrosine kinase response to tyrosine kinase activation, thus appearing to dictate downstream signaling cascade response. (Andl and Rustgi, 2005) Known downstream signaling pathways include activation of the MAPK and Ras/ERK molecular signaling cascades by a mechanism which involves grb2/mSOS recruitment (Edery et al, 1994). The multidocking intracellular portion of the RET gene appears to be both vital to tyrosine kinase function as well as downstream signaling due to the number of signaling molecules that interact there(eg. Shc, Src, FRS2, IRS1, Gab1/2, and Enigma) (Santoro et al, 1995a; Arighi et al, 1997; Lorenzo et al, 1997) Competitive Shc versus Frs2 recruitment at Tyr 1062 has been shown to mediate increased cell-survival (Lundgren et al, 2006) An alternative mechanism appears to rather signal via PI3K and Akt upregulation following recruitment of grb2/gab2 (Alberti et al, 1998). These downstream adaptor protein functions are particularly important as they play a vital

function in effecting the multiple diverse roles played by the RET receptor during development.

The unfolding of RET due to extracellular domain mutations (eg C620S) affects polarity which may result in tyrosine kinase activation (Carlomagno et al, 2006).

Mutation in the Tyrosine-kinase rich region (eg. in 95% of MEN 2B patients), on the other hand, alters the substrate specificity of RET tyrosine kinase and appears to induce a different set of downstream signaling genes from that carrying the MEN 2A mutation. This appears to function via the sic protein (Pelicci et al, 2002) which is known to induce downstream activation of the Ras/mitogen-activated protein- and P13K/Akt signaling cascades (Salvatore et al, 2001;Wong et al, 2005). It may well have bearing on the speed and aggressiveness of the oncogenic process as well as influencing the penetrance of the MEN 2 phenotype. Both the phosphorylated and non-phosphorylated forms have been shown to have a pre-organized activation loop and are competent to bind ATP and substrate (Knowles et al, 2006).

Recruitment at the phosphorylated Tyr 1062 intracellular site has been shown to be particularly important as it is recruited and activated by both the Ret adaptor proteins (Shc and Frs2) activated by MEN2A or by MEN2B , as well as via the rearranged Ret oncoproteins (eg Ret/ptc2) involved in papillary and other thyroid carcinomas (Arighi et al, 1997). This function does not take place in isolation, but is activated by binding to a ligand complex formed by the glial cell line-derived neurotrophic factor (GDNF) (Jing et al, 1996) and its ligand co-receptor, the GDNF-family receptor-alpha (GFRα) receptors. These receptors are GPI linked thus permitting dimerization (Baloh et al, 1997) and are known to involve lipid rafts to promote GDNF interaction (Saarma, 2001). In addition, further RET/ GDNF signaling pathways appear to be partly controlled by Heparan sulfate interactions (Wang et al, 2007).

Demonstration of Tyrosine kinase activity upregulation by the RET/PTC fusion gene in papillary thyroid carcinoma (PTC) (Grieco et al, 1990) is a further known mechanism of increased RTK activity resulting in oncogenesis. In this case, RET activation which results from gene dimerization is mediated through coiled-coil motifs in the NH_2 terminus of the chimeric protein(Croyle et al, 2008). Following the Chernobyl disaster (ionizing radiation)in Russia, papillary thyroid tumors occurring in children show a high prevalence of RET fusion gene rearrangements with at least 11 different gene fusions being described (the majority being a RET/PTC3 rearrangement) (Rabes and Klugbauer, 1998). Other less frequent rearrangements are also implicated in PTC and include the RET/PTC1 and some more novel rearrangements (eg RET/PTC5). Activating RET mutations are not confined to PTC but are also associated with other thyroid carcinomas (Nikiforov et al, 1997).

The RET/PTC fusion gene occurs as a result of a balanced intrachromosomal inversion with fusion of the RET 3' portion to the 5' portion of various genes (eg *BRAF, RET*, or *RAS* genes of the MAPK system) to form RET/PTC and similar chimeras (Adeniran et al, 2006). As a result, the tyrosine kinase RET domain then becomes controlled by 5' fused regulatory sequences of other genes with dimerization potential (Klugbauer and Rabes, 1999). *RET* receptor tyrosine kinase gene activation then occurs leading to initiation of oncogenesis.

Other genes (eg EGFR) may also be involved in RET kinase activation, signaling, and the resultant growth stimulation within the final oncogenic pathway. The epidermal growth factor receptor (EGFR) has been implicated because of experimental evidence whereby the kinase inhibitor PKI166 has been shown to decrease RET/PTC kinase autophosphorylation and activation of downstream signaling cascades in thyroid cells (Croyle et al, 2008).

Additional factors influencing gene penetration and the signaling pathways involved in metastasis and invasion are less clear, but it would appear that factors such as gene methylation may play an epigenetic role by altering the phenotype without affecting the genotype.

IV. Mechanisms of RET activation

Abnormal disulphide homodimerization of RET is the most likely explanation of gene activation following cysteine-substitution mutations at RET codons 609, 611, 618, 620, 630 and 634 (Chappuis-Flament et al, 1998; Takahashi et al, 1999). The induction of a disulfide-linked homodimerization particularly in exon 11, (codon 634 point mutations) (Asai et al, 1995; Santoro et al, 1995b) due to genetic mutations results in oncogenesis due to the removal of a half of the intermolecular bond, thus allowing an abnormal bond with a second mutant molecule to occur, resulting in decreased RET at the plasma membrane (Arighi et al, 2004).

One of the problems in the hypothesis is that it does not explain the MEN2-HSCR association and why certain commonly occurring RET variations (eg S767R and P1039L) fail to show RET malfunction (Pelet et al, 1998), whereas others (eg R231H) do (Bordeaux et al, 2000).

This observation raises the role of RET ligands (eg GDNF) in the survival and differentiation of developing neurons but also raises the case for a possible second intracellular mechanism (Geneste et al, 1999) in the pathogenesis of MTC. An alternative explanation is the possible trapping of mutant RET molecules in the endoplasmic reticulum in the MEN2- HSCR association or the modifying effect of secondary inter-related pathways.

In this regard, other genes (eg EGFR, VEGF) have also been shown to play a role in RET kinase activation, signaling, and the resultant growth stimulation occurring within the final oncogenic pathway. One example is the EGFR, which has been implicated because of experimental evidence whereby the kinase inhibitor PKI166 has been shown to decrease RET/PTC kinase autophosphorylation and activation of downstream signaling cascades in thyroid cells (Croyle et al, 2008). In addition, VEGF and angiogenesis are also important factors in advanced tumor biology and serum vascular endothelial growth factor receptors (sVEGF-C) levels have been shown to have a strong correlation nodal metastases in advanced PTC (Yu et al, 2008). Crosstalk between the vascular endothelial growth factor (VEGF A/VEGF2) and the GDNF/RET signaling pathways has

also been reported (Tufro et al, 2007), suggesting a possible link in their function. Many of the tyrosine kinase inhibitors currently under study are multiple tyrosine kinase inhibitors and include VEGF and EGFR tyrosine kinase inhibition

V. Potential Molecular targets in MTC and thyroid tumours

Understanding the biological role of the RET proto-oncogene in thyroid carcinoma is an important area of biomedical research which leads not only to better understanding of the pathophysiological changes involved in oncogenesis but also determines the optimal management and identifies molecular targets thus allowing the development of novel therapeutic approaches.

Current understanding of RET function suggests additional molecular mechanisms and raises the case for a possible second intracellular mechanism (Geneste et al, 1999) in the pathogenesis of certain MTC carcinomas.

VII. Mutation testing and prophylactic thyroidectomy

There appears to be fairly consistent associations between genotype and phenotype in MTC. The assessment of risk by RET gene mutation analysis and the ability to predict potential carrier states in family members with genotype-phenotype correlations as well as the evidence for an age-related progression of MTC, is becoming a reality. In general, MEN 2A mostly associated with variations in the 6 cysteine radicals in the extracellular domain (with varying degrees of risk) and 95% of MEN 2B patients are associated with a point mutation (Methionine□threonine) in exon 16(M918/T) of the intracellular domain. This molecular knowledge has led to the introduction of prophylactic surgery and removal of the target organ (total thyroidectomy) in affected individuals (particularly children) (Skinner, 2003), thus preventing the onset of cancer.

VIII. Therapeutic implications of molecular RET mechanisms in Cancer

A clear need for new therapeutic approaches exists due to the chemo and radio-insensitivity of the majority of these tumors, particularly when metastatic. Understanding of the molecular mechanisms involved in oncogenesis and potential molecular target sites has, in turn, led to exciting new therapeutic options by targeting of the RET tyrosine kinase action in the treatment of MEN type 2 and MTC (Ball, 2007).

A. Risk level and codon-directed surgery in MTC and FMTC

Effective preventative management depends on early identification of gene carriers and prophylactic thyroidectomy prior to any clinical or biochemical abnormalities.

Machens and Dralle, have stratified in 2007 the MTC risk into three categories according to the mutation-related aggressiveness, giving rise to a concept of "codon-directed" timing of surgery. A recent long term follow-up study of 46 RET gene carriers categorized the risk to children and young adults (aged 4 - 21 years) into level 1 (low risk) and level 2 mutations (intermediate to high risk) (Frank-Raue et al, 2006). Level 1 mutations (n=11) appeared to be associated with variations in codons 790, 791, 804 and 891 and had a high incidence of cure. By way of contrast, 5 (14%) of the 35 level 2 patients (mutations of codons 618, 620, 630 and 634), had ongoing disease. On long term follow-up (mean 6.4 yrs), 2 of those with 634 mutations, developed other MEN manifestations (viz: hyperparathyroidism and bilateral Phaeochromocytoma). This latter risk would probably increase with time and long-term follow-up is necessary. It is now generally accepted that RET 609 mutations should also be added to Level 2 (Machens and Dralle, 2007b). Level 3 mutations include those at 883 and the 918 positions

However, it appears to be important to separate the sporadic from familial MTC as Elisei and colleagues in 2008 observed a significantly ($p<0.0001$) higher representation of non-cysteine codons in the sporadic group as opposed to the familial group which were mostly cysteine-related. Furthermore, there appears to be a difference in prevalence between various population groups in terms of the frequency of RET mutations encountered. Recent increase in exon 13-15 mutations in a German series (Frank-Raue, 2007), does not appear to be necessarily reflected in certain other population groups (Dvorakova et al, 2008). In addition, a study of MTC in Sardinia has shown a 59% prevalence of the V804M mutation (against the expected 5% of other European populations) (Pinna, 2007). This demonstrates the importance of the genetic background in evaluating the RET gene in these patients.

Codon-risk protocols are of considerable value but not completely watertight and the age of MTC onset does not always appear to be consistent. Recent advances into the significance of RET proto-oncogene signaling and the molecular pathways of RET signal transduction in the development of MTC, and oncogenesis have created new potential treatment modalities with exciting new possibilities for management.

B. Novel molecular targets in treatment of thyroid carcinomas

Current molecularly based strategies for MTC include tyrosine kinase inhibitors, gene therapy of significant RET mutations and the promotion of cellular death (Petrangolini et al, 2006), as well as monoclonal antibodies against oncogenic proteins (Drosten and Putzer, 2003) and, nuclease-resistant factors that both recognize and inhibit RET (de Groot et al, 2006). A number of multiple kinase inhibitor drugs have shown experimental activity and are currently entering clinical trials.

Of particular current interest in this regard, is the development of the RET-targeting orally administered tyrosine kinase inhibitors (eg: ZD6474; RPI-1 (Cuccuru et al, 2004) BAY 43-9006 (Carlomagno et al, 2006). These agents are mostly multi-kinase inhibitors and include effects on VEGF as well as affecting certain other kinases

which results in further angiogenesis inhibition (Petrangolini et al, 2006).

Experience with ZD6484 (a specific RET, Epidermal growth factor, VEGF tyrosine kinase inhibitor) and other multi-kinase inhibitors (eg sorafinib) are currently reporting encouraging early results (Gupta-Abramson et al, 2008) in metastatic tumors. Sorafinib appears to be particularly interesting as it inhibits a wide spectrum of kinases known to be active in thyroid tumours (including Raf kinase, VEGF, platelet-derived growth factor receptor, and RET tyrosine Kinases). This is bourne out by a reported rapid response to sorafenib/tipifarnib therapy in a patient with an exon 11 RET mutation. The agent NVP-AST487 (an N,N'-diphenyl urea) has been shown to block tumor growth and Calcitonin gene expression in cell lines with RET activating mutations by inhibiting RET autophosphorylation and downstream signaling.

Early results with these modalities appear encouraging with one research group reporting a statistically significant 51% tumour inhibition, a 210 % increase in apoptotic cells, a 47% loss of cellularity and a 37% decrease in micro vessel density (Petrangolini et al, 2006).

Similar results are also being reported for Axitinib (AG-013736), a potent VEGF 1, 2, and 3 inhibitor, which has been experimentally shown to reduce transplanted MTC by 89% in an animal model (Johanson et al, 2007) and is active against all histologic subtypes of advanced thyroid cancer (Cohen et al, 2008).

On the other hand, not all tumors respond and certain studies show that these agents may be effective in those without the usual RET mutations (Keno-Stuart et al, 2007). These observations are supported by observed differences in RET ultrastructure (Knowles et al, 2006), and raises questions as to the possibility of a secondary oncogenic mechanism in certain tumors.

In addition to these TK inhibitors, an array of monoclonal antibodies has been introduced as targeted treatment in the treatment of head and neck, breast and lung cancers. They target receptors or ligands in cellular proliferation and inflammation pathways and prevent phosphorylation by blocking the ATP-binding domain. This results in inhibition of signal transduction required for upregulation of function.

IX. Conclusion

Molecular targeting definitely appears to be a step forward in the management of MTC and other thyroid carcinomas. On the other hand, it would appear that more work needs to be done in this area to streamline the molecular-directed treatment and identify those cases most likely to benefit from this form of treatment.

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