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Genetic abnormalities of somatostatin receptors in pituitary tumors

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LOH

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ABSTRACT

Somatostatin exerts antisecretive and antiproliferative effects on different endocrine cells by acting through a family of G protein-coupled receptors that includes 5 subtypes (SST1-5). Normal human pituitary and pituitary adenomas have been shown to express almost all SST subtypes, with the exception of SST4. Consistent with the observation that octreotide and other somatostatin analogs bind to SST2 and SST5 with high affinity, these genes have been screened for quantitative/qualitative abnormalities in tumors removed from patients with poor responsiveness to somatostatin analogs treatment. Data obtained in GH-secreting adenomas suggested that resistance to octreotide was frequently associated with low expression of SST2 mRNA, although other authors failed to confirm this finding. To date, the only mutational change involving SST2 and SST5 is the Arg to Trp substitution in codon 240 of the SST5 gene that was found in one acromegalic patient resistant to octreotide. Similarly, loss of heterozygosity at SST5 gene locus in pituitary adenomas has been described in individual tumors. In recent years, molecular studies investigated the possible association of gene polymorphisms and susceptibility to diseases and/or resistance to drugs. As far as polymorphic variants of SST genes are concerned, a possible role of SST5 C1004T and T-461C alleles in influencing GH and IGF-I levels in patients with acromegaly has been proposed. Nevertheless, polymorphic variants in SST2 and SST5 genes seem to have a minor, if any, role in determining the different responsiveness to somatostatin analogs in patients with acromegaly.

INTRODUCTION

The identification of somatostatin (SS) and the subsequent development of somatostatin analogs (SS-analogs) provided important information on the pathophysiology of neuroendocrine system and significantly modified the medical approach to patients with neuroendocrine tumors, including pituitary and gastroenteropancreatic endocrine tumors (Lamberts et al. 2002). SS mediates a variety of biological effects, the most important occurring at the pituitary (inhibition of GH and TSH secretion) and gastroenteropancreatic (GEP) levels (inhibition of insulin, glucagon, secretin secretion, inhibition of hydrochloric acid production and intestinal liquid absorption). In addition to hormone secretion inhibition, somatostatin has anti-proliferative and anti-angiogenic properties that have been largely investigated both on cell lines (Garcia de la Torre et al. 2002, Florio et al. 2003, Dagupta 2004) and GH-secreting tumors (Luciani et al. 2005, Ferrante et al. 2006). Three decades after SS identification, 5 receptor subtypes (SST1-5) involved in the transduction of SS were identified and cloned. The expression of SST, generally with the exclusion of SST4, is a hallmark of neuroendocrine cells that is generally maintained during tumoral transformation (Hofland et al. 2001, Reubi et al 2001, deHerder et al. 2003).

SS-analogs, that are largely used in clinical practice to visualize primary and metastatic GEP lesions and to control hormone hypersecretion, bind to SST2 and SST5 with high affinity (Hofland&Lamberts 2004). The majority of pituitary tumors, particularly GH-secreting (GH-omas) and TSH-secreting adenomas, and GEP tumors (e.g. gastrinomas, insulinomas, VIP-omas, PP-omas and carcinoids) responds to somatostatin analog with a decrease, or even normalization, of the hypersecreted hormones with consistent amelioration of signs and symptoms of the disease (Caron et al. 2001, Lamberts et al. 2002, Oberg 2002, Freda et al. 2005). In contrast to the frequent occurrence of desensitization to somatostatin action in patients with GEP tumors, in patients with pituitary tumors resistance to SS is a rare event (10-20%) occurring at the beginning of the

treatment, generally due to low expression of SST2 and associated with the absence of *gsp* oncogene (Landis et al 1989, Barlier et al 1999, Corbetta et al. 2001, Park et al 2004, Taboada et al, 2007). The mechanisms responsible for the lack of hormonal control by SS are unknown, probably because of the multiplicity of the involved events (Reubi et al., 1989; Barlier et al., 1999; Jaquet et al., 2000, Lamberts et al. 2002).

As far as the ability of SS to control tumor growth is concerned, evidences are accumulating that SS-analogs are effective not only in reducing GH and IGF-I levels within the normal range in about 50-60% of acromegalic patients, thus reducing the morbidity and mortality rate of these patients, but also in controlling pituitary tumor growth (Stewart 2000, Melmed et al. 2005). In particular, recent studies carried out on untreated acromegalic patients reported that treatment with SS-analogs caused a greater than 30% tumor shrinkage in about 70% of patients (Bevan et al. 2002, Melmed et al. 2005). The mechanisms mediating the antiproliferative action of somatostatin and the receptor subtypes involved in this process have been only partially characterized. In particular, studies on several cell lines expressing SST1, 2, 4 and 5 showed that SS causes cell cycle arrest (Cheung et al., 1995, Buscail et al., 1995; Florio et al., 1996; Cattaneo et al., 1996, Cordelier et al., 1997, Patel et al., 1997, Sharma et al., 1999). More recently, it has been shown that SST5 trigger both cytostatic and cytotoxic responses in pituitary tumors, respectively (Luciani et al. 2005, Ferrante et al. 2006).

In this review we will summarize the results of different studies carried out in acromegalic patients looking for genetic abnormalities affecting the different SST subtypes potentially involved either in GH-secreting adenoma pathogenesis or in resistance to medical treatment.

Mutation(s) in the somatostatin receptor gene(s)

Several years ago, our group described the first mutation of a receptor of the SST family in one acromegalic patient resistant to treatment with the somatostatin analog octreotide (Ballarè et al., 2001). Acromegaly is a chronic disease characterized by elevated GH and IGF-I levels,

generally owing to a GH-secreting pituitary adenoma. When inadequately treated, acromegaly results in significant morbidity and greatly increased mortality. Because only about 50-60% of patients are effectively cured by the surgical removal of the adenoma (Ludecke & Abe, 2006), medical therapy has an important role in the management of these patients. The introduction of new drugs, especially somatostatin analogs, has greatly contributed to improve the prognosis of acromegaly (Melmed et al., 1998, Melmed 2006).

The commercially available long-acting somatostatin analogs, such as long acting octreotide and lanreotide, bind with high affinity to SST2, SST5, and to a lesser extent SST3 (Reisine et al., 1995; Patel 1997; Patel et al., 1997). Activation of these receptors generates a series of intracellular events, including inhibition of adenylyl cyclase and reduction of cytosolic calcium (Reisine et al., 1995; Patel et al., 1997). Moreover, somatostatin exerts direct antiproliferative effects on cell lines by triggering signal transduction pathways which are not completely defined (Cheung et al., 1995, Buscail et al., 1995; Florio et al., 1996; Cattaneo et al., 1996, Cordelier et al., 1997, Patel et al., 1997, Sharma et al., 1999, Luciani et al. 2005, Ferrante et al. 2006). However, in some cells which express SST2 and SST5, somatostatin reduces the activity of mitogen activated protein kinases (MAPK), a family of protein serine/threonine kinases considered key molecules in transducing growth factor proliferative signals (Patel et al., 1997; Cattaneo et al., 1996; Cordelier et al., 1997, Hubina et al. 2006). Somatostatin analogs lower GH levels in about 90% of acromegalic patients. However, in 60% of all patients IGF-I and GH are not lowered to levels considered beneficial to normalize life expectancy (Freda et al. 2005, Melmed 2006). In addition, somatostatin analogs block the growth of the tumoral mass and may indeed slightly reduce tumor size in up to 50% of patients (Melmed et al., 2005).

The molecular basis of the different sensitivity to somatostatin remains largely unknown. There is a general agreement that patients harbouring tumors with mutations of the $G_{s\alpha}$ gene that constitutively activate adenylyl cyclase (referred to as *gsp* mutations) (Landis et al., 1989) are highly sensitive to the inhibitory action of somatostatin (Spada et al., 1990; Barlier et al., 1998,

Barlier et al. 1999). Conversely, because no mutations of SST2 and SST5 genes are usually found in resistant patients (Petersenn et al., 2000, Corbetta et al. 2001), resistance has been attributed to a reduced expression of SST2 or SST5 (Reubi et al., 1989; Barlier et al., 1999; Jaquet et al., 2000; Corbetta et al. 2001, Park et al 2004, Taboada et al, 2007). To date, the only mutational change involving a somatostatin receptor is the germ-line R240W mutation in the SSTR5 gene, that was found in one acromegalic patient resistant to octreotide (Ballarè et al., 2001). This mutation, that replaces an Arg with Trp, is located in the 22-residue region of Lys224 to Arg245 within the third cytoplasmic loop of the receptor (Figure 1A). This region represents the cytoplasmic extension of the α -helix 6 and is a consensus sequence identifying a potential site for coupling with G proteins (Patel 1997; Patel et al., 1997). In both human and rat SST3–5, this arginine is highly conserved. The replacement of Arg240 with Trp, an aromatic and highly hydrophobic amino acid, is predicted to alter critical electrostatic interhelical interactions, leading to altered G protein coupling. Indeed, several single amino acid substitutions in other G protein-coupled receptors resulting in altered signalling have been localized in this motif of the third cytoplasmic loop (Allen et al., 1991; Parma et al., 1993).

The expression of the mutant receptor in stably transfected CHOK1 cells has been demonstrated to induce profound alterations in somatostatin signalling (Figure 1B,C,D). In particular, the expression of the mutant receptor totally abrogated the inhibitory action of somatostatin on cell growth and MAPK activity induced by SST5 activation and was associated with a higher rate of cell proliferation (Figure 1B&C). Indeed, the mutant receptor increased serum stimulated MAPK activity, suggesting that replacement of Arg 240 by Trp confers to the receptor the ability to couple with G proteins different from those activated by the WT SST5 and able to signal to the MAPK cascade via either G_{α} (such as Gq) or $\beta\gamma$ subunits (Iglese et al., 1995; van Biesen et al., 1996).

The relative contribution of SST2 and SST5 in the control of GH secretion in acromegalic patients is still unclear. In fact, although several evidence point to a predominant role of SST2

both receptors are probably required for hormonal control because the activation of SST2 and SST5 results in a synergistic effect on GH release (Shimon et al., 1997, Jaquet et al., 2000, Ren et al. 2003). This view is also supported by the observation that these receptors may form heterodimers with enhanced functionality (Rochville et al., 2000 a&b). As far as the expression of receptor subtypes in GH-secreting adenomas is concerned, it is well documented that SST5 is the most abundantly expressed receptor (Greenman and Melmed 1994, Corbetta et al. 2001, Hofland et al. 2004, Park et al. 2004, Taboada et al. 2007), SST5 mRNA levels being several fold higher than SST2 mRNA (Jaquet et al., 2000, Corbetta et al. 2001, O'Toole et al. 2006). This ratio was even higher in the adenoma harbouring the SST5 mutation, which showed a selective loss of SST2 mRNA, as frequently occurs in poorly responsive tumors (Reubi et al., 1989; Barlier et al., 1999; Jaquet et al., 2000, Corbetta et al. 2001), suggesting that this mutation, together with the low expression of SST2, may be responsible for the resistance to octreotide observed in this patient.

Loss of heterozygosity at SST5 gene locus in pituitary adenomas

Several studies reported loss of heterozygosity (LOH) on various chromosomes in functioning and non-functioning pituitary adenomas (Pei et al. 1995, Bates et al. 1997, Simpson et al. 1999, Asa & Ezzat 2000, Clayton et al. 2000, Asteria et al. 2001, Buch et al. 2003, Simpson et al. 2004). In particular, in about a third of invasive tumors LOH on chromosomes 10q26, 11q13 and 13q12-14 have been described. Recently, LOH at the SST5 gene locus (located at chromosome 16p13.3) has been evaluated in a series of somatotroph and thyrotroph adenomas (Filopanti et al. 2004) taking advantage of the Pro335Leu substitution (C1004T) that is a common polymorphism previously reported in the Danish and British populations. By this approach, 2 tumors (1 somatotroph and 1 thyrotroph adenoma) had LOH at the SST5 locus, while 11 retained the two alleles.

Although SST5 cannot be considered as a tumor suppressor gene, it is well established that the activation of SST2 and SST5 by somatostatin exerts a crucial role in inhibiting hormone secretion and cell proliferation of normal and adenomatous pituitary (Lamberts et al. 1991, Hofland & Lamberts 2003). It is therefore conceivable that the reduced receptor expression or the preferential presence of allelic variants may render these cells less sensitive to the inhibitory action of somatostatin.

Likewise previous studies that failed to identify a second hit in pituitary tumors with LOH (Pei et al. 1995, Bates et al. 1997, Simpson et al. 1999, Hodo et al. 2003), no mutational changes in the retained allele were found in the tumors with LOH at the SST5 locus. Despite the absence of mutations, it is worth noting that the tumors retained the Leu335 variant. The Pro to Leu substitution is a non-conservative amino acid change located in the C-terminal intracellular tail of the receptor and mutation analysis of this domain has shown that it is involved in the interaction with adenylyl cyclase and in mediating receptor desensitisation and internalisation (Hukovic et al. 1998, Sharma et al. 1999)

The phenotype of tumors with LOH at SST5 gene locus deserves some considerations. Contrary to what is observed in the majority of patients with TSH-secreting adenomas, somatostatin analogs, even at high dosage, were ineffective in controlling TSH and thyroid hormone hypersecretion in the patient with LOH. Moreover, this patient had a very aggressive tumor that required a second surgery due to a rapid tumor re-growth. Taking into account that the induction of SST5 mRNA has been shown to represent a crucial step for the inhibition of TSH secretion and cell proliferation caused by thyroid hormone in the TtT-97 murine thyrotroph tumor (Lames et al 1997), it is tempting to speculate that LOH at the SST5 locus, resulting in low SST5 mRNA, together with the retention of a variant receptor, may contribute to the aggressive phenotype of this tumor and resistance to octreotide. As far as somatotroph adenomas were concerned, LOH at the SST5 locus was associated with a normal responsiveness to octreotide,

this finding being consistent with the predominant role of SST2 in the inhibition of GH secretion (Jaquet et al. 2000).

SST2 and SST5 gene polymorphic variants in acromegalic patients

In recent years, molecular studies investigated the possible association of gene polymorphisms and susceptibility to diseases and/or resistance to drugs. In particular, a recent study reported the association of polymorphisms in SST5 gene and bipolar affective disorder in the Danish and British population, consistent with the hypothesis that reduced somatostatinergic tone or altered heterodimerization of SST5 and dopamine D2 receptor might be involved in the pathogenesis of this disorder (Nyegaard et al. 2002). Moreover, a polymorphism located in human SST2 promoter responsible for reduced SSTR2 transcription has been identified in human pancreatic cancer cells (Torrisoni et al. 2001).

The reported single nucleotide polymorphisms located in promoter element-binding sites or causing nonsynonymous amino acid substitutions in SST2 and SST5 genes have been recently analyzed in a series of GH-secreting adenomas (Filopanti et al. 2005). By this approach, any significant differences in SST2 and SST5 allele frequencies between acromegalic patients and controls were found (Table 1). As far as the impact of SST2 polymorphisms on the clinical phenotype was considered, the two single nucleotide substitutions detected in the promoter sequence at positions -83 and -57 from the major transcription initiation site were not associated with differences in clinical characteristics, hormonal profile, or responsiveness to somatostatin analog therapy. This data are in contrast with the notion that -83 g substitution is associated with a reduced SST2 transcription at least in pancreatic cell lines (Torrisoni et al. 2001) and indicate that, although the poor responsiveness to somatostatin analogs in acromegalic patients have been correlated to the reduced SST2 expression by the corresponding tumor tissues (Reubi et al.,

1989; Barlier et al., 1999, Jaquet et al. 2000, Saveanu et al. 2001, Hofland LJ & Lamberts SW 2004), this phenomenon is not due to polymorphic variants in SSTR2 gene promoter.

Regarding SSTR5 polymorphism, patients with C1004 allele (P335) showed IGF-I levels significantly lower than patients homozygous for 1004T (335L) (Figure 2A) and a tendency to lower GH levels. This substitution is a common polymorphism causing a nonconservative amino acid change (*i.e.* proline to leucine) and it is located in the C-terminal intracellular tail of the receptor, and mutation analysis of this domain has shown that it is involved in the interaction with adenylyl cyclase and in receptor desensitization and internalization (Sharma et al. 1999). Moreover, this variant has been recently associated with bipolar affective disorder in the British population, consistent with the hypothesis that reduced somatostatinergic tone or altered heterodimerization of SSTR5 with dopamine D2 receptor might be involved in the pathogenesis of this disorder (Nyegaard et al. 2002, Rocheville et al. 2000). Therefore, it is tempting to speculate that the variant receptor may confer a low sensibility to the hypothalamic somatostatin tone, resulting in an impaired negative feedback and higher IGF-I levels in acromegalic patients. Conversely, the lack of differences in the responsiveness to somatostatin analogs between the different genotypes is likely related to the supraphysiological doses of peptide reached during octreotide or lanreotide administration.

The study of C1004T and T-461c SSTR5 haplotypes revealed that GH levels were lower in P+/T- patients (*i.e.* C1004 homozygotes and heterozygotes and C-461 homozygotes) than in P-/T+ patients (*i.e.* T1004 homozygotes and T-461 homozygotes and heterozygotes). This association, together with the observation that in patients having them both present or absent (P+/T+ or P-/T- haplotypes) GH levels were superimposable, suggested opposite actions of SSTR5 C1004 and T-461 alleles on GH secretion. Taking into account that T-461c polymorphism is included in a sequence recognized by Hmx1, a transcription factor that belongs to a family of homeodomain proteins (Amendt et al. 1999), it has been hypothesized that this polymorphism might affect SSTR5 transcription.

CONCLUSIONS

In the last years several studies investigated the impact of alterations in genes coding for the somatostatin receptor subtypes in the pathogenesis of pituitary tumors, particularly GH secreting adenomas. Due the relevant role of both SST2 and SST5 in controlling GH secretion and somatotroph growth, these two receptor subtypes have been considered candidate genes for the poor responsiveness to somatostatin analogs that characterizes a subset of patients with acromegaly. However, no molecular events responsible for the low expression of somatostatin receptors that is associated with the resistance to somatostatin action have been identified. Indeed, mutations in the coding sequence of these receptors and/or loss of heterozygosity in loci where these receptor are located seem to be very rare events. Moreover, analysis of SST2 and SST5 gene polymorphisms seem to suggest that these variants are not associate with different responsiveness to somatostatin analogs in patients with acromegaly. It is well established that the identification of somatostatin and its five receptor subtypes has provided important information on the pathophysiology of pituitary tumors and that the development of somatostatin analogs has significantly modified the medical approach to patients with pituitary tumors. However, at present it remains to be defined whether genetic abnormalities or variations in somatostatin receptors are relevant in pituitary tumors formation and clinical outcomes.

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Figure legends

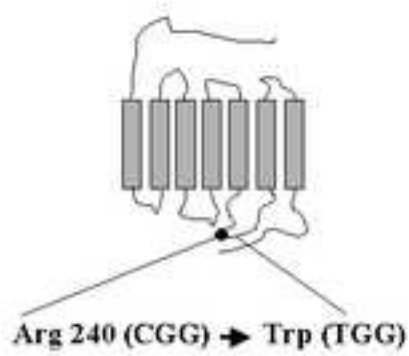
Figure 1. A, R240W mutation is located in the third intracellular loop of SST5, this region representing the cytoplasmic extension of the α -elix 6 and being potentially involved in coupling with G proteins; B. In basal condition, R240W clones showed a higher proliferation rate as evaluated by a Cell Counter after 72 h incubation in the presence of FCS 10%; C, Incubation with the somatostatin analog octreotide (1 nM for 24 h) caused a significant reduction of FCS-induced proliferation of WT cells. Conversely, in mutant cells octreotide caused a significant increase in growth rate; D, In CHO cells with WT SST5, 1 nM somatostatin-28 (SS-28) induced a significant inhibition of FCS-stimulated MAPK activities, whereas in R240W mutant cells the peptide did not reduce but even increased this activity by 2- to 3-fold (Ballarè et al. 1998).

Figure 2 Basal circulating levels of IGF-I (A) and GH (B) in 66 acromegalic patients categorized by SSTR2 and SSTR5 genotypes and haplotypes as follow: P+ subjects were c1004 homozygotes and heterozygotes; P- subjects were t1004 homozygotes; T+ subjects were t-461 homozygotes and heterozygotes; T- subjects were c-461 homozygotes; P+/T+ subjects had c1004 and t-461 alleles; P-/T- did not have both alleles; and P+/T- had only c1004 allele and P-/T+ conversely. Mean \pm SEM. §, Student's *t* test, $P = 0.02$. Mean \pm SEM; *, Kruskal-Wallis test, $P < 0.05$, Dunn's post test on P+/T- and P-/T groups (Filopanti et al 2005)

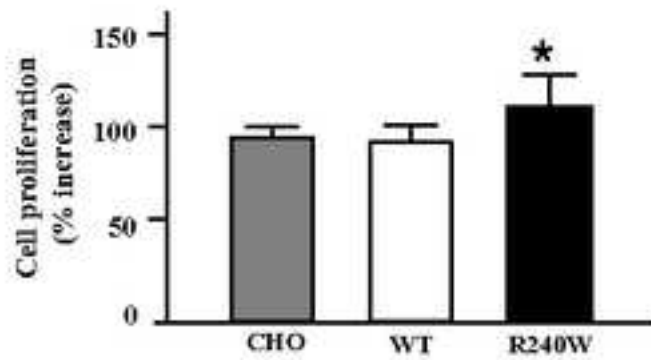
Table 1. SST2 and SST5 gene polymorphic variants in acromegalic patients

	Acromegalic (%)			Control (%)		In vivo/ in vitro phenotype
	+/+	+/-	-/-	+/+	+/-	
SST2 polymorphisms						
t80c	100	0	0	100	0	N/A
c-57g	65.5	34.5	0	72.7	27.3	No effect on sst2 expression in pancreatic cell lines
a-83g	53.9	46.1	0	54.5	45.5	Reduced sst2 expression in pancreatic cell lines
SST5 polymorphisms						
c1004t	40.9	59.1	0	42.4	57.6	Bipolar affective disorder
c325t	0	0	0	0	0	N/A
t-461c	30.3	69.7	0	34.8	65.2	N/A

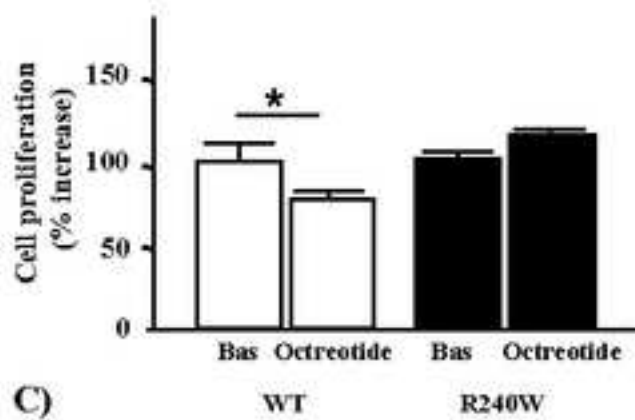
N/A, not available



A)



B)



C)

D)

