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SHORT COMMUNICATION

Mutation analysis of the c-*mos* proto-oncogene and the endothelin-B receptor gene in medullary thyroid carcinoma and phaeochromocytoma

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Summary The characteristic tumours of MEN 2 are medullary thyroid carcinoma (MTC) and phaeochromocytoma. Somatic *RET* mutations have been found in only 23-40% of sporadic MTC and 10% of sporadic phaeochromocytomas. Thus, we sought other genes which may play a role in the pathogenesis of these tumours. We carried out direct sequence analysis of human c-*mos* and human *ENRB* in a series of sporadic MTC and phaeochromocytomas to determine if somatic mutations in these two genes could account for some of the sporadic MEN 2-related tumours in which no *RET* mutations are detected. No somatic mutations were found.

Keywords: MEN 2; *RET*; c-*mos*; endothelin-B receptor; medullary thyroid carcinoma; phaeochromocytoma

Multiple endocrine neoplasia type 2 (MEN 2) is an autosomal dominantly inherited cancer syndrome associated with germline mutations in the *RET* proto-oncogene, which codes for a receptor tyrosine kinase expressed in tissues and tumours of neural crest origin (Bolino *et al.*, 1995; Carlson *et al.*, 1994; Donis-Keller *et al.*, 1993; Eng *et al.*, 1994, 1995*c*; Hofstra *et al.*, 1994; Mulligan *et al.*, 1993, 1994, 1995). MEN 2 is characterised by the presence of MTC and phaeochromocytoma. Somatic *RET* mutations have been detected in 23-40% of sporadic MTC in series comprising ten or more tumours (Eng *et al.*, 1994, 1995*b*; Hofstra *et al.*, 1994; Komminoth *et al.*, 1995; Zedenius *et al.*, 1994) and 10-20% of sporadic phaeochromocytomas (Beldjord *et al.*, 1995; Eng *et al.*, 1994, 1995*a*; Lindor *et al.*, 1995).

The human c-*mos* proto-oncogene encodes a serine-threonine protein kinase expressed at high levels in germ cells (reviewed in Yew *et al.*, 1993). It is required for meiosis and plays a role in the initiation of oogenesis (Colledge *et al.*, 1994; Sagata *et al.*, 1988). Transgenic mice ectopically overexpressing *mos* develop medullary thyroid carcinomas (MTC) and phaeochromocytomas (Schulz *et al.*, 1992). The endothelin-B receptor gene (*ENRB*) codes for a G-protein-coupled receptor, which is expressed in several tissues, including the kidneys, adrenal medulla and phaeochromocytomas (reviewed in Davenport, 1996; Davenport *et al.*, 1994). Recently, germline mutations in *ENRB* have been found in patients with Hirschspung disease (HSCR) (Puffenberger *et al.*, 1995), a common congenital condition characterised by the lack of enteric ganglia leading to intestinal obstruction (Okamoto and Ueda, 1967). Up to 40% of patients with HSCR have mutations in the *RET* proto-oncogene (Attié *et al.*, 1995).

To determine if mutations in the c-*mos* or *ENRB* genes play a role in the pathogenesis of the sporadic tumours in which no *RET* mutations have been detected, the coding sequence of c-*mos* and *ENRB* was examined in DNA from ten sporadic MTC and ten sporadic phaeochromocytomas which do not have known somatic *RET* mutation.

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Materials and methods

Tumours

The ten MTCs and nine of ten phaeochromocytomas studied here have been described previously (Eng *et al.*, 1994, 1995*a*,*b*). The remaining phaeochromocytoma was a unilateral tumour occurring in a patient without first- or second-degree relatives with these syndromes, known phaeochromocytoma or MTC, or other stigmata of MEN 2/von Hippel-Lindau disease. Genomic DNA was extracted as described by Mathew *et al.* (1987).

Polymerase chain reaction (PCR) and DNA sequence analysis

PCR was performed, using 50-100 ng of template genomic DNA and red hot *Thermus icelandicus* DNA polymerase, according to the manufacturer's recommendations (Advanced Biotechnologies, Surrey, UK) in the presence of 1.5 mM magnesium chloride.

A total of 30 to 40 cycles of amplification were carried out at 95°C for 1 min, 59°C (c-*mos*) or 60°C (*ENRB*) for 1 min and 72°C for 1 min, followed by a final 10 min cycle at 72°C. For c-*mos*, primer pairs used were Hu Mos 1F (5'-TCTTCATTCACTCCAGCGG-3') and Hu Mos 1R (5'-AAGTCGCCTTGTACACCGAG-3'), Hu Mos 2F (5'-GGTGTGCTTGCTGCAGAG-3') and Hu Mos 2R (5'-CGCCATAGATGACTTGGTGT-3'), Hu Mos 3F (5'-CCTAGGGACCATCATCATGG-3') and Hu Mos 3R (5'-GTGTCTGGAAGCACAGCAGA-3') or Hu Mos 4R (5'-GGACGGGCGCAGGTCGTAGGCCAC-3'), and Hu Mos 4F (5'-TCAGTGAGCAGGATGTCTGTAA-3') and Hu Mos 5R (5'-CTTGACCAAGTTTTCAGTCAGC-3'). For *ENRB*, the primer pairs used were ETB-0F (5'-CACACCCCTTCCAGAACG-3') and ETB-1R (5'-CGCCCTTTACCTTGTAGACATT-3') [exon 1], ETB-2F$_a$ (5'-CTGCTGGCAGAGGACTGG-3') and ETB-I3R (AAGGAGTGGGGAACAGGG-3') [exons 2 and 3], ETB-I4F (5'-GCTATGAGTAAAATGAGCCAT-3') and ETB-I5R (5'-CTGCCTATAAAAAAGATCGATGG-3') [exon 5], ETB-5F (5'-CAGGATTCTGAAGCTCACTCTTT-3') and ETB-I6R (5'-TGTTTAAAAAAATCATCCATGA-3') [exon 6], and ETB-I6F (5'-ATACAAAGAAAGTCAGAACCCTGG-3') and ETB-8R (5'-TTTTTGTTTTGTTTTGGCAAAT-3') [exon 7]. Primers for exon 4 have been previously described (Puffenberger *et al.*, 1995).

PCR products were gel and column purified (Eng *et al.*,

1994). Twenty aliquots of 100 ng of PCR product served as the template for cycle sequencing (Cyclist kit or the Cyclist Exo-Pfu DNA sequence kit, Strategene). Sequence variants were confirmed by digestion with an appropriate restriction enzyme.

Results

No somatic mutations were detected in the coding sequence of c-*mos* or the endothelin-B receptor gene among ten sporadic MTC and ten sporadic phaeochromocytomas. In addition, all PCR products were the expected sizes, suggesting no deletions or splicing variants which could have been missed by sequence analysis.

A novel sequence polymorphism was detected within the kinase domain of c-*mos*: a G (A1) to T (A2) conversion, changing an alanine to a serine at codon 105. This causes loss of a *Blp*I restriction site. The A1 and A2 alleles occurred at frequencies of 0.85 and 0.15, respectively, among 122 Caucasian chromosomes.

Discussion

Although the tumours that developed in transgenic c-*mos* mice resembled those of MEN 2, suggesting that the human homologue may be involved in MEN 2-related tumours, no somatic c-*mos* mutations or rearrangements could be found in human MTC or phaeochromocytomas. However, gene amplification in the tumours could not be excluded with our analyses, and sufficient DNA was not available for gene dosage studies. The lack of c-*mos* mutations in human tumours might be because in transgenic mice, the *mos* constructs were transcribed from the Moloney murine sarcoma virus LTR, a relatively strong promoter which may also cause ectopic patterns of expression; or because of species-specific differences. Finally, it is possible that c-*mos* may play a role in rare MTC and phaeochromocytomas but the numbers examined precluded detection. In light of our data, it is interesting to note that *in vitro*, many deletion or missense mutations of conserved residues, except for one 10-residue deletion, in *Xenopus mos* result in the elimination of biological and kinase activity (Fukasawa *et al*., 1995).

The finding of germline mutations of *ENRB* in HSCR and its expression pattern suggested that this gene would be a good candidate for involvement in the pathogenesis of MTC and phaeochromocytoma. However, no mutations were detected. Thus, mutation of neither human c-*mos* nor *ENRB* appears to play a major role in the tumorigenesis of MTC and phaeochromocytomas.

A novel sequence polymorphism in the kinase domain of c-*mos* was detected. The polymorphisms could serve as a useful marker in the region of human c-*mos* on chromosome subband 8q12, and may in addition help in the understanding of computer-modelled structure–function relationships of kinase domains.

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References

ATTIÉ T, PELET A, EDERY P, ENG C, MULLIGAN LM, AMIEL J, BOUTRAND L, BELDJORD C, NIHOUL-FÉKÉTÉ C, MUNNICH A, PONDER BAJ AND LYONNET S. (1995). Diversity of *RET* proto-oncogene mutations in familial and sporadic Hirschsprung disease. *Hum. Mol. Genet.*, **4,** 1381–1386.

BELDJORD B, DESCLAUX-ARRAMOND F, RAFFIN-SANSON M, CORVOL J-C, DE KEYSER Y, LUTON J-P, PLOUIN P-F AND BERTAGNA X. (1995). The *RET* proto-oncogene in sporadic pheochromocytomas: frequent MEN 2-like mutations and new molecular defects. *J. Clin. Endocrinol. Metab.*, **80,** 2063–2068.

BOLINO A, SCHUFFENECKER I, LUO Y, SERI M, SILENGO M, TOCCO T, CHABRIER G, HOUDENT C, MURAT A, SCHLUMBERGER M, TOURNIAIRE J, LENOIR GM AND ROMEO G. (1995). *RET* mutations in exons 13 and 14 of FMTC patients. *Oncogene*, **10,** 2415–2419.

CARLSON KM, DOU S, CHI D, SCAVARDA N, TOSHIMA K, JACKSON CE, WELLS SA, GOODFELLOW PJ AND DONIS-KELLER H. (1994). Single missense mutation in the tyrosine kinase catalytic domain of the *RET* protooncogene is associated with multiple endocrine neoplasia type 2B. *Proc. Natl Acad. Sci. USA*, **91,** 1579–1583.

COLLEDGE WH, CARLTON MBL, UDY GB AND EVANS MJ. (1994). Disruption of c-*mos* causes parthenogenetic development of unfertilized mouse eggs. *Nature*, **370,** 65–68.

DAVENPORT AP. (1996). Distribution of endothelin receptors. In *Endothelins in Biology and Medicine*, Miller R, Pelton JT and Huggins J. (eds) CRC Press: Florida (in press).

DAVENPORT AP, MAGUIRE JJ AND KARET FE. (1994). Endothelin receptors and their subtypes. In *Endothelin: Role in Health and Disease*, Gulati A. (ed.) pp. 17–30. Harwood Academic Publishers: Amsterdam.

DONIS-KELLER H, DOU S, CHI D, CARLSON KM, TOSHIMA K, LAIRMORE TC, HOWE JR, MOLEY JF, GOODFELLOW P AND WELLS SA. (1993). Mutations in the *RET* proto-oncogene are associated with MEN 2A and FMTC. *Hum. Mol. Genet.*, **2,** 851–856.

ENG C, SMITH DP, MULLIGAN LM, NAGAI MA, HEALEY CS, PONDER MA, GARDNER E, SCHEUMANN GFW, JACKSON CE, TUNNACLIFFE A AND PONDER BAJ. (1994). Point mutation within the tyrosine kinase domain of the *RET* proto-oncogene in multiple endocrine neoplasia type 2B and related sporadic tumours. *Hum. Mol. Genet.*, **3,** 237–241.

ENG C, CROSSEY PA, MULLIGAN LM, HEALEY CS, HOUGHTON C, PROWSE A, CHEW SL, DAHIA PLM, O'RIORDAN JLH, TOLEDO SPA, SMITH DP, MAHER ER AND PONDER BAJ. (1995*a*). Mutations of the *RET* proto-oncogene and the von Hippel–Lindau disease tumour suppressor gene in sporadic and syndromic phaeochromocytoma. *J. Med. Genet.*, **32,** 934–937.

ENG C, MULLIGAN LM, SMITH DP, HEALEY CS, FRILLING A, RAUE F, NEUMANN HPH, PFRAGNER R, BEHMEL A, LORENZO MJ, STONEHOUSE TJ, PONDER MA AND PONDER BAJ. (1995*b*). Mutation of the *RET* proto-oncogene in sporadic medullary thyroid carcinoma. *Genes Chrom. Cancer*, **12,** 209–212.

ENG C, SMITH DP, MULLIGAN LM, HEALEY CS, ZVELEBIL MJ, STONEHOUSE TJ, PONDER MA, JACKSON CE, WATERFIELD MD AND PONDER BAJ. (1995*c*). A novel point mutation in the tyrosine kinase domain of the *RET* proto-oncogene in sporadic medullary thyroid carcinoma and in a family with FMTC. *Oncogene*, **10,** 509–513.

FUKASAWA K, ZHOU R, MATTEN WT, ARMSTRONG AJ, DAAR I, OSKARSSON M, SATHYANARAYANA BK, MACLVOR L, WOOD TG AND VANDE WOUDE GF. (1995). Mutagenic analysis of functional domains of the *mos* proto-oncogene and identification of the sites important for MAPK activation and DNA binding. *Oncogene*, **11,** 1447–1457.

HOFSTRA RMW, LANDSVATER RM, CECCHERINI I, STULP RP, STEELWAGEN T, LUO Y, PASINI B, HOPPENER JWM, PLOOS VAN AMSTEL HK, ROMEO G, LIPS CJM AND BUYS CHCM. (1994). A mutation in the *RET* proto-oncogene associated with multiple endocrine neoplasia type 2B and sporadic medullary thyroid carcinoma. *Nature*, **367,** 375–376.

KOMMINOTH P, KUNZ EK, MATIAS-GUIU X, HIORT O, CHRISTENSEN G, COLOMER A, ROTH J AND HEITZ PU. (1995). Analysis of *RET* proto-oncogene point mutations distinguishes heritable from nonheritable medullary thyroid carcinomas. *Cancer*, **76,** 479–489.

LINDOR NM, HONCHEL R, KHOSLA S AND THIBODEAU SN. (1995). Mutations in the *RET* protooncogene in sporadic pheochromocytomas. *J. Clin. Endocrinol. Metab.*, **80,** 627–629.

MATHEW CGP, SMITH BA, THORP K, WONG Z, ROYLE NJ, JEFFREYS AJ AND PONDER BAJ. (1987). Deletion of genes on chromosome 1 in endocrine neoplasia. *Nature*, **328,** 524–526.

MULLIGAN LM, KWOK JBJ, HEALEY CS, ELSDON MJ, ENG C, GARDNER E, LOVE DR, MOLE SE, MOORE JK, PAPI L, PONDER MA, TELENIUS H, TUNNACLIFFE A AND PONDER BAJ. (1993). Germline mutations of the *RET* proto-oncogene in multiple endocrine neoplasia type 2A. *Nature*, **363,** 458–460.

MULLIGAN LM, ENG C, HEALEY CS, PONDER MA, FELDMAN GL, LI P, JACKSON CE AND PONDER BAJ. (1994). A *de novo* mutation of the *RET* proto-oncogene in a patient with MEN 2A. *Hum. Mol. Genet.*, **3,** 1007–1008.

MULLIGAN LM, MARSH DJ, ROBINSON BG, SCHUFFENECKER I, ZEDENIUS J, LIPS CJM, GAGEL RF, TAKAI S-I, NOLL WW, FINK M, RAUE F, LACROIX A, THIBODEAU SN, FRILLING A, PONDER BAJ AND ENG C. (1995). Genotype–phenotype correlation in MEN 2: report of the International *RET* Mutation Consortium. *J. Intern. Med.*, **238,** 343–346.

OKAMOTO E AND UEDA T. (1967). Embryogenesis of intramural ganglia of the gut and its relation to Hirschsprung disease. *J. Pediatr. Surg.*, **10,** 437–443.

PUFFENBERGER EG, HOSODA K, WASHINGTON SS, NAKAO K, DE WIT D, YANAGISAWA M AND CHAKRAVARTI A. (1995). A missense mutation of the endothelin B receptor gene in multigenic Hirschsprung's disease. *Cell*, **79,** 1257–1266.

SAGATA N, OSKARSSON M, COPELAND T, BRUMBAUGH J AND VANDE WOUDE GF. (1988). Function of c-*mos* proto-oncogene product in meiotic maturation in *Xenopus* oocytes. *Nature*, **335,** 519–525.

SCHULZ N, PROPST F, ROSENBERG MP, LINNOILA RI, PAULES RS, KOVATCH R, OGISO Y AND VANDE WOUDE GF. (1992). Pheochromocytomas and c-cell thyroid neoplasms in transgenic c-*mos* mice: a model for the human multiple endocrine neoplasia type 2 syndrome. *Cancer Res.*, **52,** 450–455.

YEW N, STROBEL M AND VANDE WOUDE GF. (1993). *Mos* and the cell cycle: the molecular basis of the transformed phenotype. *Curr. Opin. Genet. Devel.*, **3,** 19–25.

ZEDENIUS J, WALLIN G, HAMBERGER B, NORDENSKJÖLD M, WEBER G AND LARSSON C. (1994). Somatic and MEN 2A *de novo* mutations identified in the *RET* proto-oncogene by screening of sporadic MTCs. *Hum. Mol. Genet.*, **3,** 1259–1262.