***BRAF* and endocrine tumors: mutations are frequent in papillary thyroid carcinomas, rare in endocrine tumors of the gastrointestinal tract and not detected in other endocrine tumors**

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Abstract

The tumorigenesis of sporadic endocrine tumors is still not fully understood. Activating point mutations of the serine/threonine kinase gene *BRAF* located on 7q34 are found in a wide range of malignancies, with the highest frequency (66%) occurring in malignant melanomas. Melanomas are tumors of neural-crest-derived cells as are medullary thyroid carcinomas, pheochromocytomas and paragangliomas. *BRAF* has not been examined in endocrine tumors of the diffuse neuroendocrine system or of neural-crest-derived cells.

We examined 130 endocrine tumors of the pancreas, parathyroid gland, adrenal medulla, paraganglia, lung and gastrointestinal tract as well as follicular and c-cell-derived thyroid tumors. We found a high rate of V599E mutations in papillary thyroid carcinomas (47%), one V599E mutation in a well-differentiated gastric endocrine carcinoma (malignant carcinoid), but no activating *BRAF* mutations in all other endocrine tumors examined. These results point towards different pathways in tumorigenesis of endocrine tumors of various localizations and only rare involvement of the MAP kinase (MAPK) pathway in a subset of malignant neuroendocrine tumors.

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Introduction

Activating mutations of the serine/threonine kinase gene *BRAF* located on chromosome 7q34 have recently been reported in a wide range of malignancies, with the highest frequency (66%) occurring in malignant melanomas (Davies *et al.* 2002). The described mutations are clustered in exons 11 and 15 and are located in the G-loop or activation segment of the gene. It was shown that transient transfection with V599E *BRAF* leads to a 10-fold increase of basal kinase activity and downstream activation of the MAP kinase (MAPK) pathway (Davies *et al.* 2002). The V599E mis-sense mutation in exon 15 is found in more than 90% of mutated malignant melanomas (Davies *et al.* 2002). Interestingly, thus far no analyses of endocrine tumors

have been conducted (with the exception of thyroid tumors), although there are several indications pointing towards a possible role of *BRAF* in this type of tumor also. First, like melanomas (Dupin & Le Douarin 2003), a subset of endocrine tumors including pheochromocytomas (PCCs), paragangliomas (PGLs) and medullary thyroid carcinomas (MTCs) evolve from neural-crest-derived cells (Pasini *et al.* 1996). Secondly, on a molecular genetic level, activation of tyrosine kinases (*Ret* mutations in MTCs and PCCs, *Ret/PTC* translocations or TM3/NTRK1 rearrangements producing the TRK oncogene in papillary thyroid carcinomas (PTCs) are known to be of importance at least in a subset of endocrine tumors. Thirdly, chromosomal gains of 7q34 have repeatedly been described in endocrine tumors, possibly an alternative mechanism leading to

increased *BRAF* activity due to increased protein expression. More specifically, comparative genomic hybridization (CGH) studies have demonstrated gains of 7q in up to 66% of follicular thyroid carcinomas (FTCs) (Roque *et al.* 1999, Perissel *et al.* 2002), 30% of pancreatic endocrine tumors (PETs) (Speel *et al.* 1999, 2001, Stumpf *et al.* 2000), 10% of parathyroid adenomas (PTAs) (Agarwal *et al.* 1998), gastrointestinal neuroendocrine tumors (GI-NETs) (Zhao *et al.* 2000), bronchial neuroendocrine tumors (B-NETs) (Zhao *et al.* 2000) and PGLs (Edstrom *et al.* 2000) and 5% of papillary thyroid carcinomas (PTC) (Singh *et al.* 2000). We therefore investigated a series of 130 endocrine tumors of different organs for *BRAF* alterations in exons 11 and 15.

Materials and Methods

Tumor samples

Fresh frozen tissue and paraffin blocks were obtained from the files of the Department of Pathology, University Hospital Zurich. A total of 130 tumors were examined including 25 PETs, 10 PTAs, 16 PCCs (all without *Ret* mutations), 18 PGLs, 8 B-NETs and 6 GI-NETs (carcinoids, CDs), 25 MTCs (including one multiple endocrine neoplasia type 2a (MEN2a)-associated tumor, 7 tumors with somatic *Ret* exon 16 mutations and 13 tumors without *Ret* mutation), 7 FTCs and 15 PTCs. Tumor samples and corresponding normal tissue had been snap-frozen in liquid nitrogen after surgical removal and stored at -80°C . Where no fresh tissue was available, paraffin blocks of tumor and non-neoplastic tissue were used. These tissue samples had been fixed in 4% buffered formalin and embedded in paraffin according to standard protocols.

Controls

Tumor DNA of a paraffin-embedded malignant melanoma with known *BRAF* V599E mutation was used as positive control; blood DNA of a healthy individual served as negative control.

Denaturing gradient gel electrophoresis (DGGE)-based mutation analysis

DNA from fresh frozen tissue was extracted using the Purgene-kit (GentraSystems, Minneapolis, MN, USA) according to the manufacturer's recommendations. Where no frozen tissue was available, tumor and normal tissue were micro-dissected from 10 μ m tissue sections of paraffin blocks and the DNA extracted as previously described (Perren *et al.* 1998).

PCR using genomic DNA as template was carried out in a 50ul mixture of 1×PCR buffer (Perkin Elmer Europe,

Table 1 Primers used

BRAF 11neuFgc 5'-*TTTTCTGTTTGGCTTGACTGA-3'
 BRAF 11 Rgc 5'-gcgcgCGAACAGTGAATATTTCCTTTGAT-3'
 BRAF 15 Fgc 5'-cgcggcTCATAATGCTTGCTCTGATAGGA-3'
 BRAF 15 Rgc 5'-*GGCCAAAAATTTAATCAGTGA-3'

*cgcccgccgccccgcgcccggcccgcgcccccgcccgaaataataaa

Rotkreuz, Switzerland) containing 400 ng of template DNA, 200 μ M dNTP (Roche), 1 μ M of each intron-based primer (Table 1) and 1 μ l Taq Polymerase (Ampli Taq Gold, Perkin Elmer Europe). A 'touch-down' procedure was used consisting of denaturation for 5 s at 95 °C, annealing for 60 s at temperatures decreasing from 60 to 55 °C during the first 11 cycles (with 0.5 °C decremental steps in cycles 2 to 11), and ending with an extension step at 72 °C for 60 s. Ten cycles with an annealing temperature of 55 °C and 15 cycles with an annealing temperature of 45 °C followed with extension times of 90 s. After a final extension for 10 min at 72 °C, heteroduplex formation was induced by initial denaturation for 10 min at 98 °C followed by incubations at 55 °C for 30 min and 37 °C for 30 min. For DGGE, 10 μ l of the PCR product in 3 μ l Ficoll-based loading buffer were loaded onto 10% polyacrylamide gels containing a urea-formamide gradient in 0.5 $\times$ Tris-acetate TAE-EDTA. The amplicons were electrophoresed at 60 °C and 100 V for 16 h. DNA strands were visualized using silver staining as described previously (Komminoth *et al.* 1994). All samples were additionally cycle sequenced using an automated sequencer (Model 373A, Applied Biosystems, Foster City, CA, USA) and the Sequencher v.3.1.1 (Gene Codes Corp., Ann Arbor, MI, USA) software.

Results

BRAF exon 15 mutations were detected in 7 of 15 PTCs (47%). All mutations consisted of a thymidine to adenine exchange at nucleotide 1769 resulting in a valine to glutamate substitution of residue 599 (V599E). A representative DGGE gel and the sequence result is shown in Fig. 1.

In all 7 PTCs the V599E mutation was somatic in nature, thus it was absent in the germline DNA of the patients. Exon 11 mutations were absent in the 15 PTCs tested.

One gastric, well-differentiated endocrine carcinoma (malignant carcinoid) with liver metastasis showed a somatic *BRAF* V599E mutation, no alterations were detected in the remaining 5 gastrointestinal CDs.

No alterations indicative for mutations of *BRAF* exon 11 and 15 were identified in any of the 25 PETs, 10 PTAs, 16 PCCs, 18 PGLs, 25 MTCs, 7 FTCs and 8 bronchial CDs tested. The results are summarized in Table 2.

Figure 1 Top, DGGE pattern of *BRAF* exon 15. Samples marked '+' show an abnormal banding pattern. Bottom, the corresponding sequence analysis shows a heterozygous V599E mutation. Tu: Tumor; No: normal non neoplastic tissue.

Discussion

We describe a high rate of somatic *BRAF* exon 15 mutations in PTCs of classical type. All these *BRAF* mutations were somatic as the sequence of the corresponding normal tissues was wild-type. These results are

in agreement with reports, published during completion of this study, which described the same type of *BRAF* exon 15 mutation in 36–69% of PTCs (Cohen *et al.* 2003, Fukushima *et al.* 2003, Kimura *et al.* 2003, Namba *et al.* 2003, Nikiforova *et al.* 2003, Soares *et al.* 2003, Xu *et al.* 2003, Puxeddu *et al.* 2004, Soares *et al.* 2004, Trovisco *et*

Table 2 Mutations present in endocrine tumors

Tumor	No. of tumors	Mutation		
		Braf 11	Braf 15	Ret 16
PET*	25	0/22	0/25	n.a.
CD lung	8	0/8	0/8	n.a.
CD gi	6	0/6	1/6	n.a.
PTA	10	0/10	0/10	n.a.
PCC	16	0/16	0/16	0/13
PGL	18	0/18	0/18	n.a.
PTC	15	0/15	7/15	n.a.
FTC	7	0/7	0/7	n.a.
MTC**	25	0/25	0/24	7/20
Total***	130	0/127	8/129	

PET, pancreatic endocrine tumor (*including 3 MEN1-associated PETs); CD lung, bronchial neuroendocrine tumors (carcinoids); CD gi, gastrointestinal neuroendocrine tumors (carcinoids); PTA, parathyroid adenoma; PCC, pheochromocytoma; PGL, paragangliomas; PTC, papillary thyroid carcinoma; FTC, follicular thyroid carcinoma; MTC, medullary thyroid carcinoma (**including 1 MEN2a-associated MTC); ***numbers do not add up, some exons could not be amplified by PCR.

al. 2004, Xing et al. 2004). Thus, the pathway of PTCs appears to be elucidated since either a tyrosin kinase receptor (*Ret*/*PTC* translocation, *TRK* mutation) or the downstream tyrosin kinase *BRAF* are affected on a genetic level.

BRAF is a member of the MAPK pathway inducing a mitogenic response upon stimulation of tyrosine kinase receptors (Liebmann 2001). In colon carcinomas, *BRAF* mutations were only found in tumors without *K-RAS* mutations, indicating an equivalent tumorigenic effect (Davies et al. 2002, Rajagopalan et al. 2002). A similar exclusive correlation was observed in PTCs for *BRAF* mutations and *Ret*/*PTC* translocations. Kimura et al. (2003) and Soares et al. (2003) showed no overlap between tumors with *Ret*/*PTC* translocation, *BRAF* or *RAS* mutations in 78 and 50 PTCs respectively, indicating that in PTCs the two alterations lead to very similar downstream effects. Our series included one PTC with an inactivating *PTEN* mutation and concomitant loss of *PTEN* protein expression (Dahia et al. 1997, Gimm et al. 2000), but no *BRAF* exon 15 mutation. As *BRAF* is known to be downstream of G protein-coupled receptor (GPCR) signaling (Liebmann 2001), the inactivating mutation of the tumor suppressor *PTEN* could lead to a similar downstream effect.

The work presented is the first study examining *BRAF* in endocrine tumors other than PTCs and FTCs. We detected one somatic *BRAF* V599E mutation in a metastasizing endocrine tumor of the stomach (malignant carcinoids). This is evidence for a rare involvement of the MAPK pathway in these tumors. The fact that a malignant endocrine tumor bears this mutation might be indicative for an event of tumor progression. Interestingly, we previously described a similar phenomenon of a PET harboring a *PTEN* mutation (Perren et al. 2000) which also turned out to be of a malignant phenotype.

The absence of *BRAF* exon 11 and 15 mutations in all other examined types of endocrine tumors — including the neural-crest-derived MTC, PGL and PCC — seems interesting. In addition to MEN2-associated familial MTC, PCC and PTA, a subset of sporadic forms of the first two tumors are also known to harbor activating *Ret* tyrosine kinase receptor mutations (Komminoth et al. 1995, van der Harst et al. 1998). However, we did not detect any *BRAF* mutation in sporadic MTCs, PCCs and PGLs. The *Ret* mutation status of the MTCs and PCCs of this study was known and 7 MTC samples with somatic *Ret* M918T mutation as well as one MEN2a (C634Y)-associated MTC were included (Table 2). Somatic or germline *Ret* mutations were absent in all 13 informative PCC samples. Therefore *BRAF* mutations are absent in these tumors irrespective of *Ret* mutation status. These data are indirect evidence that signaling of the constitu-

tively active forms of mutated *Ret* differs from *Ret*/*PTC* translocation induced signaling. In neural-crest-derived tumors, pathways other than the MAPK downstream pathway of *Ret* seem to be of importance. On the other hand, FTCs are more likely to harbor *RAS* mutations than PTCs; however, we and others could not detect any *BRAF* V599E mutation in FTCs.

In summary our study confirms a high rate of *BRAF* V599E mutations in PTCs. We detected a single V599E mutation in a metastasizing gastric endocrine carcinoma which might point towards a role of the MAPK pathway in malignant transformation of these tumors. *BRAF* mutations were absent in all neural-crest-derived tumors including MTCs, PCCs and PGLs. As a subset of these tumors is known to contain activating *Ret* mutations, our results indicate that pathways other than the MAPK pathway are downstream targets of *Ret* mutations in these tumors.

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