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Oncogene and Growth Factor Expression in MEN 2 and Related Tumors

Jeffrey F. Moley,* Göran K. Wallin,* Michele B. Brother,* Michael Kim,* Samuel A. Wells, Jr,* and Garrett M. Brodeur†

Pheochromocytomas occur sporadically or in individuals affected by inherited syndromes including multiple endocrine neoplasia (MEN) type 2A and 2B, neurofibromatosis, and the von Hippel-Lindau syndrome (vHL). Medullary thyroid carcinomas (MTCs) also occur sporadically or as part of MEN 2A, MEN 2B, and familial MTC. Little is known of the molecular genetic background of these tumors. We have shown previously that activation of the N-ras, H-ras, and K-ras oncogenes does not occur in these tumors, but that deletions of the short arm of chromosome 1 are extremely common (> 60%) and may indicate loss of a suppressor gene in the chromosomal region 1p31-36. We have examined the structure and expression of N-myc, c-myc, L-myc, c-mos, nerve growth factor (β -NGF), and the low affinity nerve growth factor receptor (LNGFR) in a series of pheochromocytomas and MTCs from patients with hereditary and sporadic diseases. Southern analysis, using radiolabeled DNA probes, revealed no evidence of amplification or rearrangement of these genes in any normal or tumor tissues except for loss of heterozygosity at the L-myc locus (1p32) in 9 pheochromocytomas from patients with MEN 2A or MEN 2B, in 5 of 11 non-MEN pheochromocytomas, and in 3 of 24 non-MEN MTCs. Gene expression at the RNA level was examined by Northern analysis or ribonuclease protection assay (RPA) using radiolabeled DNA or cRNA probes. C-myc transcripts were detectable at low levels in all tumors tested. L-myc and c-mos transcripts were not detectable in any tumor, and N-myc transcripts were detectable at low levels in all tumors by Northern analysis or RPA. LNGFR transcripts were not detectable in four tumors (two vHL pheochromocytomas, two sporadic pheochromocytomas), but were present at low levels in all other tumors. High levels of gene transcripts, or abnormally sized transcripts, were not seen in any tumor. Deletion of chromosome 1p remains the most consistent and significant molecular genetic abnormality yet identified in these tumors. (Henry Ford Hosp Med J 1992;40:284-8)

Medullary thyroid carcinomas (MTCs) and pheochromocytomas can occur sporadically or in patients with certain familial predisposition syndromes. In multiple endocrine neoplasia (MEN) type 2A and type 2B, patients inherit a susceptibility to the development of MTCs and pheochromocytomas. Familial MTC (FMTC) is another type of MTC which is inherited as an autosomal dominant trait in which patients develop MTCs only. Pheochromocytomas also occur in patients with von Hippel-Lindau (vHL) syndrome and neurofibromatosis type 1. Genetic linkage studies have mapped the predisposing gene in MEN 2A, MEN 2B, and FMTC to the pericentromeric region of chromosome 10 (1-6); however, the predisposing gene(s) has not yet been identified.

The molecular genetic events underlying tumor formation in MEN 2A and MEN 2B are not understood. Unlike hereditary retinoblastoma, deletion or loss of heterozygosity (LOH) in the region of the predisposing locus is found rarely in pheochromocytomas and MTCs from these patients (7-9). It is possible that malignant transformation in the MEN 2 syndromes is caused by other mechanisms, such as oncogene activation and/or the inactivation of a tumor suppressor gene at a different chromosomal locus. We and others have reported chromosome 1

LOH in pheochromocytomas and MTCs from patients with MEN 2A and MEN 2B (10-14), and it is possible that this region harbors a tumor suppressor gene whose inactivation is important in tumorigenesis. Evidence of oncogene activation in these tumors has not been found as yet. We examined *ras* gene sequences in sporadic and hereditary MTCs and pheochromocytomas and found no mutations or deletions (10,15). In this report, we examine the oncogenes *N-myc*, *c-myc*, *L-myc*, and *C-mos*, nerve growth factor (β -NGF), and the low affinity nerve growth factor receptor (LNGFR) in MTCs and pheochromocytomas. *N-myc*, *c-myc*, and *L-myc* were examined because they frequently are amplified or overexpressed in other tumors of neural crest origin (16-18). *C-mos* was examined because transgenic mice which express the *c-mos* gene develop pheochromocytomas and MTCs (19). LNGFR was examined because it is involved in the

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differentiation of a rat pheochromocytoma cell line, and because abnormalities of this receptor have been noted at the DNA, RNA, and protein levels in a closely related neural crest-derived tumor, neuroblastoma (20,21).

Materials and Methods

Tumors and constitutional tissue were obtained at the time of diagnostic or therapeutic surgery. Tumor specimens were frozen immediately in liquid nitrogen and subsequently thawed only for preparation of DNA and RNA. These studies have been approved by an institutional human studies committee.

DNA studies

We examined normal and tumor DNA from 18 patients with pheochromocytomas (7 MEN 2A, 2 MEN 2B, 7 sporadic, 2 vHL) and 27 patients with MTCs (14 MEN 2A, 7 MEN 2B, 6 sporadic) for abnormalities of the *N-myc*, *c-myc*, and *L-myc* genes. We examined normal and tumor DNA from 11 patients with pheochromocytomas (5 MEN 2A, 1 MEN 2B, 5 sporadic) for abnormalities of the β -NGF gene. The LNGFR gene was examined in normal and tumor DNA from 13 patients with pheochromocytomas (7 MEN 2A, 4 sporadic, 2 vHL), and 10 patients with MTCs (6 MEN 2A, 2 MEN 2B, 2 sporadic). The *c-mos* gene was examined in normal and tumor DNA from 12 patients with pheochromocytomas (6 MEN 2A, 4 sporadic, 2 vHL), and five patients with MTCs (3 MEN 2A, 1 MEN 2B, 1 sporadic). DNA was prepared from tumors by standard detergent proteinase K lysis followed by organic extraction and extensive dialysis. Following appropriate digestion, electrophoresis, and Southern transfer (22), filters were hybridized with plasmid probes or purified plasmid DNA inserts labeled by the nick translation (23) or random hexamer primer (24) technique. For *N-myc*, *c-myc*, and *L-myc*, the DNA was digested with Eco RI; for β -NGF, the DNA was digested with Bgl II; and for *c-mos*, the DNA was digested with Bam HI.

RNA studies

We examined the proto-oncogenes *N-myc*, *c-myc*, *L-myc*, and *c-mos*, and the LNGFR at the RNA level in pheochromocytomas and MTCs. *N-myc* expression was examined in 10 pheochromocytomas (3 MEN 2A, 1 MEN 2B, 4 sporadic, 2 vHL) and two MTCs (1 MEN 2B and 1 sporadic). *C-myc* expression was examined in four pheochromocytomas (2 MEN 2A, 2 sporadic, and 2 MTCs [1 MEN 2B and 1 sporadic]). *L-myc* expression was examined in 10 pheochromocytomas (3 MEN 2A, 1 MEN 2B, 4 sporadic, and 2 vHL) and one MTC (MEN 2B). *C-mos* expression was examined in nine pheochromocytomas (2 MEN 2A, 1 MEN 2B, 4 sporadic, and 2 vHL). LNGFR expression was examined in 13 pheochromocytomas (3 MEN 2A, 2 MEN 2B, 6 sporadic, 2 vHL) and one MTC (MEN 2B). Total RNA preparation was done by the method of Chomczynski and Sacchi (25). The size and amount of mRNA for *N-myc*, *c-myc*, *L-myc*, *c-mos*, and LNGFR were analyzed by Northern analysis of total cellular RNA. Thirty micrograms of RNA were denatured and electrophoresed on a formaldehyde-containing 0.8% agarose gel. The RNA was transferred to nitrocellulose filters in 10X

SSC, and filters were then baked at 80 °C. Filters were prehybridized for several hours and then hybridized with purified plasmid DNA inserts labeled by the random hexamer primer technique (24). Filters were then washed and exposed to film for one to three days.

Probes

For *N-myc* DNA and RNA studies, we used a full-length *N-myc* cDNA which is subcloned into the plasmid vector, Bluescript (Stratagene, San Diego, CA) (26). For *c-myc*, we used a cDNA clone which was obtained from Dr. R. Eisenman (27). A genomic *L-myc* clone was obtained from Dr. J. Minna (28). For *c-mos*, we used a plasmid clone from Dr. G. Vande Woude which contains the entire coding sequence (the gene has a single 1.4 kb reading frame) (29). A full-length cDNA clone of LNGFR was obtained from Dr. M. Chao (30). The β -NGF probe was obtained from Dr. A. Ullrich (31). Controls using a β -actin probe (32) were performed to demonstrate that expected amounts of undergraded RNA were transferred to the filters.

Ribonuclease protection assays

Ribonuclease protection assays (RPAs) were done to look for low-level expression of *N-myc* and *c-mos*. In these assays, portions of the *N-myc* and *c-mos* cDNAs were subcloned into Bluescript (Stratagene, San Diego, CA), a plasmid vector which contains T3 and T7 RNA polymerase promoters flanking the restriction site polylinker, into which the cDNA fragments were subcloned. Antisense cRNA fragments of specific size (200 to 300 base pairs) were then generated, incorporating ³²P UTP label. These fragments were mixed with the test DNA, and the solution was then treated with RNase to digest any single stranded fragments. Aliquots were electrophoresed on denaturing acrylamide gels, which were then exposed to film.

Results

DNA studies

There was no evidence of deletion, amplification, or rearrangement of the *N-myc*, *c-myc*, *L-myc*, *c-mos*, β -NGF, or LNGFR genes in any tumor studied. For these probes, all tumors demonstrated the expected normal band pattern and intensity. However, LOH at the *L-myc* locus (1p32) (33) was identified in 9 pheochromocytomas in MEN 2A and MEN 2B patients, compared to only 2 of 7 sporadic pheochromocytomas. We also found *L-myc* LOH in 1 of 2 vHL pheochromocytomas. *L-myc* LOH was noted in 3 of 24 informative MTCs from patients with MEN 2A. LOH on chromosome 1p has been reported by us previously (10,11). The β -NGF locus also maps to the short arm of chromosome 1; however, none of the 11 individuals studied were informative, using two different probe-enzyme combinations previously reported to have a high percentage of informativeness (34).

RNA studies

Using Northern analysis, *N-myc* transcripts were detected in 6 of 10 pheochromocytomas (4 sporadic, 2 vHL) (Fig 1). These transcripts were of expected size. Transcripts (Fig 1) were not

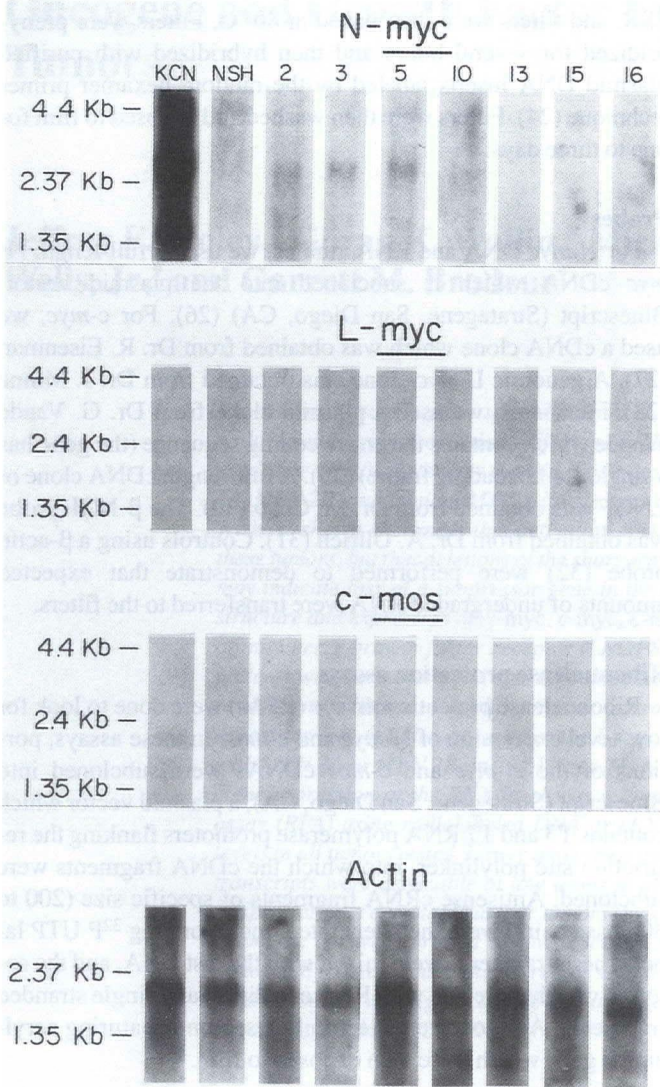


Fig 1—Northern analysis of tumor RNA. Autoradiogram of a nitrocellulose filter containing total cellular RNA from cell lines and tumor RNA, probed with radiolabeled *N-myc* cDNA probe (top), *L-myc* probe (second from top), *c-mos* probe (third from top), and β -actin probe (bottom). For description of probes, see text. KCN: neuroblastoma cell line which amplified and overexpresses *N-myc*; NSH: neuroblastoma cell line which does not amplify *N-myc*. 2-16: representative pheochromocytoma RNAs. 2, 3, 5, 13: sporadic tumors. 10: MEN 2A tumor. 15, 16: von Hippel-Lindau tumors. Normal-sized *N-myc* transcripts are present in tumors 2, 3, and 5. No transcripts are seen for *N-myc* in other tumor RNAs, and no expression was detected for *c-mos* or *L-myc* in any tumor.

seen in one MEN 2B and three MEN 2A tumors. Using the ribonuclease protection assay, low levels of transcription of *N-myc* were found to be present in all pheochromocytomas and MTCs tested (Fig 2) including an MEN 2A pheochromocytoma (case 8) in which Northern analysis did not show expression. *N-myc* expression was detected in two MTCs tested by ribonuclease protection assay (one MEN 2B, one sporadic). *C-myc* expres-

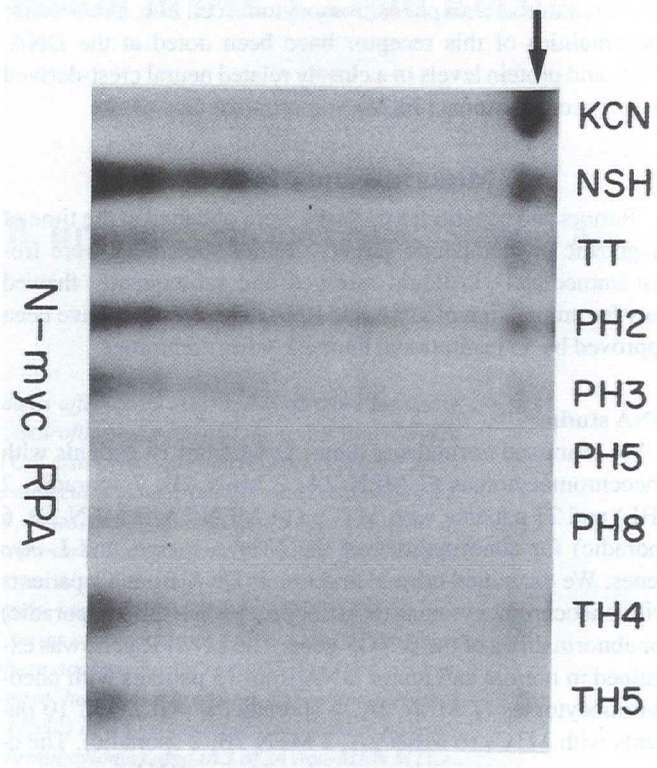


Fig 2—Ribonuclease protection assay for *N-myc* expression. Total cellular RNA was incubated with radiolabeled *N-myc* antisense cRNA probe of defined length. Single-stranded RNA was then digested with RNase, and the remaining protected double-stranded fragments electrophoresed on a denaturing 6% acrylamide gel, which was then exposed for 1 hour. Arrow: location of protected fragment, which is present in all tumor and cell line lanes. KCN: neuroblastoma cell line which overexpresses *N-myc*. NSH: neuroblastoma cell line which does not amplify *N-myc*. PH2, 3, and 5: sporadic pheochromocytomas. PH8: MEN 2A pheochromocytoma. TH4: sporadic MTC. TH5: MEN 2B MTC.

sion was detected by Northern analysis in all six tumors tested, though at very low levels (data not shown). *L-myc* and *c-mos* transcripts were not detectable in any tumor by Northern analysis (Fig 1). Ribonuclease protection assay also failed to detect any expression of *c-mos* in two tumors tested. *LNGFR* transcripts were not detectable in five tumors (1 MEN 2A, 2 MEN 2B, 2 sporadic pheochromocytomas), but were present in all other tumors (Fig 3). High levels of gene transcripts were not seen in any tumor (Fig 3).

Discussion

Because the predisposition gene for the MEN 2 syndromes in the pericentromeric region of chromosome 10 has not been cloned and characterized as yet, it is not possible to completely rule out the possibility that inactivation of the normal inherited allele results in tumor formation in these patients. However, re-

cent reports have noted allelic loss on chromosome 10 in tumors of only 4 of 86 informative cases (7-9). This contrasts with the situation in hereditary retinoblastoma in which tumors that occur in predisposed individuals achieve homozygosity for a defective RB-1 allele in the majority of cases (35,36). It is possible that malignant transformation in the MEN 2 syndromes is caused by other mechanisms, such as oncogene activation or the inactivation of a tumor suppressor gene at a different chromosomal locus.

The data presented here add to the growing body of information regarding the molecular biology of pheochromocytomas and MTCs. We (15) and others (9) previously reported that *ras* gene mutations do not occur in these tumors. Overexpression of the proto-oncogene *N-myc* has been reported in 6 of 21 MTCs analyzed by *in situ* hybridization (37), and a recent study demonstrated expression (without gene amplification or rearrangement) of *c-myc* and *c-fos* in six pheochromocytomas, suggesting that these cells are in a state of growth stimulation (38). In this study, we evaluated the possible role of the *N-myc*, *c-myc*, *L-myc*, and *c-mos* genes in the development of these tumors and have found no evidence that their activation plays a role. At present, there is little evidence that oncogene activation plays a role in the development of these tumors. This contrasts with other related tumors of neural crest origin, such as neuroblastoma or melanoma, in which activation of specific oncogenes is seen with substantial frequency (15,39,40).

The pattern of hyperplasia of selected target organs preceding the development of overt neoplasia in the MEN 2 syndromes suggests that abnormal regulation of growth and differentiation may be important in the growth and development of these tumors. The NGFR pathway is required for the survival of sympathetic and sensory neurons and is known to cause differentiation of the rat pheochromocytoma cell line PC12 into cells resembling sympathetic neurons (41-43). There is evidence that the NGFR pathway is critically important to the development and differentiation of normal chromaffin cells, the cell of origin of pheochromocytomas. Multiple defects in the NGFR pathway have been described in neuroblastoma (21), a tumor which is embryologically related to pheochromocytoma. In this report, we found no abnormalities of the β -NGF or LNGFR genes at the DNA level, and we found absence of detectable expression of LNGFR in the majority of pheochromocytomas tested. No evidence of abnormal expression, such as abnormal transcript size, was noted; however, it is possible that the lack of expression of this receptor may contribute to the maintenance of the undifferentiated state in pheochromocytomas and MTCs.

We have reported that deletion of the short arm of chromosome 1 occurs in all pheochromocytomas from patients with MEN 2A and MEN 2B and in a substantial number of sporadic tumors and MTCs (10,11). This is the most consistent abnormality so far described in tumors from patients with MEN 2A and MEN 2B. The consistent finding of this deletion suggests the presence of a tumor suppressor gene in this region. Deletion of this gene may be the second "hit," which results in the development of pheochromocytomas in patients with MEN 2A and MEN 2B, and may also contribute to the development of MTCs in MEN 2A and of sporadic pheochromocytomas and MTCs.

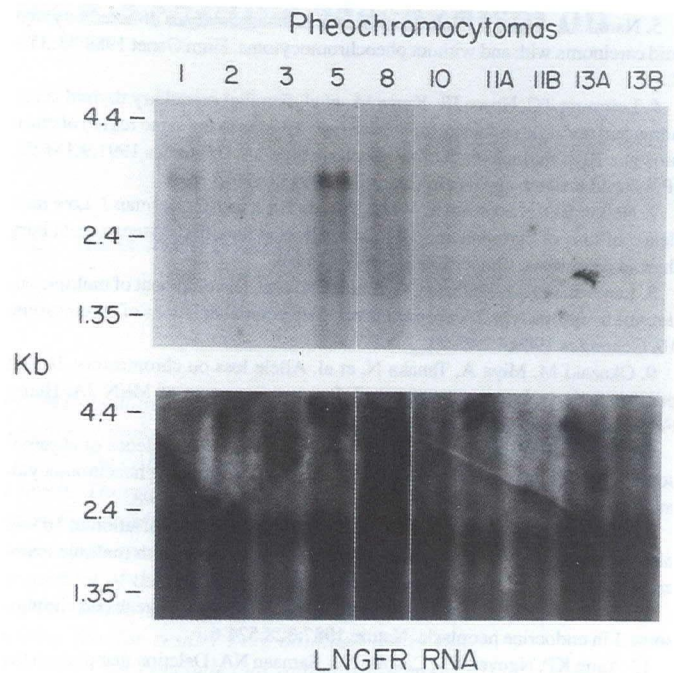


Fig 3—Northern analysis of LNGFR expression. Autoradiogram of nitrocellulose filter containing total cellular RNA from pheochromocytoma RNA probed with radiolabeled LNGFR c-DNA probe (top) and radiolabeled β -actin probe (bottom). Tumors 1, 8, and 10: MEN 2A pheochromocytomas. Tumors 2, 3, 5, 13A, and 13B: sporadic pheochromocytomas. Tumors 11A, 11B: MEN 2B pheochromocytomas (A and B designate different tumors from the same patient). Tumors 1 and 5 demonstrate moderate expression of normal-sized LNGFR transcripts (3.8 kb). Tumors 2, 3, and 10 have low levels of LNGFR transcripts. In tumors 8, 11A and 11B, and 13A and 13B, no transcripts were detectable.

Despite extensive studies by us (10,11,15) and others (9,37, 38), little evidence for activation of oncogenes or growth factors has been found as yet in pheochromocytomas and MTCs. Deletions of chromosome 1p remain the most consistent and significant molecular genetic abnormality yet identified in these tumors.

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