EXPRESSION OF MOUSE Tbx2 GENE IN NORMAL AND MALIGNANT MELANOPHORES BY RT-PCR

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ABSTRACT

Objective: To observe the expression of mouse Tbx2 gene in normal and malignant melanophore. Methods: The normal and malignant cells were used to extract total RNA. The expression of the Tbx2 gene was detected by RT-PCR. Results: No expression of the Tbx2 gene in the normal melanocytes was noted, but all malignant cells showed expression of the Tbx2 gene. Conclusion: Tbx2 plays a critical role during the development of the malignant cells.

Key words: Gene amplification, Gene expression, Malignant cell, Tbx2

Tbx2 is a member of a recently discovered gene family of transcription factors, and the new mouse genes that contain the T-box domain have been named T-box 1–6 (Tbx1 through Tbx6) after the Brachyury or T gene that encode proteins ranging in size from 400 to >900 amino acids.[1, 2] Members of this gene family share a motif of 200 amino acids which in the T gene product has been shown to exhibit sequence-specific, DNA-binding activity.[3] Recently the T protein has been shown to activate expression of a reporter gene through binding to the T consensus sequence, confirming its role as a transcription factor.[4] The cloning and characterization of T gene homologs in a variety of other vertebrate species provides strong evidence for the conservation of yet unknown target gene involved in mesoderm development.[5] Chapman and associates[6] have shown that six murine T-box genes exhibit overlapping but unique patterns of expression during embryogenesis, indicating that expression of these gene is temporally and spatially regulated. This suggests that, similar to Pax gene, T-box genes play important roles in development of the malignant cells. Little is known about expression of mouse Tbx2 gene in normal and malignant cells. To determine whether the Tbx2 gene is a candidate for involvement in malignant cells, we report here that expression of mouse Tbx2 in the malignant melanocytes by RT-PCR.

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MATERIALS AND METHODS

Reagents

The enzymes of AMV, Taq DNA polymerase and dNTPs were purchased from Promega Co.

Cell Lines

Eleven cell lines utilized in this study were kindly provided by Dr. Goding. The normal melanophore line K1735 and 10 malignant melanophore lines Mel a I, Mel a II, Mel a III, Mel a ras, Mel c, Mel b a, Melb sci, Mel a, B16 and HM96 were maintained in RPMI 1640 medium containing 10% fetal bovine serum, penicillin and streptomycin in a humidified 37℃ incubator with 5% CO_2.

RNA Isolation, Reverse Transcription and PCR

Isolation of total cellular RNA procedures.[7] RNA was isolated with RNATRIZOLTM reagent (Gibco/BRL). RNA integrity was assessed through electrophoresis by denaturing agarose gels containing formaldehyde and the concentration was determined by UV absorption prior to reverse transcription and PCR. For the RT-PCR isolation of the Tbx2 cDNA, total cell RNA was subjected to

reverse transcription with AMV reverse transcriptase followed by a first strand cDNA synthesis. All PCR primers (Table 1) (Mouse Tbx2 primers and G3PDH primer as positive control) were designed according to the published sequence of Gene Bank (MMU15566) and produced from Marie Curie Cancer Research Institute. PCR was performed in the volume of 100 μl containing 500 ng upstream and downstream primer each, 1.5 mmol/L $MgCl_2$, and 4 U Taq DNA polymerase. The parameters of PCR amplification of RT-PCR were first cycle: 95℃ 5 min, when at 70℃, Taq was added, and then at 50℃–75℃ 45 sec and 72℃ 1 min and other cycles: 95℃ 40 sec, 50℃–75℃ 45 sec and 72℃ 1 min from 2 to thirty cycles. After thirty cycles of amplification, the PCR products (10 μl) were subjected to electrophoresis on 1.5% agarose gel and followed by ethidium bromide staining.

RESULTS

The Expression of Mouse Tbx2 Gene in Normal and Malignant Cells by RT-PCR

Total cellular RNA of the normal melanophore line K1735 and malignant melanophore lines Mel a I, Mel a II, Mel a III, Mel a ras, Mel c, MELb a, Melb sci, Mel a, B16and HM96 were extracted and the expression of mouse Tbx2 was examined using RT-PCR technique. The PCR products were analyzed on ethidium bromide-stained agarose gels. In order to prevent the possibility of technical failure, we simultaneously amplified the Tbx2 gene with the G3PDH gene. In these conditions, the Tbx2 were successfully amplified and exhibited the expected size. No expression of the Tbx2 gene in the normal melanocyte was noted, but the ten malignant melanocytes showed expression of the Tbx2 gene. These data are presented in Figure 1.

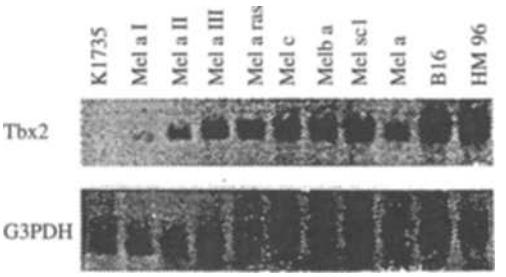

Fig. 1. The expression of mouse Tbx2 gene in normal and malignance melanophore by RT-PCR

Table 1. The Tbx2 gene primers for Tbx2 detection with PCR

Primer set	Nucleotide sequence	Position	Length (bp)
Tbx2	5'-GAT GAG AGA GAG CCA AGC TGG-3'	28–46	
	3'-TCC GTT CGG AGA CGG CCT GG-5'	171–190	163
Tbx2	5'-GAT GAG AGA GAG CCA AGC TGG-3'	28–46	
	3'-TGG TCA AGG TGT TCG ACC-5'	374–391	364
Tbx2	5'-TGG TTC ATT ATC ACG GAG CCC A-3'	1839–1860	
	3'-TCC TGC ACA TGT CGT GTC TTA-5'	2229–2249	411

DISCUSSION

Almost 70 years ago, a mutant mouse having a short blunt-ended tail was recovered in the progeny from an X-ray-induced mutagenesis study.[8] The mutation responsible for this phenotype was named Brachyury (short tail), or simply T for tail. In homozygotes, the T mutation was found to express a more severe phenotype resulting in embryonic death during midgestation with abnormalities in mesoderm-derived tissue. Recently mutation in two of the human T-box genes, Tbx3 and Tbx5, have been shown to be responsible for two human dysmorphoric syndromes, Holt Oram[9] and ulnar-mammary syndrome.[10] The T-box family of gene products is defined by the highly conserved, DNA-binding region known as the T-box. The conservation of this region over hundreds of millions of years of evolution argues for the conservation of function, at least in terms of DNA binding. However, the protein regions outside the T-box are divergent, suggesting different specificities for potential interaction with other protein effector. By determining the sites of potential function as well as potential interrelationships in this putative transcription factor family, Bollag[15] has laid the goundwork for a comprehensive study of the evolution and function of the recently discovered mouse T-box genes, Tbx1-Tbx5. A comprehensive phylogenetic analysis of all known T-box genes[11] indicates the existence of at least 4 ancestral T-box gene sequences at the onset of vertebrate evolution that gave rise to the 6 mouse T-box genes Tbx1 through Tbx5 and the T locus. Two pairs of T-box genes arose through duplication events that occurred later during vertebrate evolution: Tbx2 and Tbx3 derive from a relatively recent common

ancestral gene, as do Tbx4 and Tbx5. In contrast, the T and Tbx1 loci have existed as separate entities throughout vertebrate evolution. When the results of mapping studies are combined with the phylogenetic data, it appears that a prevertebrate tandem duplication event gave rise to the Tbx2/3 and Tbx4/5 precursor genes, and that later, a whole-cluster duplicative transposition event gave rise to the Tbx2/Tbx4 cluster on one chromosome and the Tbx3/Tbx5 cluster on a second chromosome, with two cross-cluster cognate gene pairs.[12] The retention of linkage between cluster members over a long period of time suggests that this linkage may have functional significance by analogy with the HOX system. The expression patterns that we have documented for Tbx1-5 appear to reflect their evolutionary relationships. Tbx1, which is distantly related to the other 4 genes, has an expression pattern that is unique in most tissues, suggesting divergent functions. The most similar patterns of expression are between the cognate loci, Tbx2 and Tbx3, and Tbx4 and Tbx5, reflecting their relatively recent divergence from common ancestral genes, and providing a further regulation. In fact, the overlapping expression patterns may indicate a high degree of functional overlap between the cognate gene pairs, although our examination reveals differences in the temporal and spatial domains even in areas of overlapping expression. Perhaps most of the functional diversification of cognate gene pairs revealed are areas of expression unique to each gene: expression of Tbx3 in the blastocyst, Tbx2 in the pinnae, and the complementary expression of these genes in the epithelial and mesenchymal components, respectively, of the mammary gland primordia. Among several chordate species examined, homologs of T appear to have retained both similar expression patterns and similar function.[12] T-box gene homologs other than T have now been found in several other vertebrate species including man,[12] as well as nonvertebrate species such as C. elegans and Drosophila.[13] In insects, T homologs have been shown to specify the development of posterior structure including the hindgut, possible indicating a phylogenetic relationship between posterior structure in insects and vertebrates.[14] The Drosophila omb gene, which is a direct ortholog of Tbx2 is involved in optic lobe development.[13] Tbx2, 3 and 5 expression in the developing mouse eye could indicate the conservation of an ancient functional role for the ancestral Tbx2/3/4/5 gene in eye development. Previously, Tbx2 message has been detected by Northern blotting in lung and kidney and at lower levels in the heart and ovary.[15]

Melanocytes afford a particularly attractive system for understanding the molecular mechanisms operating to achieve the commitment and differentiation of a cell lineage. Melanocytes originate in the neural crest as a dispersed population of melanoblasts which migrate primarily to the hair follicles and epidermis, before differentiating into mature, pigmented cells. In addition to being responsible for skin, hair and eye colour,[16] melanocytes also perform an essential function in the generation of action potentials in the inner ear.[17] Moreover, in response to UV irradiation, skin melanocytes increase the production of the pigment melanin which is then transferred to the surrounding keratinocytes as a protection from UV-induced DNA damage.[18] Because melanocytes are not essential for viability, and because pigmentation is an obvious phenotypic character, around 70 genes which affect the melanocyte lineage have been identified by genetic analysis, of which over 20 have now been cloned. These include genes which play crucial roles in melanocyte commitment, survival or differentiation, such as the c-kit[19] and the endothelin B receptors,[20] and the transcription factors microphthalmia,[21] Pax3,[22] and Sox10,[23] as well as genes encoding melanogenic enzymes, such as tyrosinase and tyrosinase-related protein-1 (TRP-1) which map to the albino and brown loci respectively.[24] No expression of Tbx2 gene was reported in melanocytes, possibly because these cells are present only as a dispersed population and are consequently difficult to detect. We were aware that RT-PCR was an extremely sensitive tool for the extraction of specific cDNAs and may sometimes result in the detection of rare messages. As such we wished to confirm the expression of Tbx2 in a range of cell types derived from the normal melanocyte and nine malignant melanocytes. RNA was therefore prepared from cell lines and analysed by RT-PCR. The result (Figure 1) revealed that no expression of Tbx2 gene in the normal melanocyte was noted, but all malignant melanocytes showed expression of Tbx2. The study by Carreira et al. indicated that the brachyury-related transcription factors is a repressor of the melanocyte-specific TRP-1 promoter.[25] Our data showed that Tbx2 plays a critical role during the development of the malignant melanophore. Although we have yet to examine the requirements within Tbx2 for transcription regulation of the malignant melanophore, it is possible that Tbx2 may play roles as both activators and repressors depending or their responsiveness to different signal transduction pathways. Since the repertoire of signaling pathways which may be active is likely to be a different cell type, the role of individual members of the T-box family may vary between tissues or during development. Future work will be directed towards exploring these issues.

REFERENCES

[1] Brook WJ, Cohen SM. Antagonistic interactions between wingless and decapentaplegic responsible for

dorsal-ventral pattern in the drosophila leg. Science 1996; 273:1373.

[2] Zhang J, King ML. Xenopus VegT RNA is localized to the vegetal cortex during oogenesis and encodes a novel T-box transcription factor involved in mesodermal patterning. Development 1996; 122:4119.

[3] Kispert A, Herrmann BG. The brachyury gene encodes a novel DNA binding protein. EMBO 1993; 12: 3211.

[4] Kispert A, Koschorz B, Herrmann BG. The T protein encoded by Brachyury is a tissue-specific transcription factor. EMBO 1995; 14:4763.

[5] Rashbass P, Cooke LA, Herrmann BG, et al. A cell autonomous function of Brachyury in T/T embryonic stem cell chimeras. Nature 1991; 353:348.

[6] Bamshad M, Lin RC, Law DJ, et al. Mutations in human TBX3 altr limb, apocrine and genital development in ulnar-mammary syndrome. Nature Genet 1997; 16:311.

[7] Easty DJ, Guthrie k, Maung CJ, et al. Protein B161 as a new growth factor: expression of B61 and up-regulation of its receptor epithelial cell kinase during melanoma progression. Cancer Res 1995; 55:2528.

[8] Dobrovolskaia-Zavadskaia, N. Sur la mortification spontanee de la queue chez la souris nouveau-nee et sur l'existence d'un caractere (facteur) hereditaire <<non viable>>C.R. Seanc Soc Biol 1927; 97:114.

[9] Basson CT, Bachinsky DR, Lin RC, et al. Mutations in human cause limb and cardiac malformation in Holt-Oram syndrome. Nature Genet 1997; 15:30.

[10] Bamshad M, Lin RC, Law DJ, et al. Mutations in human TBX3 altr limb, apocrine and genital development in ulnar-mammary syndrome. Nature Genet 1997; 16:311.

[11] Agulnik SI, Garvey N, Hancock S, et al. Evolution of vertebrate T-box genes by tandem duplication and cluster dispersion. Genetics 2000; (in press).

[12] Agulnik SI, Bollag RJ, Silver LM. Conservation of the T-box gene family from Mus musculus to Caenorhabditis elegans. Genomics 1995; 25: 214.

[13] Pflugfelder GO, Roth H, Poeck B, et al. A homology domain shared between Drosophila optomotor-blind and mouse Brachyury is involved in DNA binding. Biochem Biophys Res Commmun 1992a; 186:918.

[14] Kispert S, Koschorz BG, Leptin M, et al. Homologs of the mouse Brachachyury gene are involved in the specification of posterior terminal structures in Drosophila, Tribolium, and Locusta. Genes Dev 1994; 8:2137.

[15] Bollag RJ, Siegfried JA, Cebra-Thomas J, et al. An ancient family of embryonically expressed mouse genes sharing a conserved protein motif with the T-locus. Nature Genet 1994; 7:383.

[16] Silvers WK. The coat colors of mice. New York: Springer-Verlag, 1979; p12–24.

[17] Steel KP, Barkway C. Another role for melanocytes: their importance for stia vascularis development in the mammalian inner ear. Development 1997; 107:453.

[18] Fitzpatrick TBG, Szabo M, Seiji, et al. Biology of the malanin pigmentation system, In: TB. Fitzpatrick, et al. Dermatology in General Medicine, 2nd ed. New York: McGraw-Hill, 1979; p131-163.

[19] Nocka KS, Majumder B, Chabot P, et al. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice. Genes Dev 1989; 3:816.

[20] Baynash AGK, Hosoda A, Giaid JA, et al. Interaction of endothelin-3 with endothelin-B receptor is essential for development of epidermal melanocytes and enteric neurons. Cell 1994; 79:1277.

[21] Hodgkinson CAKJ, Moore A, Nakayama, et al. Mutations at the mouse microphthalmia locus are associate with defects in a gene encoding a novel basic helix-loop-helix-zipper protein. Cell 1993; 74:395.

[22] Baldwin CTNR, Lipsky CF, Hoth T, et al. Mutation in PAX3 associated with Waardenburg syndrome type I. Hum Mutat 1994; 3:205.

[23] Pingault VN, Bondurand K, Kuhlbrodt DE, et al. SOX10 mutations in patients with Waardenburgs-Hirschprung disease. Nature Genet 1998; 18:171.

[24] Jackson, IJ. A cDNA encoding tyrosinase-related protein maps to the brown locus in mouse. Proc Natl Acad Sci USA 1988; 85:4392.

[25] Carreira S, Dexter TJ, Goding CR, et al. The Brachyury related transcription factor Tbx2 is a repressor of the melanocyte-specific TRP-1 promoter. J Mol Bio (in press).