SHORT REPORT

EXPRESSION OF PAX 3 ALTERNATIVELY SPliced TRANSCRIPTS AND IDENTIFICATION OF TWO NEW ISOFORMS IN HUMAN TUMORS OF NEURAL CREST ORIGIN

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The developmental gene PAX 3 is expressed in the early embryo, in developing muscle and elements of the nervous system, including the brain. Since no one has investigated the expression of the isoforms of PAX 3 in the neuroectodermal tumors melanoma and small cell lung cancer (SCLC), we have carried out a comprehensive screening for the expression of the isoforms PAX 3a–e using RT-PCR in human melanoma cell lines, primary human ocular and secondary cutaneous melanomas. We have identified 2 new isoforms of PAX 3, g and h, which we have isolated, cloned and sequenced. Sets of primers for each isoform were designed and their specificity was confirmed by sequence analysis of the products. The isoforms PAX 3a–e were detected in all human cutaneous melanoma cell lines (8/8), but only PAX 3c (1/2) and PAX 3d (2/2) in ocular melanoma cell lines. The same PAX 3 isoforms were detected in more than 80% of human cutaneous melanomas: PAX 3a and b (15/17), PAX 3c (14/17), PAX 3d (16/17) and PAX 3e (15/17). In contrast the results for 7 SCLC cell lines were PAX 3a (0/7), PAX 3b (1/7), PAX 3c (3/7), PAX 3d (6/7), PAX 3e (2/7); 8/8 cutaneous melanoma cell lines and 8/8 ocular melanoma tissues, together with 14/17 cutaneous melanoma tissues screened, expressed the new isoform PAX 3g. All 8 cutaneous melanoma cell lines expressed PAX 3h, but it was not detectable in any of the tumour tissues (0/20). Neither of the 2 ocular melanoma cell lines expressed the 2 new isoforms. Comparison of the different ampiclon staining intensities on a gel suggests that PAX 3c and PAX 3d are the predominant transcripts expressed, with relatively lower expression of PAX 3e and PAX 3h. We propose that these and the 2 new isoforms we have discovered may be important in oncogenesis and differential diagnosis of melanomas or SCLC.

Key words: PAX 3; isoforms; melanoma; small cell lung cancer

We have reported that insulin growth factor (IGF) II and PAX 3/FKHR cooperate in the oncogenesis of RMS. Subcutaneous injection of C2C12 cells transfected with IGF-II and PAX 3/FKHR into nude mice produced tumors that grew more rapidly, showed increased angiogenesis and lower apoptosis, than tumors from cells transfected with these genes singly, PAX 3 alone or IGF-II in combination with PAX 3.11 In this study, PAX 3 but not PAX 7 was found to be expressed in melanoma cell lines and tumor tissue.

Recent reports have described human PAX 3 encoding 2 additional exons downstream of exon 8.12 Furthermore, Tsukamoto et al.13 have reported 2 additional truncated transcript isoforms of PAX 3. In this study, we have isolated and cloned 2 new isoforms, PAX 3g and PAX 3h, which lack the complete coding region of exon 8.

No previous study has specifically examined the expression of all of the known PAX 3 isoforms in neural crest-derived human tumor tissues. Using RT-PCR with specific primers to detect each of the PAX 3 transcript isoforms, we have screened human melanoma and small cell lung cancer (SCLC) cell lines and melanoma tumor tissues, including 8 primary ocular melanomas and 12 cutaneous metastases, for their expression. Such an investigation may indicate a possible role of the isoforms of PAX 3 in differential diagnosis, tumor progression and tumorigenesis.

MATERIAL AND METHODS

Cell lines and tumor samples

A JR1, embryonal rhabdomyosarcoma cell line expressing PAX 3 (used as positive control), 8 cutaneous melanoma cell lines, 2 ocular melanoma cell lines and 7 SCLC cell lines were cultured in DMEM with 10% FCS until they reached approximately 70% confluence.

Tissue samples consisted of 8 primary ocular melanomas and 12 cutaneous melanoma metastases. Five further total RNA samples from cutaneous melanomas were generously provided by the Cancer Tissue Bank Research Centre, School of Biological Sciences at the University of Liverpool in Liverpool, United Kingdom. Two samples of normal human skin were used as negative controls.

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Extraction of total RNA and RT-PCR analysis

Total RNA was extracted from different cell lines and tissue specimens using an RNA extraction kit (Promega, Madison, WI). Tumor tissue was cut into small (≈ 1 mm) pieces and placed into a sterile eppendorf tube containing 1 ml of prechilled denaturing solution. The tissue was disrupted with a homogenizer until no fragments of tissue or clumps of cells were visible. The samples were then processed as for cell lines. RNA (1 μg) from each cell line was reverse-transcribed into cDNA using a 2-strand cDNA synthesis kit (Roche, Lewes, East Sussex, UK). The procedure for RNA extraction and cDNA synthesis was as per the manufacturer’s instructions. cDNA (1 μl) from each cell line and tumor was amplified by PCR. The human S14 ribosomal protein RNA was amplified in parallel and used as an external control. To avoid any
nonspecific amplification of targets from DNA, primers were designed to amplify across exon boundaries (Fig. 1). The nucleotide sequences of primers used are shown in Table I. PAX 7 expression was detected using specific primers spanning the region from exon 7 (E7F) to exon 8 (E8R) of the PAX 7 gene, amplifying a 148 bp product. In the case of the PAX 3 isoforms, PAX 3e was detected using primers E7F/I5R, PAX 3d using primers E8F/I9R and PAX 3e using primers E8F/I10R, amplifying 553, 241 and 294 bp, respectively. The primers E8F/I10R also amplify a 465 bp fragment representing PAX 3d containing intron 9. PAX 3a and PAX 3b were detected using primers E3F/E5R amplifying 684 and 277 bp amplicons, respectively. The primers used for the detection of the 2 newly identified PAX 3 isoforms were E7F/I9R (PAX 3g) and E7F/I10R (PAX 3h).

The PCR procedure was as described previously. Briefly, it was carried out for 30 cycles (94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min) with a final incubation at 72°C for 10 min. This was carried out for 30 cycles (94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min) with a final incubation at 72°C for 10 min. This was the linear region of the graph (cycle number proportional to amount of DNA produced). The PCR products (5 μl) were run on a 1% agarose 1 × TBE gel and stained with ethidium bromide.

Manual sequencing by dideoxy chain termination

RT-PCR was performed on 1 μg of total RNA from the melanoma cell line, XP44, using the primers E8F/I9R (PAX 3d), E8F/I10R (PAX 3e), E7F/I9R (PAX 3g) and E7F/I10R (PAX 3h; Table I). The PCR products were separated on a 1% agarose/1 × TAE gel. The desired DNA bands were excised from the gel and were purified using the Wizard PCR Preps DNA purification system (Promega). The gel-purified PCR amplicons were sequenced using the Promega fmol DNA sequencing kit (Q.4100) using 30 pmol of each respective forward or reverse primer and 0.5 μl 35S-dATP (> 37 TBq/mmol, SJ 1304, Amersham, Buckinghamshire, U.K.). The sequence ladders were generated using denaturing polyacrylamide gel electrophoresis and autoradiography (Kodak X-OMAT film).

Isolation and cloning of PAX 3g and PAX 3h

Briefly, to amplify full-length cDNA of each PAX 3 isoform, a forward primer (PF) to a noncoding region in the promoter of PAX 3, 45 bp from the transcription start site, was used in conjunction with reverse primers I9R and I10R. PCR products were ligated into pCR 2.1TOPO cloning vector (Invitrogen, La Jolla, CA). Recombinant clones were screened for using primers E7F/I9R (PAX 3g) and E7F/I10R (PAX 3h). Approximately 500 ng of plasmid DNA was sequenced for 25 cycles with the ABI Prism Big Dye Termination Cycle Sequencing Reaction Kit (Applied Biosystems, Foster City, CA) using M13 forward and reverse primers. PCR products were analyzed on an Applied Biosystems V1 ABI 377 automated sequencer.

RESULTS

Sequence analysis

Primers were designed to amplify DNA sequences specific to each of 5 PAX 3 isoforms. This was confirmed for the isoforms PAX 3d and PAX 3e by sequence analysis of the amplicons generated by primers E8F/I9R and E8F/I10R, respectively. In the case of PAX 3d, intron 8 is spliced and translation continues from exon 8 to exon 9. This encodes an alternative C-terminus shown in Figure 2(c). For the isoform PAX 3e, introns 8 and 9 are spliced and translation proceeds from exon 8 to exon 9 and exon 10. The amino acid sequence encoded by the additional exons 9 and 10 is shown in Figure 2(d). The primers for PAX 3e also generated an additional larger amplicon, which on sequence analysis was found to contain the 171 bp intron 9. This may represent PAX 3d, wherein intron 9 is retained (PAX 3d + intron 9; Fig. 3).

In this study, we detected expression of PAX 3 but not PAX 7 in melanoma cell lines (Fig. 3a) and melanoma tissues (data not shown). Screening of melanoma cell lines with the primers E7F/I9R and E7F/I10R, as well as amplifying the predicted amplicons, amplified 2 corresponding lower-molecular-weight bands. An example is shown for cutaneous melanoma cell lines screened with primers E7F/I9R (Fig. 3b). To confirm they were new isoforms of PAX 3 and not artifacts of the PCR reaction, both were sequenced. Full-length cDNAs of the new isoforms were amplified using primers to the 5′ UTR and 3′ UTR of PAX 3 and the 2 products were cloned and sequenced (Fig. 2a and b). Both lacked the complete 247 bp coding region for exon 8. In the case of PAX 3g, exon 8 and intron 8 are spliced and translation proceeds from exon 7 to exon 9 (Genbank accession number Ay 251279). This encodes a different C-terminal amino acid sequence from that encoded by exon 9 in PAX 3d (Fig. 2e). In the case of PAX 3h, exon 8, intron 8 and intron 9 are spliced and translation proceeds from exon 7 to exon 9 and exon 10 (Genbank accession number Ay 251280). Again, this codes for a different sequence from that encoded by exons 9 and 10 in PAX 3e (Fig. 2f). Comparison of the new exon 9 sequence in PAX 3g with the peptide databases did not reveal any obvious homology. In contrast, the sequence GFKSF, identical to the last 5 amino acids in exon 10 in PAX 3h, was found in the C-terminal region of a PAX 6 truncated splice variant isolated from the rat small eye strain (rSey; Fig. 2g).

RT-PCR screening

RT-PCR screening was undertaken to investigate whether PAX 3 transcript isoforms were expressed in cell lines derived from melanoma and SCLC. RT-PCR was used to screen cell lines from cutaneous melanomas (n = 8), ocular melanomas (n = 2) and SCLCs (n = 7), together with tissues comprising 8 ocular and 17 cutaneous melanomas. Figure 3(c) shows an example of RT-PCR result for ocular melanoma. The embryonal rhabdomyosarcoma cell line JR1, which is known to express PAX 3, was used as a positive control. Normal human skin was employed as a negative control.

Overall expression and detection status is summarized in Table II. Semiquantification of the results showed intense amplicon staining (+ +) for PAX 3b in 6/8 cutaneous melanoma cell lines compared with only 1/8 for PAX 3a. Neither PAX 3a nor PAX 3b was detected in the 2 ocular melanoma cell lines or in 6/7 SCLC cell lines. PAX 3b was just detectable (+) in the remaining SCLC.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence of the oligonucleotide</th>
<th>PAX 3e mRNA nucleotide position</th>
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<tr>
<td>PAX 7 E7F</td>
<td>5′-GCCACAGCTTTCGAGGCAGAC-3′</td>
<td>412</td>
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<tr>
<td>PAX 7 E8R</td>
<td>5′-AAGTGAGCTGAGCTGAAAGG-3′</td>
<td>692</td>
</tr>
<tr>
<td>E3F</td>
<td>5′-TGAAGGACGTGAGAGCCGAGG-3′</td>
<td>997</td>
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<tr>
<td>E5R</td>
<td>5′-TTGAGGAGGAGGAGGAGGAGG-3′</td>
<td>1029</td>
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<td>5′-GAGGCCCGTCTGCGTAGCTGAGC-3′</td>
<td>1259</td>
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<tr>
<td>E8F</td>
<td>5′-GCTGAGAGCTTACGGCTAGCTGAGC-3′</td>
<td>1288</td>
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<tr>
<td>I9R</td>
<td>5′-CAGAGAGGATCTTCTGATACGGCTGAGC-3′</td>
<td>2026</td>
</tr>
<tr>
<td>I10R</td>
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<td>2250</td>
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<tr>
<td>S14 3F</td>
<td>5′-GCCAGACAGAAAGGAATCT-3′</td>
<td></td>
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<tr>
<td>S14 4R</td>
<td>5′-CAGCTGCAAGGAGGCTTCTGTC-3′</td>
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<tr>
<td>PF</td>
<td>5′-TGCGCCCTACCTCAGTAGCTTGGGAGG-3′</td>
<td>-45</td>
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</table>
EXPRESSiON OF PAX 3

PAX 3c and PAX 3d appear to be the predominant isoforms expressed overall. Intense staining (+ +) for both isoforms was seen in all 8 cutaneous melanomas cell lines. The majority of cutaneous melanomas, 11/17 for PAX 3c and 12/17 for PAX 3d, also showed intense amplicon staining. All 8 ocular melanomas gave intense amplicon staining for PAX 3d and 5/8 for PAX 3c. This was in contrast to 2 ocular melanoma cell lines in which PAX 3c (1/2) and PAX 3d (2/2) were only just detectable (Table II).

For SCLC cell lines, staining was not as intense as for the melanomas but PAX 3c (3/7) and PAX 3d (6/7) still were the predominant isoforms expressed. PAX 3e appears to be expressed at relatively low levels compared with the other isoforms, being just detectable (+) in most of the tumor tissues screened and at an intermediate status (+) in 7/8 cutaneous melanoma cell lines.

As regards the 2 new isoforms PAX 3g and PAX 3h, the former appears to be more widely and strongly expressed, being detectable in 8/8 cutaneous melanoma cell lines, 1/2 ocular melanoma cell lines, 14/17 cutaneous melanomas and 8/8 ocular melanomas. PAX 3h, in contrast, was detected in 8/8 cutaneous melanoma cell lines (although in the majority expression was low), but was undetectable in melanomas or any of the other cell lines or tumors examined. Since PAX 7 was not detected in any of the cell lines or tumor tissue studied here, none of the data (except a representative gel in Fig. 3a) is presented or discussed in this article.

DISCUSSION

Apart from the identification of 2 novel isoforms, several other important findings have emerged from this study. PAX 3 promotes oncogenesis in vitro and in vivo in mice and can inhibit myogenic differentiation in tissue-cultured C2C12 myoblasts.10 PAX 3 also plays a role in the migration, proliferation and survival of cells in early muscle development.16 PAX 3 may exert an ant apoptotic effect through the Bcl family of apoptosis inhibitors. PAX 3 and PAX 3/FKHR can transcriptionally activate the Bcl-2 promoter and ectopic overexpression of PAX 3, PAX 3/FKHR or Bcl-2 can rescue cells from antisense-induced apoptosis.17 Many of the characteristics outlined above are features involved in the development and progression of tumors.

PAX 3 expression has been reported in melanoma cell lines and tumor tissue.18–20 PAX 3 is involved in the development pathway leading to the genesis of the mature pigment producing melanocyte from a nonpigmented melanoblast precursor cell originating in the trunk neural crest. In addition, PAX 3 activates expression of the gene encoding the microphthalmia-associated basic helix-loop-helix leucine zipper transcription factor (Mitf). Mitf in turn positively regulates the melanocyte-specific tyrosinase TRP-1 and TRP-1 has a clearly defined role in melanogenesis.22 Furthermore, Galibert et al.23 have observed that PAX 3 is identical to the previously described melanocyte-specific factor MSF, which regulates TRP-1 through 2 melanocyte-specific sequences in the TRP-1 promoter. It is possible, therefore, in the progression of melanoma that deregulation of PAX 3 expression has occurred, uncoupling it from its role in melanogenesis and allowing its oncogenic properties to predominate.

Several alternative transcripts of PAX 3 have recently been described. Two PAX 3 isoforms designated PAX 3a and PAX 3b include the first 4 exons then truncate prematurely at sequences in intron 4.13 Both lack the entire homeodomain and C-termi nus and probably have significantly different functions from the other PAX 3 isoforms. PAX 3c, the isoform originally reported for PAX 3,12 retains intron 8 and translation proceeds from exon 8 for 5 codons
into intron 8 before reaching a stop codon. In PAX 3d, intron 8 is spliced and translation proceeds from exon 8 to exon 9. Similarly with PAX 3e, introns 8 and 9 are spliced and translation proceeds from exon 8 to include exons 9 and 10 (Fig. 1). Barber et al.\textsuperscript{12} have isolated a mouse cDNA where exon 9 is directly joined to exon 5 and have designated it Pax 3f, but we did not detect expression of

![FIGURE 3](image-url)
this isoform in any human cell lines or tumor tissues (data not shown). Sequence analysis of the 2 new isoforms cloned and sequenced by us demonstrated that they both lacked part of the transcription activation domain coded for by exon 8; in keeping with the established nomenclature, they have been designated PAX 3g and PAX 3h. The predicted C-terminal amino acid sequences coded for by exons 9 and exons 10 in PAX 3g and PAX 3h were different from those coded for by the same exons in PAX 3d and PAX 3e. A comparison of the new sequences with the peptide databases revealed that the last 5 amino acids GKFSF, coded by exon 10 in PAX 3h, occur also in the C-terminal region of a Pax 6 truncated splice variant isolated from the rSey.24 This implies biologic significance. As is the case with PAX 3g and PAX 3h, the splicing in the Pax 6 truncation mutant creates a novel C-terminal peptide sequence, and it is within this sequence that the peptide sequence GKFSF is found (Fig. 2g). The deletion of exon 8 in the new isoforms may affect their transactivation and oncogenic properties. Interestingly, investigators have demonstrated that the COOH-terminal transactivation domain plays a key role in regulating the function of the PAX 3 homeodomain. It was found that replacement of the PAX 3 transactivation domain with the unrelated VP16 transactivation domain enabled PAX 3 to transactivate homeodomain-specific sequences (as is the case with PAX 3/FKHR) as well as to transform fibroblasts.25 It has been reported that the oncogenic potential of PAX 3-FKHR requires the PAX 3 homeodomain recognition helix, but not the PAX 3 paired box DNA binding domain.26 Further studies will be required to determine whether the deletion of exon 8 in the 2 new isoforms has a similar deregulatory and oncogenic effect. In addition to those functions mentioned above, PAX 3 may also function through interaction with other proteins27,28 and it is possible that these novel C-terminal amino acid sequences of PAX 3g and PAX 3h modulate such interactions.

In the present study, RT-PCR was used to screen for expression of PAX 3 transcript isoforms in primary ocular and secondary cutaneous melanomas and SCLCs and cell lines derived from them. There was little difference in numbers of cutaneous melanomas expressing PAX 3a and PAX 3b, although there was a difference in amplicon staining intensity. The significance of these expression patterns and what role PAX 3a and PAX 3b play in tumorigenesis and cancer progression are unknown. Tsukamoto et al.13 found PAX 3b to be expressed in most of the normal tissues they examined, whereas PAX 3a was expressed only in cerebellum, esophagus and skeletal muscle. Considering the difference in their structure from other PAX 3 isoforms, namely, their lack of a transcription transactivation domain, one possibility is that they may interact in some way with the full-length isoforms of PAX 3 and alter their functions, therefore acting in a regulatory manner. There is no evidence for this, although truncation mutations in the C-terminal region have been reported for PAX 6 in patients with aniridia.29 Furthermore, these mutants are found to act in a dominant negative fashion when coexpressed with wild-type PAX 6 in transient transfection assays and when expressed in transgenic mice.29,30 Similar results have been found with PAX 5. Cotransfection of the alternative truncated isoform PAX 5d with the full-length isoform PAX 5a was found to act in a dominant negative fashion in transcription assays. Interestingly, cotransfection of PAX 5e, an additional truncated isoform, with PAX 5a had the opposite effect and increased transactivation activity.31 PAX 3c and PAX 3d appear to be the predominantly expressed isoforms overall, with not only the largest number of cell lines and tumors showing detection, but also the highest intensity of band staining. PAX 3e, in most cases, was expressed at relatively low levels. Interestingly, we have not detected PAX 3e in 2 murine melanoma cell lines B16 F10 and B16 4A5 (data not shown). This is in agreement with the findings reported by Barber et al.12 who found that only the human gene contains the consensus GT donor nucleotides at the 5′ end of the putative intron 9 sequence. Since PAX 3e is expressed at relatively low levels in the cell lines and melanoma tissues we have screened, it is doubtful whether it plays a significant role in their formation and progression. On the other hand, PAX 3e may account for some of the phenotypic differences observed between mice and humans with dominant mutations in PAX 3, such as those seen in Splotch mutant mice and Waardenburg’s syndrome in man.12

The results suggest that the isoforms of PAX-3, particularly PAX 3c and PAX 3d, may play a significant role in the development and progression of melanoma and SCLC. PAX 3a and PAX 3b may function by regulating the transactivation properties of the full-length isoforms, as do the truncated isoforms of PAX 5 and PAX 6. This may be true also for the new isoforms PAX 3g and PAX 3h, although it is possible that these too may have specific transactivation properties, as they still retain a major proportion of their transactivation domains. In Wilms’ tumor, a children’s nephroblastoma that is believed to arise as a consequence of the metanephric mesenchyme failing to differentiate,32 the WT1 protein is a transcription regulator involved in the regulation of cell growth and differentiation and, like PAX 3, is subject to alternative splicing. Previous studies have indicated that each isoform has distinct transcription regulatory properties, and alterations in the relative amounts of the various WT1 isoforms may be a significant factor in the etiology of sporadic Wilms’ tumor.33–35 Importantly, PAX 3 isoform expression may be restricted to neural crest-derived tumors, such as melanoma, neuroblastoma (data not shown) and SCLC. The neural crest origin of the latter may be in doubt according to a study by Anbazhagan et al.,36 who found that SCLC microarray gene expression profiles resembled non small cell lung tumors more closely than carcinoids, which in turn resembled astrocytomas. Nevertheless, SCLCs do express neuroendocrine hormones and PAX 3.

Several members of the PAX gene family are expressed in tumors and in the corresponding embryologic tissue from which these tumors are derived. For instance, Pax 2 and Pax 8 are expressed in embryonic mouse kidney, childhood Wilms’ tumors and human adult renal cell carcinomas.37–40 Interestingly, Pax 5, expressed in the neural tube and the developing murine brain but...
undetectable in neonatal cerebral neoplasms, is expressed in both medulloblastomas and glioblastomas. 1, 2 Although PAX 3 has been reported to have oncogenic properties, further studies will be required to establish its role in the tumors studied here. Recently, Scholl et al. demonstrated that > 70% of melanoma cells died after downregulating PAX 3 expression with specific PAX 3 antisense oligonucleotides, which suggests that PAX 3 may be a target for future therapy.

REFERENCES


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