BMP Antagonist DAN and Uveal Melanoma Progression

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Submitted
Summary

Members of the BMP family and their antagonists have been implicated in morphogenesis and cancer development. Functional studies from our group revealed that BMP7 can act as a potential therapeutic compound in the treatment of eye melanoma. Using transcriptional profiling of TGFβ superfamily members in uveal melanoma cells, we identified differential expression of the BMP antagonist DAN upon challenge with BMP7. The BMP antagonist DAN encodes a secreted protein in the cysteine knot family. The family of DAN (differential screening-selected gene aberrative in neuroblastoma also known as NO3) encompasses tumor suppressor genes that can act as inhibitors of TGFβ and Wnt pathways.

In the present study, we analyzed DAN expression in primary uveal melanoma tissue of enucleated cancer patients with complete follow-up by real-time PCR and immunohistochemistry. The level of DAN mRNA expression in uveal melanoma significantly correlated with patient survival. Uveal melanoma with low/undetectable DAN mRNA expression (≤0.01) is significantly correlated with poor survival compared to patients with higher DAN mRNA expression. Immunolocalization studies revealed strong expression in non-cancerous tissue of retina but low or no expression of DAN in the tumor. Furthermore, a trend was noticeable between low DAN expression in tissue sections and poor survival of these patients. Differential DAN expression may, therefore, be involved in the pathogenesis of uveal melanoma and metastasis. Expression of DAN in primary tumors may thus represent a new prognostic factor for survival of uveal melanoma patients.
Introduction

Uveal melanoma is the most common primary malignant intraocular tumor in adults, with a varying annual incidence of 6–12 per million in Caucasians. The therapy of uveal melanoma remains problematic due to a high rate of metastatic dissemination, irrespective of the success of treatment of the primary tumor. Metastases occur mainly in the liver (87%), lungs (46%) and bone (29%).

Members of the bone morphogenic protein (BMP) subfamily of signaling proteins are involved in many developmental processes. Knockout studies have shown that BMP7 is essential for early morphogenesis of the eye and kidney. BMP7 knockout mice revealed deficient ocular growth, due to severe epithelial development disturbances. Recent evidence suggests that BMP7 plays a role in a functional system in the eye that modulates and balances the expression of ECM proteins (collagen IV, laminin, fibronectin) in trabecular meshwork cells of the trabecular system. Disturbances of this balance may result in primary open angle glaucoma (POAG). Recent functional studies from our group provide evidence for an inhibitory role of BMP7 in uveal melanoma progression and metastasis. These BMP signals can be modulated by the activity of transforming growth factor beta (TGFβ) or BMP-antagonists.

Extracellular BMP antagonists include noggin, chordin, follistatin and follistatin-related gene (FLRG), ventroptin, twisted gastrulation(Tsg), and the Dan/cerberus family of genes: the tumor suppressor Dan, gremlin and its rat homolog drm, the protein related to Dan and Cerberus (PRDC), caronte, Dante (Dte) and sclerostin. Despite the initially observed role of these antagonist in BMP-mediated processes, a number of BMP antagonists also strongly inhibit Wnt signalling including sclerostin and DAN. In this study we identified differential screening-selected gene aberrative in neuroblastoma (DAN, also known as NO3) as a target gene that is induced by BMP7. DAN encodes a secreted protein in the cysteine knot super family. Nakamura et al. found that endogenous DAN mRNA was up-regulated during retinoic acid-induced neuroblastoma differentiation, indicating that DAN might be involved in neuronal differentiation. During some stages of embryogenesis DAN has been found during eye development. Biochemical analyses have demonstrated that DAN bound directly BMPs, inducing BMP2 and -4, but also acted as an inhibitor of the Wnt pathway, that is of great importance in uveal melanoma development. Initially DAN has been identified as a tumor suppressor in malignant fibroblast cells. Its tumor suppressor actions and its ability to antagonize Wnt functions underscore DAN’s importance in cancer development. Its potential role in uveal melanoma has remained elusive.

In this study we examined and compared DAN expression in clinical samples of normal eyes and human uveal melanoma at transcriptional and protein level and established its role as prognostic factor.
Primary uveal melanoma tumors:
In this study, 28 enucleated eyes, due to uveal melanoma, were included (conformed to requirements of Declaration of Helsinki). Three melanomas were treated by enucleation after failed ruthenium-106 plaque radiotherapy combined with transpupillary thermotherapy. The other 25 eyes were primarily enucleated because of a large uveal melanoma. Median age of the patients, 14 females and 14 males, at time of enucleation was 61.5 years (range 42-84 years). The maximal follow-up was 115 months. Tumor diameter varied from 7 mm to 18 mm (mean 12.5 mm, STD 2.9). After histopathological analysis, 10 tumors were classified as epitheloid, 12 as spindle cell, and 6 as mixed histology. There were 24 choroidal tumors, 2 ciliary body tumors, and 2 ciliary body melanomas with iris involvement. Tumor fragments were snap-frozen in nitrogen and stored at -80°C or paraffin embedded for immunohistochemistry.

Melanoma cell line and normal melanocytes
OCM-1 was obtained from primary uveal melanoma 26. Normal uveal melanocytes (1A, B, 2) were established in our laboratory 27. Cell line OCM-1 grew as monolayers in 10 ml/25cm² Dubecco’s modified Eagle’s medium (Gibco, Invitrogen, Breda, The Netherlands) supplemented with 10% fetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 IU/ml penicillin (Gibco), and 100 μg/ml streptomycin (Gibco). Normal melanocytes were grown as monolayer in 10 ml/25cm² HAM/F12 (Gibco) medium as described by Hu et al 28. All cells were incubated at 37°C in a humidified atmosphere with 5% CO₂.

Establishment of stable transfectants expressing the BMP7 gene
For the establishment of OCM-1 cell line with BMP7 overexpression, we used the OCM-1 FRT cell line 29. The BMP7 coding sequence (1µg construct CMV-BMP7 FRT (BMP7 cDNA)) was intergrated by targeted homologous recombination using the single FRT site as described previously 1. Stable transfectants were selected with hygromycin (400 μg/ml, Invitrogen).

Isolation of cellular RNA and real time polymerase chain reaction (RT-PCR)
RNA was isolated from cells in culture and tissue from experimentally induced tumors 29. RNA from human melanoma tissue was isolated using the RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) and proteinase K (20 mg/ml) (Qiagen). Reverse transcription was performed with random primers in the presence of RNase inhibitor (Roche Diagnostics, Almere, The Netherlands). Quantitative real-time PCR (qPCR) was performed using commercially obtained exon-specific primers for DAN (S: CAAGAACATCACCCAGATCG and AS: CAGGGACTCTGTTGGACTGTG) and Hs 99999903 m1 (β-actin), (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) on an ABI Prism 7700 sequence detection system (Applied Biosystems, Nieuwerkerk a/d Ijssel,The Netherlands). Values were normalized
with the housekeeping gene β-actin. Real-time PCR on patients tissues were repeated two times in duplicate and mean values were used for statistical analysis. For the survival analysis of DAN expression, we categorized DAN expression into 2 groups, one with low expression (≤0.01; 15 patients) and the other with high expression (0.01-0.2; 13 patients). A probability of p value < 0.05 was considered significant.

**Superarray TGFβ/BMP7 signaling pathway**

Total RNAs were isolated using TRIZOL reagent from Invitrogen (Carlsbad, CA). RNAs were then treated with DNase I (1 unit/ml) to eliminate possible genomic DNA contamination. The absence of DNA contamination was confirmed by PCR for 35 cycles with primers of GAPDH (data not shown). The apoptosis pathway-focused oligonucleotide SuperArray membranes (Cat. No. OHS012) and related reagents were purchased from SuperArray Bioscience (Frederick, MD). cRNA synthesis, labeling and hybridization were performed following the manufacturer’s instruction. Hybridization signals were detected with the chemiluminescent detection kit. Image analysis and data acquisition were performed using the web-based integrated GEArray Expression Analysis Suite provided by SuperArray Bioscience. We used the means of nine housekeeping genes to normalize the intensity of the signals.

**Immunohistochemistry**

Paraffin-embedded tissue blocks of primary uveal melanoma were used. 4-µm tissue sections were cut from each paraffin block, prepared on aminopropylethoxysilane-coated slides, and dried overnight at 37°C. Sections were deparaffinised in xylol. Endogenous peroxidase was blocked in 0.3% hydrogen peroxide in methanol at room temperature for 20 minutes. After immersion in alcohol the sections were rehydrated. Antigen retrieval was preformed by incubating the sections in a 0.1% trypsin (Sigma T-7409), calcium chloride (0.1%, anhydrous) solution in demineralised water during 20 minutes at 37°C. After antigen retrieval the slides were rinsed in phosphate-buffered saline (PBS). Sections were incubated overnight with the primary antibody, polyclonal goat anti human DAN (5 µg/ml dilution, R&D systems, Oxon, UK). The tissue sections were washed in PBS and incubated with the biotinylated rabbit-anti-goat conjugate (Dako, Glostrup, Denmark, 1:400) for 30 minutes, washed again by PBS and incubated for 30 minutes with the streptavidin-biotin-peroxidase conjugate (Dako; 1:100). After three washes in PBS, visualization of DAN was achieved after 7 minutes incubation with 3-amino-9-ethyl-carbazole in a 0.1M acetate buffer (pH 5.0) containing 0.015% hydrogen peroxide. DAN-positive cells stained red. Counterstaining was done with Mayer's hematoxylin and mounted in Kaiser’s glycerin/gelatin. The evaluation was performed with a light microscope by two independent observers. Both observers were ‘blinded’ with regard to follow-up data. Agreement was achieved in more than 91% of the cases; minor conflicting assessments were settled by mutual agreement. For the survival analysis of the immunohistochemistry staining, we categorized percentages of positive tumor
area with DAN staining: DAN 0-35%, >35% < 66%, > 66%. A probability of p value < 0.05 was considered significant. In five tumors, the evaluation of the DAN staining could not be evaluated due to lack of sufficient paraffin-embedded tumor tissue.

**Statistical analysis:**
We analyzed the correlation between DAN expressions as a prognostic factor for patient survival by a Kaplan-Meyer survival plot. We performed all statistical analysis with SPSS for windows version 12. Survival time was defined as the time from enucleation to death by metastatic disease (event) or by others (right-censored), or the patient was known to be alive. A probability of p value < 0.05 was considered significant.

![A: Expression of DAN (mRNA) analyzed with real-time-PCR in normal melanocytes, OCM1 and primary uveal melanoma tissue. B: Kaplan-Meier survival curves for cumulative survival of primary uveal melanoma patients (months) with different expression of DAN (mRNA) of the tumor. DAN expression has been categorized into 2 groups, one group with low expression (0-0.01) and the other with high expression (0.01-0.2). * <0.05](image-url)
DAN mRNA expression in human uveal melanoma tissue, normal melanocytes and uveal melanoma cell line

Figure 2: Immunolocalization of DAN in primary uveal melanoma tissue. Specific staining of DAN was observed in A: epithelium and endothelium of the cornea (magnification 20x), furthermore, positive staining was observed in B: the retina and especially the nerve fiber layer (9), outer plexiform layer (6), inner segments (4) and B retinal pigment epithelial layer (RPE) (2) (magnification 40x). C, D: Two tumors had high expression of DAN in the tumor (magnification 20x, 40x). E, F: Most tumors showed low or no staining (magnification 20x, 40x). 1 = choriocapillaris; 2 = retinal pigment epithelial layer (RPE); 3 = outer segments; 4 = inner segments; 5 = outer nuclear layer; 6 = outer...
Previously we generated human OCM-1 uveal melanoma cells that stably over-express BMP7 (OCM-1 FRT/BMP7). We further evaluated potential BMP7 target genes for uveal melanoma, using gene arrays for TGFβ/BMP signaling pathway. No clonal variability between BMP7 expressing and control cell lines was observed due to the single FRT insertion site as described previously. We observed that BMP7 induced DAN expression in vitro (basal not detectable versus BMP7 3.10^-3). As expected Id2, an established target gene that is regulated by BMP7, was induced in the OCM-1 BMP7 cells as expected (basal 0.042 versus BMP7 0.095).

Next we evaluated DAN expression in normal and tumor cell lines and patient tissues. Quantification of DAN mRNA by real-time PCR showed in relatively high amounts in the normal melanocytes (0.29), whereas low levels were found in human uveal melanoma cell line OCM-1 (0.01) (Figure 1A). In line with cell lines DAN was down regulated in primary uveal melanoma tumor tissue mRNA (figure 1A, table 1) and compared to normal tissue of the eye. Representative examples of DAN immunolocalization are depicted in figure 2. Furthermore, we investigated if DAN mRNA expression in primary uveal melanoma is related with patient survival. Low DAN mRNA expression (<0.01; 15 patients) corresponded with poor patient’s prognosis (p=0.0453) (figure 1B). In addition, a significant correlation between DAN expression (mRNA) and the established prognostic maker tumor height (distance between base of the tumor and top of the tumor which extends into the corpus vitreous) was observed.

Table 1: DAN mRNA expression with real-time PCR in primary uveal melanoma tissue

<table>
<thead>
<tr>
<th></th>
<th>LOW EXPRESSION</th>
<th>HIGH EXPRESSION</th>
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<tbody>
<tr>
<td>Number of patients (n=28)</td>
<td>n = 15</td>
<td>n = 13</td>
</tr>
<tr>
<td>Mean DAN expression (mRNA)</td>
<td>0.005274</td>
<td>0.065189</td>
</tr>
<tr>
<td></td>
<td>(0.00055-0.00875)</td>
<td>(0.011849-0.199098)</td>
</tr>
<tr>
<td>Survival time in months (mean/median)</td>
<td>67/51</td>
<td>80/80</td>
</tr>
<tr>
<td>Death</td>
<td>8/15 (53%)</td>
<td>2/13 (15%)</td>
</tr>
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**DAN immunolocalization in primary uveal melanoma tissues**

A specific positive DAN staining was observed in the retina and especially the nerve fiber layer, outer plexiform layer, inner segments and retinal pigment epithelial layer (RPE) (figure 2). The cornea stained specifically in endothelium and epithelium (figure 2). Most of the tumors...
showed low or no staining for DAN (91%), only two uveal melanomas displayed extensive staining (figure 2). Furthermore, a trend was noticeable between low DAN expression in tumor tissue and poor survival of these patients, but this was not significant (p = 0.09). The lack of significance, however may be due to the limited number of patient tissue samples that could be included in this immunolocalization study compared to the real-time PCR study.

**Discussion**

In this study we position the level of expression of DAN as a prognostic indicator for patient survival in uveal melanoma. Our study showed that low expression of DAN is significantly correlated with poor survival of uveal melanoma patients after enucleation of the affected eye. Immunolocalization of DAN in normal human eyes and uveal melanoma tissue revealed specific staining of the layers of the retina, retinal pigment epithelium, corneal epithelium and endothelium. Our data suggest that if DAN expression is low or absent, uveal melanoma might acquire a more aggressive and invasive phenotype.

Ozaki et al showed that expression of DAN was significantly reduced in malignant fibroblast cells and that overexpression of DAN inhibited proliferation of these cells \(^{25}\). In animal models, DAN not only exhibited BMP antagonist activity \(^{14,20,22,23}\), but also acted as a Wnt inhibitor \(^{18,19}\). BMP (TGFβ) and Wnt family members are involved in tumorgenesis, invasion and metastasis \(^{31-33}\). It appears, therefore, that DAN primarily acts via inhibition of BMP activity in these cancer cells \(^{20}\). Alternatively, DAN may also affect the Wnt pathway \(^{18,19}\) that can modulate cell proliferation, survival, cell behavior and cell fate \(^{34,35}\). Inappropriate activation of this pathway in response to mutations is linked to a wide range of cancers, including melanoma. Increased Wnt 5a expression has been reported in cutaneous melanomas relative to melanocytes \(^{36}\) and has also been shown to be highly correlated with enhanced motility and invasiveness in these tumors \(^{37,38}\). Zuidervaart and coworkers recently reported expression of Wnt 5a and some downstream effectors in uveal melanomas \(^{24}\) were increased in primary uveal melanomas and tumors with high expression of these proteins were associated with poorer patient survival. In absence of Wnt signals, β-catenin is targeted for degradation by phosphorylation via a complex consisting of glycogen-synthase kinase (GSK03B), axin and the APC protein. Wnt signals lead to inactivation of GSK3B thus stabilizing β-catenin levels, which then increase transcription of downstream target genes through complexes with TGFβ. A possible mechanism of progression of uveal melanoma with reference to our BMP7 study \(^{1}\) may include down-regulation of DAN. Although speculative at present, diminished DAN expression levels may result in increased Wnt signaling (Figure 3) and poor patient survival.

In conclusion our data show that DAN expression (mRNA) is significantly correlated with patient survival. Therefore, we suggest that DAN may be a new prognostic factor for uveal melanoma survival. Further studies are warranted that address DAN mechanism of action and involvement of TGFβ/BMP and Wnt family in uveal melanoma development, progression...
and metastasis.

**Figure 3:** *mechanism of induction of uveal melanoma in perspective of DAN*

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### References


