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Gene expression profiling of hereditary exencephaly in chickens

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Summary

In this preliminary study, differentially expressed genes were investigated in cranial tissues from chickens with hereditary exencephaly using cDNA microarrays containing 1152 genes and expressed sequence tags (ESTs). Genes showing twofold or greater differences at $P < 0.05$ between affected and normal cranial cells were considered to be candidates for hereditary exencephaly in chicken. Eighteen ESTs (11 known genes/homologues) were upregulated and 108 ESTs (51 known genes/homologues) were downregulated. The EST AL584231 (ROS006C9), orthologous to human *MTHFD1*, a known candidate gene for human neural tube defects (NTDs), was expressed at the same level both in normal and affected chicken cranial tissues. ESTs AL584253 (ROS006F7, *thioredoxin reductase 1*) and AL585511 (ROS024H9, *thioredoxin*), both involved in NTD pathogenic pathways in mice, were downregulated and had mean ratios of 0.41 and 0.04 for expression in affected vs. normal cells respectively. Expression differences of these two ESTs were confirmed by quantitative real-time polymerase chain reaction. These data indicate that ESTs AL584253 and AL585511 are candidates for hereditary exencephaly in chickens.

Keywords chicken, exencephaly, gene expression profiling.

Global gene expression profiling allows researchers to functionally map the genetic pathways that control growth, development and metabolism. The recent release of the chicken draft genome sequence, as well as the development of a large (578 445) collection of expressed sequence tags (ESTs), has dramatically changed the landscape for biologists wishing to use genomic tools to study the chicken (Burnside *et al.* 2005). A DNA microarray containing 1152 genes or expressed sequence tags (ESTs) isolated from 5-day-old chicken embryos with two replicate spots for each EST has been established by the Roslin Institute (http://www.ark-genomics.org/resources/chickens.php) and was used in this study because it represents pathways involved in embryonic cell differentiation, including cranium formation.

Neural tube defects (NTD), including anencephaly and spina bifida, are a group of severe congenital abnormalities in which the future brain and/or spinal cord fail to close (Greene & Copp 2005). NTDs are among the most common of all human congenital defects, yet their aetiology remains poorly understood. This is largely because of the complexity of the genetic factors regulating the intricate events involved in neuroregulation (Finnell *et al.* 2002). Biochemical and developmental pathways, mouse models, and positional comparisons have provided numerous candidate genes for the study of human NTD. In a survey of 80 studies, significant results were found for very few genes among 38 tested candidates using human populations in case–control or family based associations (Boyles *et al.* 2005). Utilizing other model organisms is beneficial for prioritizing human NTD candidate genes and to clarify the complex aetiology of this condition (Boyles *et al.* 2005).

In chicken hereditary exencephaly, which includes neurocranial defects resulting in exposure or extrusion of the brain, was originally described by Mellen (1959). Exencephaly is referred to in the OMIA database as a 'phenotype considered a defect' with unknown mode of inheritance (http://omia.angis.org.au). Divergent selection of Rhode Island Red (RIR) chickens for high and low frequency of scoliosis (based on dorsosacral vertebrate in dead embryos on day 17–21 of incubation) resulted in the frequency of scoliosis in the H line being 10-fold higher than that of the L line (40% and 4.1% respectively) (Pryszcz 1996). Frequencies of several other skeletal malformations including hereditary exencephaly were much higher in the

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H line (Pryszcz 1996). Here we report microarray results between affected chicken cranial tissues with hereditary exencephaly from the H line and unaffected normal tissues obtained from other birds of the same line.

Pieces of cranial tissues were taken from three affected and three normal birds immediately after hatching and crushed under liquid nitrogen. Fifty to 100 mg of tissue per bird were used to isolate total RNA using TRI reagent (Sigma-Aldrich, St Louis, MO, USA). Five micrograms of RNA from each bird were used as a template for reverse transcription (RT) reactions. Amino-modified first-strand cDNA was synthesized using the BD Atlas PowerScript fluorescent labelling kit (BD Biosciences, Alameda, CA, USA) and purified using QuickClean resin to remove protein contaminants. Second-strand cDNA synthesis was performed with oligo$(dT)_{15-18}$ primers using polymerase chain reaction (PCR). Resulting cDNAs from normal chickens and chickens with hereditary exencephaly were differentially labelled with Cy3 and Cy5 dyes respectively as described by the manufacturer. Removal of unincorporated dye was performed using FluorTrap Matrix (BD Biosciences). Hybridization of the Roslin Institute chicken embryonic 1152 ESTs array was performed in an Atlas Glass Hybridization chamber (BD Biosciences). Labelled cDNAs were warmed up to 50 °C GlassHyb hybridization solution (1.82 ml), transferred into hybridization chambers and hybridized overnight at 50 °C. After hybridization, the microarray slides were washed once for 10 min in GlassHyb wash solution and two times in GlassHyb wash solution with 0.1X SSC. Afterwards, the slides were rinsed in 0.1X SSC and in distilled water and then dried by centrifuging in the Beckman GS-3 centrifuge (Beckman Coulter, Fullerton, CA, USA) at 140 *g* for 6 min. The slides were scanned immediately after hybridization and washing using a ScanArray Lite scanner (Perkin-Elmer, Boston, MA, USA) to detect Cy3 and Cy5 fluorescence with excitation wavelengths of 543 and 633 nm and emission filter wavelengths of 570 and 670 nm, respectively. Laser power was kept constant for Cy3/Cy5 scans. QuantArray software (Packard BioScience, Billerica, MA, USA) was used for processing microarray images, spot location, and for creating reports of raw spot intensities. Intensity-based global normalization was performed to remove dye-specific bias, and background correction was performed by subtracting the normalized median pixel intensity of the background value from the normalized median pixel intensity of the spot. Images for each spot on the array were quantified and stored in an Excel spreadsheet, then merged with the address file for identification. A ratio of means (the ratio of the arithmetic mean intensities of each feature for each wavelength, with the median background subtracted) was calculated for every spot. The results of two independent hybridizations were statistically evaluated using a *t*-test among the four spots. Genes showing twofold or greater differences with *P*-values < 0.05 were considered to be candidates for hereditary exencephaly in chicken. First-strand cDNA for real time (RT)-PCR of selected candidate genes was synthesized using Enhanced Avian Reverse Transcriptase PCR Kit (Sigma-Aldrich). Quantitative RT-PCR was performed with the cDNA samples taken from three affected and three normal chickens as templates using the following primer pairs: AL585511 (GCACGTCCTTAGTGGCTCTC and GCCCTGGA-ACTAACACCAGA) and AL584253 (ACGAGGTGCTTCC-TATGTGG and TTTTAGTCTTCCCGGTGTGC). The chicken

Table 1 Upregulated genes/expressed sequence tags with twofold or greater differential expression between cranial tissues of affected and normal chickens[1].

Ark-Genomics ID	GenBank accession no.	Ratio	Comments
ROS037H1	AL586244	9.68	Homology to *uridine kinase-like 1*
ROS038D4	AL586270	9.085	Chicken *heat shock 70 kDa protein 4*
ROS026H8	AL585617	6.905	Chicken *40S ribosomal protein S8*
ROS046H9	AL586659	5.215	Unknown
ROS034H4	AL586098	3.72	Unknown
ROS019D1	AL585147	3.585	Homology to human *hypothetical 28.6 kDa protein T07A5.2*
ROS022F8	AL585341	3.53	Unknown
ROS013G12	AL584743	3.44	Homology to *cyclin I*
ROS025C11	AL585541	3.415	Homology to *separin* (EC 3.4.22.49)
ROS009B10	AL584437	3.405	Homology to *NADPH-cytochrome P450 reductase* (EC 1.6.2.4)
ROS021F8	AL585259	3.39	Unknown
ROS046A9	AL586616	2.955	Unknown
ROS009G9	AL584477	2.89	Unknown
ROS016C6	AL584934	2.44	Unknown
ROS030B7	AL585792	2.425	Chicken *NADH-ubiquinone oxidoreductase 24 kDa subunit*
ROS027F2	AL585649	2.415	Chicken *procollagen-lysine, 2-oxoglutarate 5-dioxygenase* (*lysine hydroxylase*) *2*
ROS036H8	AL586203	2.29	Homology to *2,3-oxidosqualene-lanosterol cyclase*
ROS048F10	AL586810	2.14	Homology to *SLC11A3 iron transporter*

[1]$P < 0.05$.

Table 2 Downregulated genes/ESTs with twofold or greater differential expression between cranial tissues of affected and normal chickens[1].

Ark-Genomics ID	GenBank accession no.	Ratio	Comments
ROS007E7	AL584314	0.46	Homology to *mitotic spindle assembly checkpoint protein*
ROS020B10	AL585182	0.455	Unknown
ROS011F3	AL584590	0.45	Chicken *nucleoporin 50 kDa*
ROS010G7	AL584548	0.445	Chicken *helix-loop-helix protein ID3*
ROS006F7	AL584253	0.41	Homology to *thioredoxin reductase 3* (*Txnrd3*)
ROS016H11	AL584981	0.405	Unknown
ROS008A9	AL584355	0.39	Chicken *DNA-binding protein inhibitor ID2*
ROS020B2	AL585177	0.385	Unknown
ROS048G2	AL586814	0.38	Homology to *adapter-related protein complex 3 sigma 2 subunit*
ROS029E11	AL585760	0.375	Unknown
ROS010H1	AL584554	0.37	Unknown
ROS015E1	AL584870	0.365	Unknown
ROS023B5	AL585375	0.365	Unknown
ROS005C9	AL584166	0.36	Unknown
ROS047F2	AL586716	0.355	Homology to *COP9 complex subunit 3*
ROS003A10	AL584055	0.355	Homology to *tubulin gamma chain* (*gamma tubulin*)
ROS037C11	AL586221	0.35	Chicken *cellular nucleic acid binding protein*
ROS004E5	AL584122	0.335	Unknown
ROS021G11	AL585272	0.335	Unknown
ROS006H5	AL584270	0.33	Unknown
ROS009E8	AL584457	0.33	Unknown
ROS005H5	AL584204	0.325	Homology to *retinoblastoma-binding protein 2* (*RBBP-2*)
ROS027G6	AL585656	0.31	Homology to *50S ribosomal protein L1*
ROS004D6	AL584117	0.31	Chicken *endoplasmic reticulum protein 29*
ROS047C3	AL586684	0.31	Unknown
ROS034F10	AL586085	0.3	Homology to *cyclin-dependent kinase 5*
ROS048H8	AL586829	0.3	Homology to *COUP transcription factor 1* (*COUP-TF1*)
ROS044B9	AL586520	0.295	Unknown
ROS008G10	AL584405	0.295	Homology to mouse *putative Rho/Rac guanine nucleotide exchange*
ROS001G12	AL583995	0.295	Chicken *vacuolar assembly protein VPS41 homologue* (*S53*)
ROS012A3	AL584616	0.285	Homology to *vimentin*
ROS007D9	AL584307	0.275	Chicken *plakoglobin*
ROS039D8	AL586320	0.27	Unknown
ROS047B6	AL586675	0.26	Unknown
ROS008C6	AL584370	0.26	Unknown
ROS047G3	AL586727	0.255	Unknown
ROS007E12	AL584318	0.25	Homology to *WD repeat domain 36*
ROS022D11	AL585324	0.245	Unknown
ROS041F9	AL586418	0.24	Homology to *isocitrate dehydrogenase (NAD) subunit gamma*
ROS003A5	AL584052	0.24	Unknown
ROS018C6	AL585062	0.24	Unknown
ROS033H11	AL586038	0.24	Unknown
ROS021G11	AL585272	0.23	Unknown
ROS047A7	AL586665	0.23	Unknown
ROS018D4	AL585070	0.22	Chicken *cullin*
ROS016H2	AL584974	0.215	Homology to *phosphatidylinositol-4-phosphate 3-kinase C2*
ROS003A4	AL584051	0.215	Homology to *protein FAM20A precursor*
ROS039B6	AL586155	0.21	Unknown
ROS039D8	AL586320	0.21	Unknown
ROS013C5	AL584689	0.21	Chicken *vacuolar proton translocating ATPase 116 kDa*
ROS026B11	AL585589	0.205	Unknown
ROS004E2	AL584121	0.185	Unknown
ROS005G11	AL584200	0.18	Unknown
ROS022E10	AL585333	0.18	Unknown
ROS048C3	AL586772	0.175	Unknown
ROS048D2	AL586782	0.175	Homology to *nucleolar GTP-binding protein 1*

Table 2 Continued.

Ark-Genomics ID	GenBank accession no.	Ratio	Comments
ROS016F5	AL584958	0.17	Chicken *lamin B1*
ROS013C8	AL584692	0.155	Homology to *protein translation factor SUI1 homologue GC20*
ROS002A8	AL584007	0.155	Homology to *synaptic glycoprotein SC2*
ROS016B6	AL584923	0.15	Chicken *secreted frizzled related protein 1*
ROS009H12	AL584488	0.15	Unknown
ROS040D7	AL586358	0.15	Unknown
ROS020B5	AL585180	0.14	Unknown
ROS045E11	AL586590	0.14	Unknown
ROS012H11	AL584662	0.135	Chicken *actin-capping protein* (*CapZ 36/32*) *alpha subunit*
ROS034B9	AL586052	0.13	Unknown
ROS014A8	AL584761	0.11	Chicken *C21orf19-like protein*
ROS004D2	AL584115	0.095	Unknown
ROS008B3	AL584358	0.095	Homology to *solute carrier family 25, member 28*
ROS011D11	AL584584	0.09	Unknown
ROS047F3	AL586717	0.09	Chicken *DnaJ homologue, subfamily C, member 9*
ROS016F5	AL584959	0.09	Chicken *translocation associated membrane protein 1*
ROS044B3	AL586517	0.085	Chicken *5′ nucleotidase*, *cytosolic IB*
ROS014A3	AL584757	0.085	Unknown
ROS027H9	AL585664	0.085	Unknown
ROS044G2	AL586547	0.085	Homology to *nuclear valosin-containing protein-like*
ROS026D10	AL585599	0.08	Unknown
ROS005B7	AL584157	0.08	Homology to *translation initiation factor eIF-2B alpha*
ROS033F5	AL586020	0.075	Unknown
ROS012F9	AL584650	0.07	Homology to *succinyl-CoA synthetase alpha subunit*
ROS018C8	AL585064	0.07	Unknown
ROS012B10	AL584624	0.065	Homology to *116 kDa U5 small nuclear ribonucleoprotein*
ROS005A6	AL584150	0.065	Unknown
ROS015A7	AL584834	0.065	Unknown
ROS016H5	AL584977	0.065	Chicken *chondromodulin-I*
ROS035H3	AL586155	0.065	Unknown
ROS017C7	AL585003	0.06	Unknown
ROS043C10	AL586482	0.06	Unknown
ROS002B1	AL584012	0.06	Chicken *lunatic fringe*
ROS017D6	AL585012	0.06	Unknown
ROS023F9	AL585422	0.06	Unknown
ROS033G11	AL586030	0.06	Unknown
ROS048D3	AL586783	0.055	Homology to *human hypothetical protein BC015183*
ROS012F2	AL584647	0.05	Chicken *oligomeric Golgi complex component 4*
ROS047B7	AL586676	0.05	Unknown
ROS005A4	AL584148	0.05	Homology to *SEL-10 protein*
ROS015A4	AL584832	0.045	Unknown
ROS003C1	AL584757	0.04	Unknown
ROS024H9	AL585511	0.04	Chicken *thioredoxin-like protein*
ROS005H12	AL584211	0.04	Unknown
ROS022H9	AL585360	0.04	Chicken *protein tyrosine phosphatase-like protein PTPLB*
ROS022A1	AL585283	0.035	Unknown
ROS013B6	AL584680	0.035	Homology to *SH3 domain-binding glutamic acid-rich-like protein*
ROS048C1	AL586770	0.02	Unknown
ROS023B8	AL585378	0.015	Homology to *HDCMA18P protein*
ROS009D11	AL584452	0.015	Chicken *133 kDa myosin-binding subunit of smooth muscle myosin phosphatase*
ROS015E12	AL584863	0.01	Chicken *ATP synthase, H+ transporting, mitochondrial F1 complex, alpha subunit, isoform 1, cardiac muscle*
ROS048G12	AL586823	0.01	Chicken *C6.1A protein*

[1]$P < 0.05$.

SPP1 (*secreted phosphoprotein 1*) gene (NM_204535; TCCTAGCAAGAGCCAAGAGG and GCCATATGCCACACTG-TCAC), formerly known as *osteopontin* (*OPN*), was used as a reference gene (Moore *et al.* 1991). The PCR amplification mix consisted of 2 μl RT product, 10 μl SYBR Green JumpStart *Taq* Ready Mix capillary formulation (Sigma-Aldrich) and 2 μl 5 mM of each primer in a final volume of 20 μl. PCR reaction conditions consisted of an initial 5-min hold at 95 °C, 40 cycles of 95 °C for 10 s, 55 °C for 10 s and 72 °C for 15 s in a LightCycler (Roche, Basel, Switzerland). A melting curve analysis was performed from 65 to 95 °C.

Analysis of the chicken embryonic microarray indicated that eighteen ESTs (including 11 known genes) were upregulated (Table 1) and 108 ESTs (including 51 known genes) were downregulated (Table 2). Interestingly, no significant difference in expression of homeoboxes was detected. Absence of associations of homeoboxes in human NTDs has been previously found (Boyles *et al.* 2005).

Whole genome-wide linkage screen for NTD in humans revealed regions of interest on HSA7 (between D7S3056 and D7S3051) and HSA10 (with peak at D10S1731) (Rampersaud *et al.* 2005). Based on these positional data, five biologically plausible candidates (*MEOX1*, *TWIST1*, *FGFR2*, *GFRA1* and *PAX2*) were identified (Rampersaud *et al.* 2005). None of these positional candidates were represented on the chicken embryo microarray (http://www.ark-genomics.org/resources/chickens.php).

Using a human pedigree with familial spina bifida, Hol *et al.* (1998) identified a G-to-A change at nucleotide 878 (878G>A) of the *methylenetetrahydrofolate dehydrogenase* (*NADP + dependent*) *1* (*MTHFD1*) gene, leading to an Arg293His substitution. A variant form of the *5,10-methylenetetrahydrofolate reductase* (*NADPH*) gene (*MTHFR*) (677C>T) is a known risk factor for NTDs in humans (Brody *et al.* 2002; Boyles *et al.* 2005). Defects in the folate pathway are a major cause of NTDs in humans (Boyles *et al.* 2005). In our study EST AL584231 (ROS006C9), orthologous to the human *MTHFD1* gene, was expressed at the same level both in normal and affected chicken cranial tissues, suggesting that this pathway is not associated with hereditary exencephaly in chickens.

The absence of mitochondrial *thioredoxin 2* (*Trx-2*) causes massive apoptosis, exencephaly, and early embryonic lethality in homozygous mice (Nonn *et al.* 2003). Trx-2 is a small redox protein containing the thioredoxin active site Trp-Cys-Gly-Pro-Cys that is localized to the mitochondria by a mitochondrial leader sequence and encoded by a nuclear gene (*Trx-2*) (Nonn *et al.* 2003). Txn in combination with Txn reductase (Txnrd) and Txn-interacting protein (Txnip) constitute the mammalian Txn pathway (Deroo *et al.* 2004). ESTs AL584253 (ROS006F7, homologous to mouse *thioredoxin reductase 1*) and AL585511 (ROS024H9, homologous to mouse *thioredoxin* and *thioredoxin 2*) were downregulated and had mean ratios of 0.41 and 0.04 of AL584253 and AL585511 respectively (Table 2). Differences in expression measured by quantitative RT-PCR revealed mean ratios of 0.37 and 0.05 respectively. Because the thioredoxin pathway is involved in NTD in mice (Nonn *et al.* 2003), we suggest that ESTs AL584253 and AL585511 are candidates for hereditary exencephaly in chickens.

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