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Expression profiling of chicken DT40 lymphoma cells indicates clonal selection of knockout and gene reconstituted cells

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ABSTRACT

The DT40 cell-line has been extensively used to create deletion mutants, one of which lacks Bruton's tyrosine kinase (Btk). Btk is a cytoplasmic tyrosine kinase important for B-lymphocyte maturation. It was previously shown that there are differences in gene expression between wild-type and Btk-deficient animals. Global gene expression profiling of the avian B-lymphoma DT40 cell-line was used as a model to differentiate among Btk knockout (KO) and Btk KO cells reconstituted with human Btk. Differences in the gene expression pattern showed statistically significant changes between parental DT40 and all the Btk KO cell populations irrespective of whether they are reconstituted or not. These results imply that in the process of generating a knockout cell-line, sub-clones are selected, which have multiple changes in their gene expression pattern ($p < 0.01$). Although other parameters could also influence the expression profile, this potentially has important implications when interpreting microarray data from gene-deleted cell-lines.

Mutations affecting Bruton's tyrosine kinase (Btk) cause disruption in B-lymphocyte development, leading to X-linked agammaglobulinemia (XLA) in humans [1,2] and the less severe X-linked immunodeficiency *(Xid)* in mice [3,4]. Mice deficient in both Btk and the related kinase Tec have an XLA-like phenotype [5]. Btk is a non-receptor tyrosine kinase belonging to the Tec family [6–8]. Btk features, from the N-terminal, a PH domain, a Tec homology (TH) and Src homology domains 3 (SH3), 2 (SH2), and 1 (SH1) [9]. Mutations affecting any of Btk's domains cause XLA and the striking feature of B-cells lacking functional Btk is that they fail to expand. Btk is highly conserved during evolution appearing in the unicellular choanoflagellate *Monosiga brevicollis* [10] and also found in *Drosophila melanogaster* [11] and other vertebrates. A sign of the conservation is the fact that the human *BTK* gene can partly substitute for loss-of-function of the *D. melanogaster Btk29A gene* [12].

Expression profiling has enhanced the understanding of cellular signaling molecules and their downstream targets in health and disease. Btk-deficiency is ideal for expression profiling, since the defect is intrinsic to the B-cell lineage. To determine effects on the transcriptome, we used the Affymetrix Genechip® microarray technology to compare avian DT40 cells with Btk KO as well as deletion mutants reconstituted with Btk. Overt differences in the expression pattern were observed. However, they did not segregate according to the presence and absence of functional Btk, but instead as a result of clonal selection. This potentially has important implications for the use of microarray analysis of cell-lines and suggests that this issue should be subject to further studies.

Materials and methods

Cell-lines: DT40 cell-lines were generated in Dr. T. Kurosaki's laboratory at RIKEN Research Center for Allergy and Immunology in Japan [13]. The cell-lines used in this study were the parental DT40, Btk knockout, B7.10; Btk KO reconstituted with human wild-type Btk, B41.13 or kinase-inactive Btk, B46.5.

Cell culture: The DT40 cells were cultured in RPMI 1640 Glutamax medium with 10% FBS, 1% chicken serum, Penicillin-Streptomycin, 4 mM glutamine, and 50 μM 2-mercaptoethanol (2-ME). DT40 and B7.10 cells were cultivated in three different fetal calf serum batches, while B41.13 and B46.5 were grown in a single serum batch. Both un-stimulated and stimulated cells were grown in the same way, starting in 1 ml serum-free medium for 15 min at 37 °C with 5% CO_2. Stimulations were during that time done with 5 μl supernatant of the hybridoma cell-line M4 (a kind gift from Dr Max D. Cooper, University of Birmingham, AL). After 15 min incubation, 49 ml medium was added and cells were cultivated for 2, 6 or 24 h with, or without, stimuli. In total the experiment was repeated ten times and replicates were pooled prior to expression profiling.

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RNA isolation and oligonucleotide array hybridization: Total RNA was extracted with RNeasy Mini Kit (Qiagen, Valencia, CA, USA). The RNA amount and the quality were measured with a NanoDrop spectrophotometer (Wilmington, USA) and an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). Five micrograms from each of the ten replicates (per sample) were pooled and hybridized to Chicken Genome arrays Genechips®; in total 28 arrays were run. The cRNA synthesis and hybridizations were performed in the BEA core facility at Department of Biosciences, Karolinska Institutet at Novum, Huddinge, Sweden.

Data and statistical analysis strategy for gene selection: In order to optimize the use of microarray chips, all the 28 samples were generated as a pool of 10 different replicates in each [14–16]. Quality control (QC) was checked using Affymetrix® Expression Console software. In order to find distinct patterns in our data set, DNA-Chip Analyzer "dChip" (www.dchip.org) was exercised [17]. dChip uses an intensity-modeling approach for data analysis and clusters microarray probe sets exhibiting similar expression profiles. Data were normalized across all the arrays using probes of 'invariant set' and 'Running median' smoothing. Model-based expression values were obtained using 'Model-based expression' method and '5th percentile of region (PM-only)' as background. The genes were filtered according to the variation across samples: 0.20 < Standard deviation/mean < 100.00. 8,835 of 38,439 probe sets satisfied the filtering criteria. In addition, the upper and lower threshold ratio was set to 1.8 while performing pair-wise comparisons. Hierarchical clustering of the gene expression profile was performed using Pearson's correlation with a threshold of $p < 0.005$ for calling significant clusters. Cluster dendrogram was generated in 'R' (www.r-project.org). Three different sets of mouse B-cell data were converted into chicken orthologs using the Netaffx® (www.affymetrix.com). Ensembl (www.ensembl.org), a public database, was used to find out the number of probe sets used by Affymetrix for each individual gene or transcript for this specific array.

Results

Global gene expression pattern in wild-type DT40 and different Btk mutants

The datasets were initially validated by the presence of internal controls, neomycin phosphotransferase, used for selection as a positive marker for Btk KO cells and Btk itself as a negative marker (Supplementary Fig. 1). The expression of neomycin phosphotransferase was influenced by cell culturing, being reduced 0.3-fold at 24 h compared to 6 h of culture, while the decreased signal from the *Btk* transcripts did not differ significantly (notably, transcripts from the transfected human *Btk* genes are undetectable by avian probe-sets). Unsupervised clustering showed that the B7.10 and the wild-type (WT) B41.13 and kinase-inactive B46.5 reconstituted variants segregated into the same arm, while the parental DT40 formed its own (Fig. 1). Thus, all KO-derived cell-lines, irrespective of reconstitution, showed a more similar expression pattern as compared to the parental DT40 cells. This led us to investigate the clonal effect of the populations used for the array study.

Five hundred and forty two probe-sets were found differentially expressed between DT40 and B7.10. Those specific probe-sets were chosen as selection markers in order to reveal the specific expression pattern in B41.13 and B46.5, respectively. A set of 49 transcripts, consisting of 62 probe-sets, was found to be very similar. Among those, Insulin like growth factor 2 mRNA binding protein 3 (IGF2BP3) was up-regulated in all the deletion mutants. On the other hand, as many as 48 transcripts were found down-regulated at the mRNA expression level (Fig. 2; Supplementary Table I). Thus, the number of transcripts that was down-regulated was much higher. The Affymetrix probe-set ID was used to track the

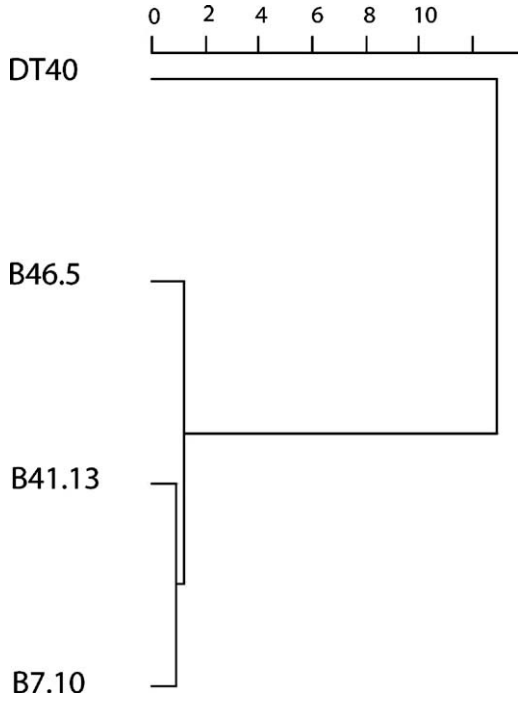

Fig. 1. Clustering pattern of DT40 and Btk KO mutants. Specific pattern of clustering among the mutants.

total number of probe-sets used for those 49 transcripts, representing 49 different genes present in our expression data. Among those, *IGF2BP3* and *USH2A* were found several times. Using the Ensembl database for *Gallus gallus,* we identified 8 transcripts in the correct orientation for *USH2A* and 4 probe-sets for *IGF2BP3* that were included on the array. Among those, 4 probe-sets for *USH2A* and 3 probe-sets for *IGF2BP3* were affected in our data sets. The locations of those specific probe-sets were identified and aligned along the corresponding gene (Fig. 3). One important observation here was that the affected probe-sets behaved in the same way. For example, in *USH2A*, out of the 8 probe-sets, those significantly reduced in Btk KO were all located at one end of the gene (Fig. 3). This distribution was highly significantly different (p value = 0.014) from a random location of the probe-sets.

The cell-lines were compared to each other in order to verify the degree of clonal selection among the deletion mutants. The number of differentially expressed transcripts in each comparison was graphically presented in the form of "inverse" Venn diagrams depicting non-overlapping transcripts (Fig. 4). In DT40 and B7.10 comparisons, the number of differentially expressed transcripts was 903 and 1938 after 6 and 24 h of cultivation, respectively. On the other hand, among the differentially expressed transcripts between Btk KO (B7.10) cells and such cells reconstituted with either WT or kinase-inactive Btk, the number was reduced to a similar extent. In each comparison, including both time points, fewer transcripts differed as to the number in DT40 versus B7.10. Thus, in the 3 comparisons made (panels C and D) an average of 705 and 1158 differentially expressed transcripts were found after 6 and 24 h, respectively.

Cross-validation of the gene expression study

A comparative strategy was taken to cross-validate the hypothesis that clonal changes were the origin of differentially expressed transcripts rather than the presence or absence of Btk. A list of 878 differentially expressed probe-sets was generated with a fold change cut-off value of 2 between DT40 and B7.10 after 6 h of cul-