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Genotype-by-age interaction and identification of longevity-associated genes from microarray data

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Abstract Microarray-based comparisons of long-lived and normal mouse strains represent a promising approach for dissecting the basis of lifespan extension in higher organisms. Recently, Boylston et al. (2006) generated a genome-wide data set that allowed expression levels of Snell (*Pit1*$^{dw/dw}$) and Ames (*Prop1*$^{df/df}$) long-lived mice to be compared with age-matched control mice across different ages (6–24 months). Longevity-associated genes were identified as those genes exhibiting differential expression between long-lived and normal mice at every age examined. In this communication, an alternative approach to identifying longevity-associated genes is suggested and applied to the data sets considered by Boylston et al. (2006). Longevity-associated genes are defined as those exhibiting significant genotype-by-age interaction with respect to expression levels of long-lived and normal mice, and a total of 63 longevity-associated genes are identified. This approach may lend greater confidence to the inference that expression of identified genes specifically underlies aging differences between long-lived and normal genotypes.

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Introduction

Long-lived strains of the laboratory mouse provide a valuable tool for dissecting and understanding factors associated with extended lifespan in mammals. In recent years, the number of known long-lived mutant mouse strains has increased appreciably (Miskin and Masos 1997; Flurkey et al. 2002; Bluher et al. 2003; Holzenberger et al. 2003; Kurosu et al. 2005). This growing collection will ultimately allow researchers to characterize differences between normal and long-lived strains at both the cellular and organismal levels. A key role in this progression will be occupied by DNA microarrays (Park and Prolla 2005; Spindler 2006). Microarrays allow transcript levels to be quantified on a genome-wide scale and are increasingly an affordable and standard means of investigating gene expression patterns. Microarrays have been used to identify expression-level differences between long-lived and normal mouse strains in several previous studies (Dozmorov et al. 2001, 2002; Boylston et al. 2004; Papaconstantinou et al. 2005; Boylston et al. 2006), and similar studies will be necessary in the future. It is therefore worthwhile to consider how these data should be analyzed in order

Table 1 Longevity-associated genes exhibiting significant genotype-by-age interaction with respect to expression levels of long-lived and normal mice

Gene Symbol	Pattern	Description
Snell (*dw/dw*)		
Pafah1b3[b]	A	Platelet-activating factor acetylhydrolase, isoform 1b, alpha 1 subunit
Pbx2[a]	A	Pre-B-cell leukemia transcription factor 2
Ppyr1[a,d]	A	Pancreatic polypeptide receptor 1
1100001G20Rik [a,d]	A	RIKEN cDNA 1100001G20 gene
Aif1[a]	A	Allograft inflammatory factor 1
2610019E17Rik[a,d]	A	RIKEN cDNA 2610019E17 gene
Cyb5r1[b]	A	Cytochrome b5 reductase 1
Laptm5[b]	A	Lysosomal-associated protein transmembrane 5
H2-Aa[a]	A	Histocompatibility 2, class II antigen A, alpha
Igh-6[b,d]	A	Immunoglobulin heavy chain 6 (heavy chain of IgM)
Syn1[b]	A	Synapsin I
Kifc2[a,d]	A	Kinesin family member C2
Btg3[a,d]	A	B-cell translocation gene 3
Cad[a]	A	Carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase
Foxa3[a,d]	A	Forkhead box A3
Mark2[b]	A	MAP/microtubule affinity-regulating kinase 2
Lamp2[b]	B	Lysosomal membrane glycoprotein 2
Amy1[b]	B	Amylase 1, salivary
Plscr2[a]	B	Phospholipid scramblase 2
Aqp4[a]	B	Aquaporin 4
Acot1[a]	B	Acyl-coenzyme A (CoA) thioesterase 1
Aox1[a]	B	Aldehyde oxidase 1
Gfm1[b]	B	G elongation factor, mitochondrial 1
Thrsp[a]	B	Thyroid hormone responsive SPOT14 homolog (Rattus)
BC031181[b]	B	cDNA sequence BC031181
Esd[b]	B	Esterase D/formylglutathione hydrolase
Abcd3[b]	B	ATP-binding cassette, subfamily D (ALD), member 3
Glo1[b]	B	Glyoxalase 1
Chpt1[a]	B	Choline phosphotransferase 1
Sdha[b]	B	Succinate dehydrogenase complex, subunit A, flavoprotein (Fp)
Atg5[a]	B	Autophagy-related 5 (yeast)
Sar1b[b]	B	SAR1 gene homolog B (S. cerevisiae)
4931406C07Rik[a,d]	B	RIKEN cDNA 4931406C07 gene
Bphl[b,d]	B	Biphenyl hydrolase-like (serine hydrolase, breast epithelial mucin-associated antigen)
1300002A08Rik[a]	B	RIKEN cDNA 1300002A08 gene
Ehhadh[a]	B	Enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase
Hsd17b4[a]	B	Hydroxysteroid (17 beta) dehydrogenase 4
Anxa8[a]	B	Annexin A8
Gstm3[a,d]	B	Glutathione S-transferase, mu 3
Dlst[b]	B	Dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)
Fabp2[b]	B	Fatty-acid-binding protein 2, intestinal
Ghr[b]	B	Growth hormone receptor
Skp1a[b]	B	S-phase kinase-associated protein 1A
Chi3l1[a]	B	Chitinase 3-like 1
Cdc2l1[c]	B	Cell division cycle 2-like 1
Ames (*df/df*)		
Limd2[a,d]	A	LIM domain containing 2
Fas[a]	A	Fas [tumor necrosis factor (TNF) receptor superfamily member]
Adrb3[a]	A	Adrenergic receptor, beta 3

Table 1 (continued)

Gene Symbol	Pattern	Description
Slc6a9[b]	B	Solute carrier family 6 (neurotransmitter transporter, glycine), member 9
2610529C04Rik[b]	B	RIKEN cDNA 2610529C04 gene
Igfals[a,d]	B	Insulin-like growth factor binding protein, acid labile subunit
Serpina3c[a,d]	B	Serine (or cysteine) peptidase inhibitor, clade A, member 3C
Ces2[a]	B	Carboxylesterase 2
Csad[a,d]	B	Cysteine sulfinic acid decarboxylase
2310047H23Rik[b]	B	RIKEN cDNA 2310047H23 gene
Mup1[a,d]	B	Major urinary protein 1
Cdc34[b]	B	Cell division cycle 34 homolog (S. cerevisiae)
Cyp2f2[b]	B	Cytochrome P450, family 2, subfamily f, polypeptide 2
Cadps2[a,d]	B	$Ca2^+$-dependent activator protein for secretion 2
Ar[a]	B	Androgen receptor
Klra19[b]	A/B	Killer cell lectin-like receptor, subfamily A, member 19
Aasdhppt[b]	A/B	Aminoadipate-semialdehyde dehydrogenase-phosphopantetheinyl transferase
Bcl9[b]	A/B	B-cell CLL/lymphoma 9

The type of pattern (A or B) indicates the specific type of genotype-by-age interaction associated with listed genes (see text and Figs. 1 and 2 for explanation)

X[a] Interaction significant ($P<0.05$) based on two-factor analysis of variance (ANOVA) model and linear models for microarray data (LIMMA) analysis

X[b] Interaction significant ($P<0.05$) based on two-factor ANOVA model, but not LIMMA analysis

X[c] Interaction significant ($P<0.05$) based on LIMMA analysis, but not two-factor ANOVA model

X[d] Differentially expressed at every age examined (criterion used by Boyleston et al. 2006)

to maximize the biological knowledge generated by this approach.

Boylston et al. (2006) recently provided a genome-wide analysis of expression differences between long-lived and normal mouse genotypes. This study utilized Affymetrix chips containing probe sets for 11,000–34,000 murine genes, representing a level of genome coverage considerably greater than that of earlier studies. Expression levels of both Snell ($Pit1^{dw/dw}$) and Ames ($Prop1^{df/df}$) long-lived dwarf mice were assayed at different ages (6–24 months), along with expression levels of corresponding age-matched control mice ($Pit1^{+/?}$ or $Prop1^{+/?}$). These data represent a key step toward understanding mouse extended lifespan phenotypes at the gene expression level. In particular, based on these data, it is possible to identify key genes that may account for aging deceleration in Snell and Ames mouse genotypes. At the same time, however, given that Snell and Ames mice exhibit multiple endocrine abnormalities, many transcriptional changes associated with these models may be unrelated to aging (Carter et al. 2002).

Longevity-associated genes were defined by Boylston et al. (2006) as genes exhibiting differential expression between long-lived and control strains with respect to *every* age considered (6–24 months). Their analysis identified a total of 205 and 785 such genes from $Pit1^{dw/dw}$ and $Prop1^{df/df}$ mice, respectively, with 49 genes common to both of these gene sets. The present communication does not dispute that these identified genes are worthwhile candidates for further investigation. However, an alternative approach is suggested and applied, yielding a narrowed list of candidates. Genes identified by Boylston et al. (2006) exhibit an *age-independent* expression difference between long-lived and normal genotypes (see Table 1 from Boylston et al. 2006). Expression of such genes could be associated with any of several phenotypic characteristics distinguishing long-lived and normal genotypes, some of which are not associated with aging directly (e.g., body size). The suggestion that expression of identified genes impacts aging, rather than some other process or characteristic, may be strengthened for cases in which genotypic expression differences are age dependent. A natural statistical approach for identifying such genes is to evaluate genotype-by-age interaction effects. This approach is applied in the

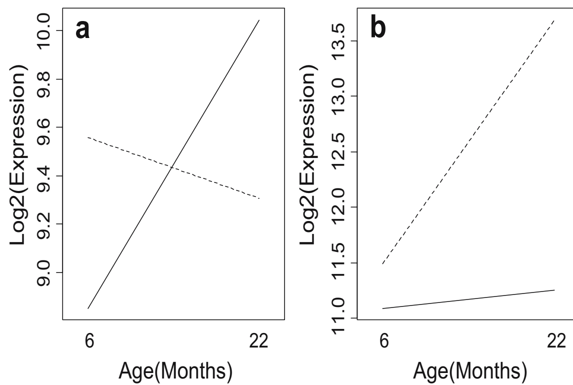

Fig. 1 Select longevity-associated genes exhibiting contrasting types of genotype-by-age interaction with respect to expression levels of Snell (*dw/dw*) long-lived mice and age-matched controls. Averaged expression profiles of *dw/dw* mice are represented by the *dashed line*, whereas those of control mice are represented by the *solid line*. In **a**, a type A expression pattern is illustrated by Foxa3 (forkhead transcription factor). In **b**, a type B expression pattern is illustrated by Gstm3 (glutathione S-transferase)

present communication, and it is suggested that identified genes are especially likely to underlie the aging processes that distinguish long-lived from normal mice.

Materials and methods

Detailed description of the experimental protocols followed in generating the data analyzed here is provided by Boylston et al. (2006). In brief, for *dw/dw* mice, four genotype-by-age treatment combinations were evaluated [*dw/dw*-6 months (n=4), *dw/dw*-24 months (n=3), control-6 months (n=4), control-24 months (n=3)]. For *df/df* mice, six genotype-by-age treatment combinations were evaluated [*df/df*-6 months (n=5), *df/df*-12 months (n=5), *df/df*-24 months (n=6), control-6 months (n=5), control-12 months (n=5), control-24 months (n=5)]. RNA was isolated from the liver tissue of experimental animals and hybridized to either MG U74Av2 (> 11,000 genes, *dw/dw* mice) or MG 430 2.0 (> 34,000 genes, *df/df* mice) oligonucleotide arrays.

Expression-level data sets processed using MAS 5.0 normalization were downloaded from Gene Expression Omnibus (series GSE3129 and GSE3150). Two approaches were used to identify genes exhibiting significant genotype-by-age interaction (at a significance level of 0.05). First, a two-factor analysis of variance (ANOVA) was applied to the expression data associated with each probe, where genotype and age were included as main-model effects along with their interaction. This approach is straightforward but requires estimation of multiple variance terms on a gene-by-gene basis, which could lead to poor performance of associated test statistics (Allison et al. 2006). A second approach, therefore, was also implemented in which genotype-by-age interactions were specified as contrasts using the linear models for microarray data (LIMMA) linear modeling package (Smyth 2004). For *dw/dw* mice, the design matrix specified only one contrast characterizing the genotype-by-age interaction between *dw/dw* and control mice at 6 and 24 months of age. For *df/df* mice, the design matrix specified three contrasts, which characterized the degree of genotype-by-age interaction between *df/df* and control mice at 6 and 12 months, 12 and 24 months, and 6 and 24 months, respectively. In both approaches described above, P values were adjusted across genes using the Benjamini–Hochberg correction (Benjamini and Hochberg 1995).

Results and discussion

A total of 63 genes were identified as exhibiting significant genotype-by-age interaction within one of

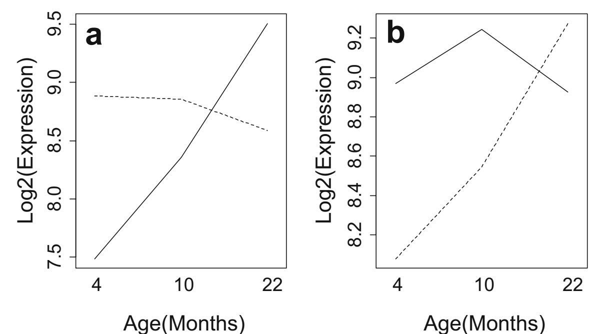

Fig. 2 Select longevity-associated genes exhibiting contrasting types of genotype-by-age interaction with respect to expression levels of Ames (*df/df*) long-lived mice and age-matched controls. Averaged expression profiles of *df/df* mice are represented by the *dashed line*, whereas those of control mice are represented by the *solid line*. In **a**, a type A expression pattern is illustrated by Fas (fatty acid synthase). In **b**, a type B expression pattern is illustrated by Ar (androgen receptor)

the two long-lived strains. The majority of these genes (45/63) were identified on the basis of expression data generated from Snell (*dw/dw*) mice, whereas the remaining (18/63) genes were identified from Ames (*df/df*) mice. The complete list of 63 identified genes is provided in Table 1 along with annotations. No genes were found to exhibit genotype-by-age interaction with respect to both *dw/dw* and *df/df* genotypes. This lack of overlap does not reflect conservative statistical methodology. The Benjamini–Hochberg correction used to adjust *P* values, for instance, is generally regarded as nonconservative, as it assumes independence of expression among different genes (Allison et al. 2006).

Two basic types of genotype-by-age interaction patterns were present among identified genes. These two patterns will be referred to as type A and B patterns, respectively, and are exemplified by select genes displayed in Figs. 1 and 2. Define *M* as the fold-change expression difference between long-lived and normal genotypes for a given gene (long-lived/ normal). Type A patterns are those for which *M* declines with increased age (see Figs. 1a and 2a), whereas type B patterns are those for which *M* increases with increased age (see Figs. 1b and 2b). For each identified gene, the type of genotype-by-age interaction pattern present is indicated in Table 1. The majority of identified genes exhibited a type B pattern (29/45 for the *dw/dw* genotype, 12/18 for the *df/df* genotype).

Identified genes were analyzed to determine whether any gene ontology terms were significantly overrepresented (Beissbarth and Speed 2004). Three main trends emerged from this analysis. First, 27 of 63 identified genes were localized to the cytoplasm ($P<0.001$), and interestingly, each of these 27 cytoplasmic genes were identified from the *dw/dw* long-lived strain. The second major trend was that many genes (18/63) were associated with transport biological processes ($P=0.034$), the majority of which (14/18) were found to exhibit a type B pattern of genotype-by-age interaction. Lastly, eight of 63 identified genes were associated with organic acid metabolism ($P=0.008$), and ten genes were associated with catalytic activity molecular functions ($P<0.036$). Several lower-level ontologies connected to these main themes were also significantly overrepresented, including specific types of metabolism [acyl-coenzyme A (CoA), fatty-acid and carboxylic-acid metabolism], and catalytic enzyme activities (lyase, carboxylic ester hydrolase, oxidoreductase, and transferase activities).

Several genes listed in Table 1 have annotations supporting a role in lifespan determination. The growth hormone receptor (Ghr) and insulin-like growth factor binding protein (Igfals), for instance, are components of the IGF-I axis (Papaconstantinou et al. 2005), which has been widely implicated in lifespan determination within several model systems (e.g., Kimura et al. 1997; Bartke et al. 2003; Tatar et al. 2003). Table 1 also includes several other endocrine-related genes, including androgen receptor (AR), adrenergic receptor beta 3 (Adrb3), thyroid hormone responsive SPOT14 homolog (Thrsp), and hydroxysteroid dehydrogenase 4 (Hsd17b4). Whereas it is not surprising that expression levels of such genes differ between $Pit1^{dw/dw}$ and $Prop1^{df/df}$ mice and age-matched controls, the age dependence of this difference is suggestive of a role in aging.

A good illustration is provided by glutathione S-transferase (Gstm3), which exhibited a type B genotype-by-age interaction pattern (see Fig. 1b). Whereas normal mice exhibited declines or maintained steady levels of this transcript with age, Gstm3 transcript abundance increased with age in *dw/dw* dwarfs. Gstm3 protects against the deleterious effects of oxidative stress, which is thought to be a key factor underlying the deleterious effects of aging (Brown-Borg 2006). The extended longevity phenotypes associated with *dw/dw* dwarfs could therefore be due in part to enhanced oxidative stress resistance due to elevated expression of Gstm3 with age (which does not occur in normal mice) (Fig. 1b).

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