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Linking codon usage bias to functional genomics in pigs

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Abstract The recent completion of a high-quality draft genome of *Sus scrofa* has enabled the detailed investigation of a variety of genomics features. There have been attempts to link genotypic variation to phenotypic variation in different animals including pigs using single nucleotide polymorphisms, copy number variation and decipher codon usage bias. The prevalence of codon usage has never been ascertained in any animal, this study therefore link codon usage bias to gene ontology enrichment. The genome CDS sequence was downloaded from Ensemble v68 *Sus scrofa* build 10.2 using BioMart (Ensembl v68). A total of 21,550 CDS with more than 50 amino acids (150 bp) were used to derive genomic codon adaptation index (proxy for codon usage bias) using an in house built perl script. Five percent low and highly codon usage biased gene were extracted. BinGO v2.44 within Cytoscape v.2.8.3 was used to identify enriched gene ontology terms using human gene annotation as background and validated by perl script. Gene ontology terms related to immune response and sensory perception were linked to lowly codon usage bias. The highly codon usage bias genes were overrepresented in gene ontology terms involved in housekeeping functions of cells. Codon usage bias controls functional genomics.

Keywords Codon usage; Gene enrichment; Functional genomic; Pigs

1 Introduction

Pigs have been important in agriculture and welfare for thousands of years. The recent completion of a high-quality draft genome of *Sus scrofa* (Groenen et al., 2012) enables the detailed investigation of a variety of genomics features. For example, there are attempts to link genotypic variation to phenotypic variation in different animals including pigs. Advances in molecular genetics from protein markers to single nucleotide polymorphisms (SNPs) and copy number variation (CNV) have shown drastic effects on phenotype (Freeman et al., 2006) however, these types of variation are unlikely to solely explain the large phenotypic diversity found at the inter and intra specific level (Paudel et al., 2013). Structural variations (SVs) like copy number variations (CNVs) have shown to play a prominent role in phenotypic evolution, adaptation and domestication of pigs (Paudel et al., 2013). Among the genetic variations, the advent of next generation sequencing methods has further allowed for a comprehensive screen of variation in codon usage bias (CUB) preference. Studying the degeneracy of genetic code, which enables most amino acids to be coded by more than one codon called 'synonymous' codon (Wright, 1990) has been done in pigs (Khobondo et al., 2015). Huge interspecific and even intragenomic variation in codon usage within and between genomes has been documented as well (Jia et al., 2009). Several biological factors such as tRNA abundance (Kanaya et al., 2001), strand specific mutational bias, replicational, transcriptional and translational selection (Hershberg and Petrov, 2008), secondary structure of proteins, mRNA structure, GC composition (Knight et al., 2001), genomic composition factors (Khobondo et al., 2015) and environmental factors (Basak and Ghosh, 2005) have been reported to influence the synonymous codon usage in various organisms. The afore mentioned factors led to two hypotheses on the evolution of codon bais; mutation bias and natural selection for translation accuracy and efficiency respectively (Sharp et al., 2005). The mutational bias hypothesis predicts that genes in the GC-rich regions of the genome preferentially use G- and C-ending codons, while those in the AT-rich regions use A- and T-ending codons (Zhang et al., 2009) as observed in mammals.

Khobondo et al. (2015) confirmed the existence of codon usage bias in the porcine genome which might suggest there is weak selection of preferred codons for translation accuracy. The codon usage bias was influenced subtle by nucleotide composition factors (GC, GC3, CDS length) among others. In the study, there was a negative correlation between genomic codon adaptation index (gCAI), a proxy of codon usage bias and GC content or GC3s. However, this finding contradicted other findings (Hershberg and Petrov, 2010) and was attributed to the difference in the genome isochore structure, ambiguity (vary with space and time) of the gene expression in mammals, or due to difference in methodology of calculating codon adaptation index (CAI) variants. The negative correlation was reported between gCAI of pig and gene length and was consistent with other reports in organism such as yeast, *Caenorhabditis elegans*, *Drosophila melanogaster*, *Arabidopsis thaliana*, *Populus tremula* and *Silene latifolia* (Qiu et al., 2011). This correlation shows that metabolic systems prefer to express those genes that are less costly (Hahn and Kern, 2005). Despite this evidence on CUB, it is not known how this phenomenon (codon usage bias) may affect gene functionality and paucity. Therefore, this study was done to relate the pig CUB (5% of each genes showing highest and lowest biasness) to gene ontologies and functional genomics.

2 Materials and Methods

2.1 Sequence data

The genome sequence used for analysis was downloaded from Ensemble v68 (*Sus scrofa* build 10.2) using BioMart (Ensembl v68). A total of 23,269 coding sequences was extracted from the female Duroc pig breed as the reference genome,. Only 21,550 CDS with more than 50 amino acids (150 bp) were included for analysis. Gene ontology (GO) terms were downloaded from Ensembl genome browser as well.

2.2 Codon indices: Genomic Codon Adaptation Index (gCAI)

Genomic codon adaptation index (gCAI) used in this study was computed earlier (Khobondo et al., 2015) as the geometric mean relative synonymous codon usage (RSCU) divided by the highest possible geometric mean of RSCU given the same amino acid (AA) sequence using an in house perl script.

Therefore, the value gCAI is a proxy for codon bias because values are normalized using codon frequencies at equilibrium, thus there is no assumption of expression bias (Khobondo et al., 2015).

2.3 Analysis tools

An in house Perl script was used to derive codon indices as described by Khobondo et al. (2015). Five percent (5%) of most and least bias genes according to gCAI were extracted and grouped in two categories (low and high bias). Because not all pig genes have associated gene names, the genes without gene names were blasted against the human Refseq mRNAs and human reference protein sequences (blastn and blastp respectively) and the best human hit was assigned as gene name. Human orthologs of porcine genes were used to perform gene ontology (GO) analysis. BinGO v2.44 (Maere et al., 2005) a plugin of Cytoscape v2.8.3 (Shannon et al., 2003) was used to identify enriched GO terms using human gene annotation as background. Hypergeometric test was used to assess the significance of the enriched terms and Benjamini and Hochberg correction was implemented for multiple comparisons. Validation of over-represented GO terms from BinGO was done using a Perl script that compared the GO terms between the two files (selected highest or lowest biased) and all GO terms downloaded from Ensembl genome browser. Statistical significance was computed using a chi- square test. In order to correct for false enrichment, P-value threshold of 0.0001 was used as significant value for GO analysis.

3 Results

3.1 High codon usage bias and Gene Ontology terms

Gene ontology analysis on the 5% high and low CUB genes using BinGO and validated by in-house Perl script found 28 and 71 GO terms to be significantly enriched in highly and lowly CUB genes, respectively. The significant GO terms covered all the three gene ontology domains of cellular components, biological processes and molecular functions. Notable associated GO terms like cell surface, plasma membrane, nucleolus, nucleoplasm and nucleus showing anatomical structures are cellular components related to biological processes. The over-representation of ribosome, actin binding for translation and holding cellular matrix (mentioned above), were expected in highly biased genes. The same apply for heme binding for oxygen supply in all

the body cells, DNA repair and transmembrane transport. Ubiquitination, phosphorylation, protein kinase binding and protein kinase activity are also highly enriched among high CUB genes (Table 1).

3.2 Low codon usage bias and gene ontology

For the lowly biased genes, there was over-representation of GO terms related to immune response (Table 2). For example, biological processes like defense response, defense to bacteria and virus, negative regulation of inflammatory cytokines and apoptotic process were significant. Antigen processing and presentation as well as lysosome which are acquired and innate immune response respectively were enriched in low CUB. Notably were the over-representations of many GO terms for organs development (liver, lung, skin, skeletal muscle, and thymus) and GO terms related to many negative regulations of some detrimental molecular functions e.g. low response to ultra violet light and blood coagulation. Olfactory GO terms like detection of chemical stimulus involved in sensory perception of smell were also enriched (Table 2).

4 Discussion

GO terms are associated with codon usage bias

GO terms such as phosphorylation involved in gene regulation mechanisms were found significantly enriched in highly biased genes. Enriched GO terms were also found to be associated with major processes such as transcription of genetic materials for gene expression, trans-membrane transport of the transcript from nucleus to cytosol and ribosome manufacture to facilitate translation. Other major term such as actin binding play a central role in many eukaryotic cells basic metabolism. It compliments cytoskeleton to shape the cells, acts in cell division, motility, contraction, adhesion, phagocytosis, protein sorting, DNA repair and signal transduction (Uribe and Jay, 2009). Many studies have established the presence of actin in the nucleus and cytoplasm and have shown that its functions are diverse in both cell components. Possible roles for nuclear actin include contribution to

Table 1 The overrepresented GO terms for the 5% highly codon biased genes of the pig genome

GO ACCESSION	CHI-SQUARED	P-VALUE	GO TERM
GO:0003779	14.8161	0.0001185	actin binding
GO:0009986	21.3826	3.76E-006	cell surface
GO:0006281	12.9777	0.0003152	DNA repair
GO:0020037	23.3437	1.36E-006	Heme binding
GO:0060749	0.019	0.00003	mammary gland alveolus development
GO:0005624	20.806	5.08E-006	membrane fraction
GO:0003676	113.4532	2.20E-016	nucleic acid binding
GO:0005730	41.2299	1.35E-010	nucleolus
GO:0005654	8.1772	0.004242	nucleoplasm
GO:0000166	131.9434	2.20E-016	nucleotide binding
GO:0005634	352.4049	2.20E-016	nucleus
GO:0055114	63.8083	1.37E-015	oxidation-reduction process
GO:0016491	48.2633	3.73E-012	oxidoreductase activity
GO:0008233	26.921	2.12E-007	peptidase activity
GO:0016310	26.921	2.12E-007	phosphorylation
GO:0005886	127.9523	2.20E-016	plasma membrane
GO:0004672	61.0469	5.57E-015	protein kinase activity
GO:0019901	18.1546	2.04E-005	protein kinase binding
GO:0030529	15.2761	9.29E-005	ribonucleoprotein complex
GO:0005840	20.5753	5.73E-006	ribosome
GO:0003735	21.0366	4.51E-006	structural constituent of ribosome
GO:0005198	14.8161	0.0001185	structural molecule activity
GO:0055085	53.909	2.10E-013	transmembrane transport
GO:0006511	12.5187	0.0004029	ubiquitin-dependent protein catabolic process
GO:0004842	14.7011	0.000126	ubiquitin-protein ligase activity

Table 2 The overrepresented GO terms of the 5% lowest codon biased genes

GO ACCESSION	CHI-SQUARED	P-VALUE	GO TERM
GO:0000187	8.4261	0.003699	activation of MAPK activity
GO:0009952	17.0015	3.74E-005	anterior/posterior pattern specification
GO:0019882	0.009208	0.0009208	antigen processing and presentation
GO:0045177	8.6621	0.003249	apical part of cell
GO:0016324	18.6789	1.55E-005	apical plasma membrane
GO:0006915	37.8925	7.48E-010	apoptotic process
GO:0008026	20.1178	7.28E-006	ATP-dependent helicase activity
GO:0030424	7.4806	2.90E-005	axon
GO:0007411	15.8045	7.02E-005	axon guidance
GO:0001568	8.8982	0.00002854	blood vessel development
GO:0030054	25.8794	3.63E-007	cell junction
GO:0016477	16.0439	6.19E-005	cell migration
GO:0000902	10.556	0.001158	cell morphogenesis
GO:0007166	22.7576	1.84E-006	cell surface receptor signaling pathway
GO:0005911	14.1308	0.0001705	cell-cell junction
GO:0008234	12.9371	0.0003221	cysteine-type peptidase activity
GO:0019221	10.3192	0.0001317	cytokine-mediated signaling pathway
GO:0006952	9.608	0.0001937	defense response
GO:0042742	5.6186	0.0001777	defense response to bacterium
GO:0050830	4.4694	0.00003451	defense response to Gram-positive bacterium
GO:0051607	4.927	0.00000264	defense response to virus
GO:0050911	210.002	2.20E-016	detection of chemical stimulus involved in sensory perception of smell
GO:0003677	250.5647	2.20E-016	DNA binding
GO:0006310	9.608	0.001937	DNA recombination
GO:0009953	5.8503	0.01557	dorsal/ventral pattern formation
GO:0009790	11.7456	0.0006099	embryo development
GO:0031012	40.535	1.93E-010	extracellular matrix
GO:0030198	13.8919	0.0001936	extracellular matrix organization
GO:0007156	22.5175	2.08E-006	homophilic cell adhesion
GO:0006955	38.6133	5.17E-010	immune
GO:0001822	13.4144	0.0002497	kidney development
GO:0030027	13.1757	0.0002836	lamellipodium
GO:0016874	14.8478	0.0001165	ligate activity
GO:0006629	24.6785	6.77E-007	lipid metabolic process
GO:0005764	0.8375	5.00E-006	lysosome
GO:0008237	20.1178	7.28E-006	metallopeptidase activity
GO:0015630	16.5226	4.81E-005	microtubule cytoskeleton
GO:0007399	14.1308	0.0001705	nervous system development
GO:0001764	13.8919	0.0001936	neuron migration
GO:0005654	19.8779	8.26E-006	nucleoplasm
GO:0017111	31.4052	2.09E-008	nucleoside-triphosphatase activity
GO:0006334	18.6789	1.55E-005	nucleosome assembly
GO:0004984	210.002	2.20E-016	olfactory receptor activity
GO:0009887	11.7456	0.0006099	organ morphogenesis
GO:0008233	59.0155	1.56E-014	peptidase activity

Continuing table 2

GO ACCESSION	CHI-SQUARED	P-VALUE	GO TERM
GO:0005543	4.5283	9.52E-016	phospholipid binding
GO:0016773	15.8045	7.02E-005	phosphotransferase activity, alcohol group as acceptor
GO:0005886	267.1531	2.20E-016	plasma membrane
GO:0010628	22.9977	1.62E-006	positive regulation of gene expression
GO:0051712	988.1238	2.20E-016	positive regulation of killing of cells of other organism
GO:0006813	22.7576	1.84E-006	potassium ion transport
GO:0046777	25.399	4.66E-007	protein autophosphorylation
GO:0046983	6.5226	4.81E-005	protein dimerization activity
GO:0019904	19.6381	9.36E-006	protein domain specific binding
GO:0006468	144.374	2.20E-016	protein phosphorylation
GO:0004713	95.6153	2.20E-016	protein tyrosine kinase activity
GO:0004725	18.9187	1.36E-005	protein tyrosine phosphatase activity
GO:0042127	20.5976	5.67E-006	regulation of cell proliferation
GO:0009411	4.4694	0.00003451	response to UV
GO:0009615	13.6531	0.0002199	response to virus
GO:0008236	19.3982	1.06E-005	serine-type peptidase activity
GO:0007165	377.3029	2.20E-016	signal transduction
GO:0001501	12.4602	0.0004157	skeletal system development
GO:0043588	5.3875	0.02028	skin development
GO:0046332	4.0154	0.00004509	SMAD binding
GO:0048538	0.02644	0.02644	thymus development
GO:0016055	12.9371	0.0003221	Wnt receptor signaling pathway

the organization of chromatin remodeling complexes, RNA processing or regulation of DNAase I function (Olave et al., 2002). In addition, actin plays a direct role in transcription by RNA polymerases, II and III (Percipalle and Visa, 2006). Ubiquitination (ubiquitin-dependent protein catabolic process, ubiquitin-protein ligase activity) is a reversible post-translational modification of cellular proteins and is known to play central roles in the regulation of various cellular processes, such as protein degradation, protein trafficking, cell-cycle regulation, DNA repair, apoptosis and signal transduction (kimura and Tanaka, 2010). Such functions are highly regulated and require high codon usage bias as witnessed in this study. For normal cell functioning, protein - protein interaction and the activities of enzymes are regulated by phosphorylation of tyrosine. More so, in all genes activation, phosphorylation plays a central role. It is not surprising that these molecular functions and biological processes are enriched in high CUB genes for cell hemostasis purposes. Taken together, most GO terms enriched in high CUB genes are important for day to day physiological functions of the cell and may be termed as ‘housekeeping’.

Functional enrichment analyses in this study do mimic Paudel et al. (2013) findings on CNVs. The enrichment of low CUB involved in the immune related genes is interesting. Genes involved in virus response such as interferon (IFN), cytochrome P450 (CYP), are usually fast evolving due to their importance for the organism to respond rapid changes in the environment. For example, members of interferon (IFN) gene families, involved in defense against viral infections (Table 2), and CYP genes, which are responsible for detoxification and drug metabolism, were found to have high CNR (Paudel et al., 2013) and very plausible in this study. This could be because these genes are less conserved and need to evolve fast to adapt to ever changing antigenic determinants, evolution of pathogen and immune evasion mechanism explored by pathogens. The low CUB usage results reported in this study concur with Paudel et al. (2013) and show that these types of genes are often found to be CN variable in pigs. The observed overrepresentation of low codon

biased in olfactory receptors (OR) was expected. *Sus scrofa* have the largest repertoire of functional OR genes in mammals whose genome has been sequenced to date (Nguyen et al., 2012), likely related to the strong dependence on their sense of smell for foraging especially in wild and in extensive production system. For efficiency of foraging different feeds, high flexibility of codon usage is thus justified. There are about 1301 porcine OR genes, nearly a third are found as copy number variable in pigs. Such large numbers of genes are less conserved and might explain the low CUB as reported in this study. These findings suggest that the wide variety of environmental conditions faced by pigs around the world have resulted in low CUB for flexibility and high CNVs. This low CUB could be because these genes are less conserved and need to evolve fast to adapt to ever changing antigenic determinants and artificially created environment for immune and olfactory receptor genes respectively.

Cell apoptosis through fas ligand (Griffith et al., 1995) being the principal mechanism by which the majority of effecter T and B lymphocytes die after clearance of an infection may justify the low CUB witnessed in this study. This might be to meet the changing infection-clearance status to regulate uncontrolled activation of lymphocytes that may result to self-destruction, limit auto reactivity and bring forth immune tolerance.

Conclusion

Functional analyses revealed high and low codon usage bias enriched with genes related to housekeeping functions and immune, sensory perception and response to stimulus respectively.

Authors' contributions

Khobondo J.O. conceived and designed the experiments, developed perl script, wrote the manuscript; Kahi A.K. discussed and improved manuscript; Ngeno K. analysed the data, improved the manuscript. All authors read and approved the final manuscript.

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