Journal of Biochemistry and Molecular Biology, Vol. 39, No. 1, January 2006, pp. 00-00

JOURNAL OF Biochemistry and Molecular Biology

Confirming Single Nucleotide Polymorphisms From Expressed Sequence Tag Datasets Derived From Three Cattle cDNA Libraries

Seung-Hwan Lee, Eung-Woo Park, Yong-Min Cho, Ji-Woong Lee, Hyoung-Yong Kim, Jun-Heon Lee[1], Sung-Jong Oh, Il-Cheong Cheong and Du-Hak Yoon*

Animal Genomics & Bioinformatics Division, National Livestock Research Institute, RDA, Suwon 441-350, Korea

[1]Division of Animal Science and Resources, Research Center for Transgenic Cloned Pigs, Chungnam National University, Daejeon 305-764, Korea

Received 25 October 2005, Accepted 21 December 2005

Using the the *Phred/Phrap/Polyphred/Consed* pipeline established in the National Livestock Research Institute of Korea, we predicted candidate coding single nucleotide polymorphisms (cSNPs) from 7,600 expressed sequence tags (ESTs) derived from three cDNA libraries (liver, M. *longissimus dorsi*, and intermuscular fat) of Hanwoo (Korean native cattle) steers. From the 7,600 ESTs, 829 contigs comprising more than two EST reads were assembled using the *Phrap* assembler. Based on the contig analysis, 201 candidate cSNPs were identified in 129 contigs, in which transitions (69%) outnumbered transversions (31%). To verify whether the predicted cSNPs are real, 17 SNPs involved in lipid and energy metabolism were selected from the ESTs. Twelve of these were confirmed to be real while five were identified as artifacts, possibly due to expressed sequence tag sequence error. Further analysis of the 12 verified cSNPs was performed using the program BLASTX. Five were identified as nonsynonymous cSNPs, five were synonymous cSNPs, and two SNPs were located in 3'-UTRs. Our data indicated that a relatively high SNP prediction rate (71%) from a large EST database could produce abundant cSNPs rapidly, which can be used as valuable genetic markers in cattle.

Keywords: Expressed sequence tag (EST), Hanwoo (Korean native cattle), Single nucleotide polymorphism (SNP)

*To whom correspondence should be addressed.
Tel: 82-31-290-1593; Fax: 82-31-290-1602
E-mail: dhyoon@rda.go.kr

Introduction

Single nucleotide polymorphisms (SNPs) in coding regions are very important genetic markers for identifying causative mutations in candidate genes affecting economically important traits in livestock. Of these SNPs, nonsynonymous SNPs (nsSNPs), which change the amino acid sequences of proteins, are the main source of phenotypic variation in important traits. Some well-defined markers such as geneSTAR marbling have been widely used in cattle breeding (http://www.geneticsolutions.com.au).

Newly identified nsSNP markers have been tested in association studies between genotype and phenotype (Vignal *et al*., 2002), and SNP markers that likely cause differences between individuals have been developed (Kruglyak, 1997; Toivonen *et al*., 2000). The characterization of nsSNPs in expressed genes can increase the likelihood of identifying alleles that have significant effects on economically important traits in cattle.

The identification of large numbers of SNPs has emerged as an important issue in numerous studies of the human and mouse genomes, and much SNP data has already been deposited in the public domain (Gu *et al*., 1998; Taillon-Miller *et al*., 1998; Buetow *et al*., 1999; Cargill *et al*., 1999; Picoult-Newberg *et al*., 1999). Recently, reliable bioinformatics tools for discovering SNPs from EST sequence data using *polybayes* (Marth *et al*., 1999) and from genomic sequence data using *polyphred* (Nickerson *et al*., 1997) have been widely used for SNP identification in livestock, at minimal cost (Buetow *et al*., 1999; Garg *et al*., 1999; Picoult-Newberg *et al*., 1999). Kim *et al*. (2003) reported extracting candidate nsSNPs from a chicken EST database using the *in-silico* pipeline containing the programs *Phred* for base-calling (Ewing and Green, 1998), *Phrap* for sequence alignment and contig assembly (Green, http://genome.washington.edu), *Polyphred* for detecting SNPs (Nickerson *et al*., 1997), and

Consed for the SNP trace view (Gordon *et al*., 1998). They selected 1,203 high-quality candidate nsSNPs using this pipeline. In addition, Hawken *et al*. (2004) reported candidate cSNPs using approximately 339,000 bovine EST sequences deposited in the National Center for Biotechnology Information (NCBI) database and confirmed a small subset of the candidate cSNPs to determine the rate of false positives.

Since ESTs represent coding sequences in the genome, nsSNPs derived from ESTs are more likely to affect phenotypes than SNPs in noncoding regions of the genome (Hawken *et al*., 2004). Therefore, we searched for nsSNPs *in silico* from EST datasets derived from three cDNA libraries to develop genetic markers related to economically important traits in cattle breeding.

Materials and Methods

cDNA library construction. Liver, intermuscular fat and *longissimus dorsi* tissues were collected from Hanwoo (Korean native cattle) steer immediately after slaughter at 24 month of age (n = 4) and immediately frozen in liquid nitrogen and store at −80°C. Total RNAs were prepared from liver, intermuscular fat and *longissimus dorsi* tissues using the TRIzol reagent (Gibco BRL Life Technologies Ltd.). RNA intensity was confirmed by examining the 28S and 18S ribosomal RNA bands on ethidium bromide stained 1.5% agarose gel. Total RNA extracted from four different Hanwoo steers were pooled and enriched for mRNA using the oligotex mRNA midi kit (Qiagen Ltd.). The cDNA library was constructed using λ ZAP® cDNA synthesis/Gigapack III gold cloning kit (Stratagene). The cDNA was prepared using 5 μg of mRNA and oligo $(dT)_{18}$ primer, size-fractionated and inserted into the Uni-ZAP XR vector using *Xho* I linker-primer and *Eco*R I adaptor. The library was stored at 4 and −80°C in 7% DMSO for long-term storage.

Plasmid isolation and cDNA sequencing. After *in vivo* mass excision with *E. coli* strain XLORL, the cDNA libraries were plated in LB plate containing ampicilin (50 μg/ml). After white/blue selection, colonies were randomly picked, and grown in 1.3 mL terrific broth in individual well of 96-deep well plates overnight at 37°C. A glycerol stock of each 96-well plate was made and stored at −80°C. Plasmid DNAs were purified by core-one plasmid HTS prep kit (CoreBiosystems). Single-pass sequencing of the 5'-termini of cDNA clones was performed using the ABI 3730XL (PE Applied Biosystems) and the ABI prism Big Dye Terminator Ver 3.1 (PE Applied Biosystems). A total of 7,600 5' EST sequences were obtained (GenBank Acc. No; CF923187-923275, CF929662-CF931317, DV782155-784555, DV791359-793759, BM986136-986212, BM929196-929223, BM888862-888942, BM929110-929189, BM956469-956615, BM967982-968040, BM967917-967981, BM967842-967916, BM967778-967841, BM967714-967777, BM967636-967713, BM967572-967635, BM967528-967571, BM967462-967527, BM967403-967456).

SNP mining from EST dataset. EST trace files were base called using *Phred* program (Ewing *et al*., 1998). Vector sequences were removed and contigs were assembled using Cross-match program and *phrap* assembler (Green 1994) based on their Smith-Waterman sequence similarity (Pivoult-Newverg *et al*., 1999). In this analysis, only 5' EST data were analyzed because coding SNPs (cSNP) are more abundant in the ESTs from 5' reads. Candidate SNPs were identified from the assembled sequences using the *polyphred* program (Nickerson *et al*., 1997). Any SNPs located 50 bp from the beginning and 50 bp at the end of assembled sequences were excluded for further analysis because of a vector sequence and low sequence quality. Also insertions and deletions were not included for the further SNP analysis. All SNPs were confirmed by visual inspection using the *Consed* trace viewer program (http://phrap.org/consed/consed.html).

Molecular confirmation of candidate cSNPs. To verify 201 putative cSNPs detected by *Phred/Phrap/Polyphred/Consed* pipeline, 17 contigs were chosen that had a BLAST (Altschul *et al*., 1990) hits against NCBI nr database with the genes involving lipid and energy metabolism (Wheeler *et al*., 2003). The selected 17 contigs had minimum average *Phred* score of 20 at the selected SNP position for all the sequences in each contig. Primer 3 out program was used for designing primers from the consensus sequence in each contig. PCR amplification was carried out in a 20 μl reaction mixture containing 1 μl of genomic DNA, 2 μl of 10 × PCR buffer, 1 μl of dNTP mix, 10 pmol of each primer and 2.5 units of Taq DNA polymerase. In general, PCR reaction was done at 94°C for 5 min, 58°C for 1 min and 72°C for 1 min (30 cycles). The verification of SNPs was determined either direct sequencing and PCR-RFLP methods using 24 unrelated Hanwoo DNA samples. SNP types were determined based on the comparison of the BLASTX result and the nucleotide sequence queries at the SNP positions.

Results

Mining SNPs from the EST datasets. ESTs have been widely used to locate SNPs in expressed genes in animals and plants. In this study, we explored cSNPs from EST sequences of Hanwoo using the Phred/Phrap/Polyphred/Consed pipeline. In total, 829 contigs were obtained after assembly using the programs Phred and Phrap. From these, 700 contigs contained two or more sequence reads (Fig. 1A). Using Polyphred, we obtained 201 high-quality candidate cSNPs from 129 contigs (Table 1). Using Consed, all SNPs were confirmed visually (Fig. 2A). Simultaneously, we also investigated the distribution of the numbers of reads and SNPs per contig. The number of reads per contig ranged from two to 30. Of the 201 SNPs, 129 had fewer than five ESTs per contig (Fig. 1B). Of the 129 contigs with SNPs, 88 (68%) contained only one SNP, 21 (16%) contained two SNPs, and 13 (10%) contained three SNPs (Fig. 2B). The average length of these contigs was 1,200 bp, and the minimal and maximal lengths of the contigs were 324 and 3,010 bp, respectively. The largest contig was assembled from 553 ESTs and contained five SNPs.

Characteristics of candidate cSNPs. Based on nucleotide

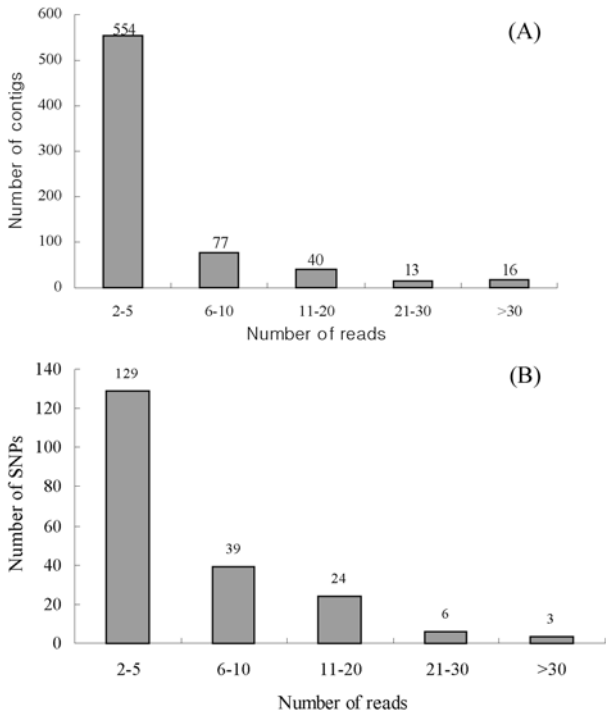

Fig. 1. Data analysis. (A) Distribution of the number of reads per contig. (B) Number of SNPs with different numbers of reads.

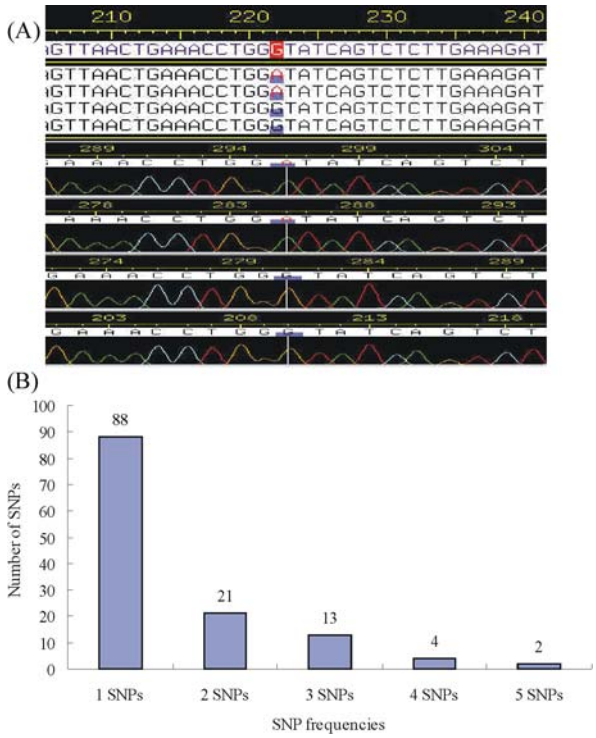

Fig. 2. (A) An example of candidate cSNP detection using the program *Consed* viewer. (B) The frequencies of SNP detection in a contig.

Table 1. Statistics of cSNPs mining from EST sequences derived from three cDNA libraries in Korean Cattle (Hanwoo)

Number of total Hanwoo EST sequences	7600 ESTs
Average length of EST sequences	650 bp
Contigs	829
Singlet	2230
Number of Contigs containing SNPs	129
Number of identified SNPs	201

substitutions, single nucleotide polymorphisms were classified as either transversions (purine ↔ pyrimidine) or transitions (purine ↔ purine or pyrimidine ↔ pyrimidine). The percentages of A ↔ G, T ↔ C, A ↔ C, T ↔ G, A ↔ T, and G ↔ C substitutions were 35, 34, 6, 11, 4, and 10%, respectively (Table 2). Of the 201 SNPs, 139 were transitions (69%) and 62 were transversions (31%), giving a transition-to-transversion ratio of 2.2 : 1, thereby confirming the 2 : 1 ratio of transitions to transversions in mammals (Cheng *et al*., 2004).

Verification of candidate cSNPs. To verify the cSNPs detected from the Hanwoo EST datasets, 17 cSNPs were selected from contigs related to energy and lipid metabolism from a BLAST search against the NCBI non-redundant protein (nr) database. Two different approaches were used to verify the cSNPs. First, PCR-RFLP was used with genomic DNA from 24 unrelated Hanwoo as templates for PCR. Twelve of 17 cSNPs were digested with the predicted restriction enzymes (contig 274 with Dde I, contig 504 with Hinf I, contig 520 with BssH II, contig 585 with Fnu4H I, contig 612 with Bsb I, contig 615 with BstB I, contig 620 with Alu I, contig 689 with Taq I, contig 710 with Aci I, contig 761 with Mbo I, contig 772 with Hha I, and contig 802 with Nia III). The other four cSNPs were subjected to sequence analysis using a public domain cow EST database (Fig. 3). The last one was failed the amplification. Consequently, 12 of the 17 cSNPs were identified as real cSNPs (71% real cSNP identification rate). Ten of the 12 verified cSNPs were located in exons and the other two cSNPs were in 3'-UTR regions (Table 3). Of these ten cSNPs, five were nsSNPs, which changed amino acids, and the other five were identified as synonymous SNPs (Table 3). We also identified nsSNPs in the genes for adipo Q protein and adipocyte fatty acid binding protein (aP2), which are related to energy homeostasis and glucose and lipid metabolism (Yamauchi *et al*., 2002).

Discussion

The identification of the genetic and biochemical mechanisms of intramuscular fat deposition is very important for cattle breeding. However, understanding the genetic basis for intramuscular fat deposition is difficult because this trait is likely controlled by a number of genes. Therefore, large-scale analysis of expressed sequence tag (EST) offers powerful new biological tools for connecting single nucleotide polymorphism

Table 2. Nucleotide substitution types for the identified cSNPs from Hanwoo EST sequence dataset

Types	Number of SNPs	Percent		Ratios*
A ↔ G	71	35%	Transition	2.2
T ↔ C	68	34%		
A ↔ C	12	6%	Transversion	1
T ↔ G	22	11%		
A ↔ T	8	4%		
G ↔ C	20	10%		

*Ratio indicates the transition over transversion ratio

in coding region with quantitative traits of cattle such intramuscular fat (Elahe *et al*., 2004; Hawken *et al*., 2004). Recently, several groups have accomplished the use of EST sequence for mining of cSNPs in human (Gu *et al*., 1998; Buetow *et al*., 1999; Picoult-Newberg *et al*., 1999; Irizarry *et al*., 2000), bovine (Hawken *et al*., 2004), porcine (Fahrenkrug *et al*., 2001), and chicken (Kim *et al*., 2003). In this study, to identify informative genes with cSNPs that affect the intramuscular fat content, we constructed three cDNA libraries from liver, *longissimus dorsi*, and adipose tissues of Hanwoo steers. In this paper, we focused on the discovery cSNPs of expressed genes using 7,600 ESTs derived from liver, *longissimus dorsi*, and intermuscular fat. Using the *Phred/Phrap/Polyphred/Consed* programs, we identified 201 SNPs in 129 contigs, with an average density of one SNP per 787 bp. This frequency is much higher than the one SNP per 2,119 bp reported for chicken (Kim *et al*., 2003). Generally, in humans, the estimated SNP detection frequency is one per 1000-2000 bp of genomic DNA, and in some regions, the frequency is as high as one per 300 bp (Wang *et al*., 1998;

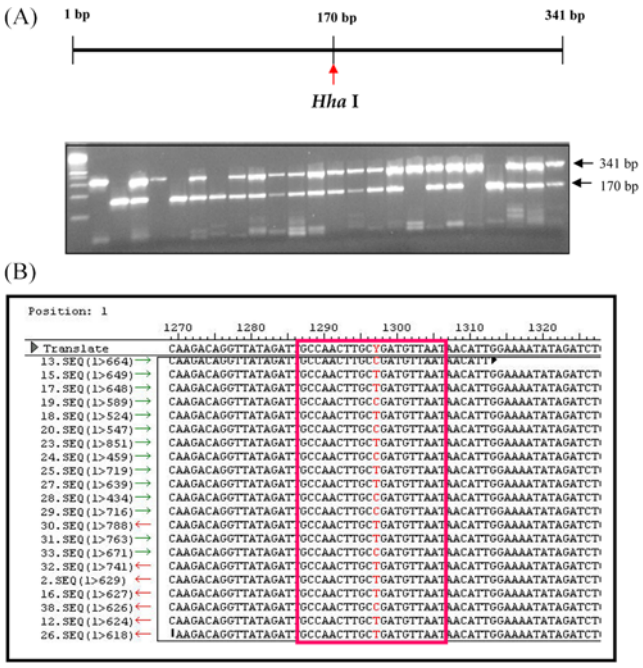

Fig. 3. Molecular confirmation of cSNPs from the Hanwoo EST dataset. (A) Examples of cSNPs in the microsomal glutathione-S-transferase gene using PCR-RFLP. (B) An example of cSNP confirmation in the leucine aminopeptidase gene using public domain cow EST data.

Dawson, 1999). Transitions (purine ↔ purine, pyrimidine ↔ pyrimidine) represented 69% of the SNPs, and transversions (purinepyrimidine) accounted for 31%, giving a transition-to-transversion ratio of 2.2 : 1 for these cSNPs in Hanwoo. Recently, chicken SNP results obtained from an EST sequence trace database resulted in a transition-to-transversion ratio of 2.3 (Smith *et al*., 2001). In contrast, Berger *et al*. (2001)

Table 3. List of 17 verified single nucleotide polymorphisms (SNPs)

Contig ID	Gene description	Location	SNP type
802	Adipocyte fatty acid binding protein (aP2)	Exon	$ATG^{met} \rightarrow GTG^{val}$
523	Dehydrogenase/3-keto acyl-CoA thiolase	Exon	$GGA^{gly} \rightarrow AGA^{arg}$
585	Myocardial vascular inhibition factor	Exon	$TCA^{ser} \rightarrow CCA^{pro}$
710	Acetyl-CoA acetyltransferase	Exon	$TGC^{cys} \rightarrow CGC^{arg}$
689	Adipo Q protein, *Bos taurus*	Exon	$UCG^{ser} \rightarrow UUG^{leu}$
772	Microsomal glutathione-S-transferase	Exon	$CGC^{arg} \rightarrow CGT^{arg}$
274	Ubiquinol-cytochrome C reductase	Exon	$CGG^{arg} \rightarrow AGG^{arg}$
520	Annexin A5, *Homo sapience*	Exon	$GAC^{asp} \rightarrow GAT^{asp}$
133	Leucine aminopeptidase, cattle	Exon	$GCT^{ala} \rightarrow GCC^{ala}$
612	Poly A binding protein	Exon	$TCA^{ser} \rightarrow TCG^{ser}$
158	Cathepsin B, *Bos taurus*	3-UTR	SNP (A → G)
815	Lim protein, *Homo sapience*	3-UTR	SNP (A → G)
504	Aminotransferase (GOT1)	3-UTR	artifact
620	ASIP, agouti protein, *Sus scrofa*	3-UTR	artifact
761	Lactate dehydrogenase B	3-UTR	artifact
615	Aldo-Keto reductase, *Bos taurus*	Exon	no SNP
659	Prion protein, *Bos taurus*		No PCR products

reported that the ratio in the fruit fly *Drosophila melanogaster* was 1 : 1.08. According to these results, mammalian genomes are more conservative than those of insects. Theoretically, if mutations are random, the transition-to-transversion ratio should be 0.5 (Vignal *et al*., 2002). However, the observed SNP data from mammalian EST sequences indicates a huge bias toward transitions. One of the biological reasons for this bias is the high rate of deamination of 5-methyl cytosine to thymidine in CpG dinucleotides (Vignal *et al*., 2002). Consequently, high frequencies of SNPs such as TC and AG are generated. When predicted a cSNP using *in silico* method, most sequence variations are due to sequencing error, which are due to single pass sequencing. Therefore, it is necessary to validate of cSNP through the PCR-RFLP and resequencing methods. We selected 17 genes with one cSNP related energy and lipid metabolism for validation. The results, 12 of the 17 cSNPs were true cSNPs. In Hanwoo breed, validation percent of cSNPs was that approximately 71%. For the human, validation studies indicate that approximately 50-82% of predicted SNP are real (Gu *et al*., 1998; Buetow *et al*., 1999; Picoult-Newberg *et al*., 1999; Irizarry *et al*., 2000). Of these 12 cSNPs, five were nonsynonymous, which is advantageous for studying the associations between genetic markers and phenotypic variation (Cheng *et al*., 2004). In conclusion, nsSNPs causing changes in the amino acid sequences of proteins are the main source of phenotypic variation in economic traits. Therefore, a number of the cSNPs collected from the Hanwoo EST analysis will be a useful genetic resource for developing genetic markers for economic traits, such as the marbling score in Hanwoo.

Acknowledgments This work was partly supported by a grant (Code No. 20050301034442016) from BioGreen 21 Program, Rural Development Administration, Republic of Korea.

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