Rarity of Somatic Mutation and Frequency of Normal Sequence Variation Detected in Sporadic Colon Adenocarcinoma Using High-Throughput cDNA Sequencing

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Abstract: We performed high-throughput cDNA sequencing in colorectal adenocarcinoma and matching normal colorectal epithelium. All six hundred three genes in the UCSC database that were expressed in colon cancers and contained open reading frames of 1000 nucleotides or less were selected for study (total basepairs/bp, 366,686). 304,350 of these 366,686 bp (83.0%) were amplified and sequenced successfully. Seventy-eight sequence variants present in germline (i.e. normal) as well as matching somatic (i.e. tumor) DNA were discovered, yielding a frequency of 1 variant per 3,902 bp. Fifty-one of these sequence variants were homozygous (26 synonymous, 25 non-synonymous), while 27 were heterozygous (11 synonymous, 16 non-synonymous). Cancer tissue contained only one sequence-altered allele of the gene ATP50, which was present heterozygously alongside the wild-type allele in matching normal epithelium. Despite this relatively large number of bp and genes sequenced, no somatic mutations unique to tumor were found. High-throughput cDNA sequencing is a practical approach for detecting novel sequence variations and alterations in human tumors, such as those of the colon.

Introduction

It is widely believed that somatic as well as germline mutations play important roles in the origin and progression of colorectal cancers (Calvert and Frucht, 2002). Many genes have been investigated for mutation to elucidate mechanisms of colorectal cancer development, with these investigations demonstrating the involvement of mutations in colorectal carcinogenesis and progression. Samuels et al. reported that PIK3CA, a catalytic subunit of the class IA phosphatidylinositol 3-kinases, was somatically mutated in 32% of colorectal cancers, resulting in the attenuation of apoptosis and facilitated tumor invasion(Samuels et al. 2004). A comprehensive study entitled, "The Tyrosine Phosphatome" was accomplished by sequencing all genes involved in tyrosine phosphorylation in a large cancer cohort consisting of 175 colorectal cancer patients(Wang et al. 2004). Most mutational studies, however, have been preoccupied with the prevalence of somatic mutations in a specific single candidate gene in relatively small colorectal cancer patient cohorts. Recently, Sjoblom et al. reported the genome-wide frequencies of somatically mutated genes in human breast and colorectal cancers(Sjoblom et al. 2006). However, the methods these used were extremely expensive, time-consuming, and labor-intensive for a typical laboratory to perform. More practical strategies, amenable to smaller laboratories with more conservative budgets, would be of great value in the continuing quest to answer questions in the fields of tumor genomics and mutatomics. To this end, we present herein a circumscribed, practical mutational study employing high-throughput cDNA sequencing in colon adenocarcinoma, in which we demonstrate the eminent feasibility and results of determining sequence variation efficiently and at low cost.

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Materials and Methods

Tissue samples

Colorectal cancer and its matching normal colonic mucosa from a patient undergoing surgical resection at the Baltimore VA Hospital after signing informed research consent was used for this study. Clinicopathological data were as follows: 75 year-old male; moderately-differentiated colorectal adenocarcinoma of the ascending colon; tumor size, $2.5 \times 1.1 \times 0.5$ cm; TNM stage (Fifth Edition of the TNM classification of the UICC, 1997), T2N0MX, without any other malignancies. Both colorectal adenocarcinoma and normal colonic epithelium (obtained at the location within the surgically resected specimen furthest from the tumor) were cut into smaller pieces and frozen in liquid nitrogen immediately after removal. A frozen aliquot of each specimen was crushed into pieces and lysed immediately in either TRIZOL reagent (Invitrogen Corp., Carlsbad, CA,) to extract total RNA, or lysis buffer of a DNeasy Tissue kit (QIAGEN Inc., Valencia, CA) to extract DNA, according to these manufacturers' instructions.

Cell lines

HeLa S3, HT29, HCT15, HCT116, LoVo, CaCo2, LS174T, LS411N, and DLD1, purchased from the American Type Culture Collection (ATCC), and KYSE30, 70, 110, 150, 220, 410, 770, 850 and OE33, obtained from Dr. Yutaka Shimada at Kyoto University in Japan (Shimada et al. 1992), were enrolled in the current study in order to validate our findings in the ATP50 gene. Culture conditions for each cell line were according to ATCC and the establisher's recommendations. All cell lines were supplemented with 10% fetal bovine serum plus an appropriate concentration of penicillin and streptomycin.

Gene selection

To increase our chances of successfully amplifying and sequencing cDNAs, we restricted our study to genes that are known to be expressed in colorectal cancer cells, based on a gene expression database at the University of California, Santa Cruz (UCSC) [http://genome.ucsc.edu/index.html]. From among this gene set, we selected a subset of genes (approximately 600) containing open reading frames (ORFs) 1000 nucleotides or shorter in length. To automate design of the large number of primer sets required, we developed an in-house primer design algorithm based on the publicly available primer design software program, Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www_slow.cgi). PCR products were designed to range from 300 to 500 bp in length. ORFs of cDNAs longer than 500 bp were divided into 2 or 3 fragments; primers were then designed with adjacent fragments overlapped, in order to completely cover these longer ORFs. Finally, for each heterozygous sequence alteration, genomic DNA primers (available on request) were designed to confirm cDNA sequencing results.

RT-PCR

Total RNA extracted from colorectal adenocarcinoma and normal colonic epithelium was reverse-transcribed using a SuperScript III First-Strand kit (Invitrogen, Carlsbad, CA), and respective cDNA pools were made. RT-PCR was performed using an AccuPrime Supermix I Kit (Invitrogen). The PCR protocol was as follows: 1 min at 96 °C followed by 35 cycles of 30 sec at 94 °C, 45 sec at 58 °C, and 1 min at 72 °C. Secondary PCR was performed on purified template from the first RT-PCR product, using the same protocol.

Sequencing

A BigDye Terminator v3.1 Kit (Applied Biosystems, Foster City, CA) was used for the sequencing reaction, and sequence products were read on an SCE 9610 automated 96-capillary sequencer (Spectruby BaseSpectrum v2.10 (SpectruMedix) and analyzed with Mutation Surveyor v2.2 (SoftGenetics LLC, State College, PA). Each time candidate sequence alterations were discovered in cDNA from colorectal cancer tissue, identical procedures were followed in matched normal epitheliam to confirm whether or not they represented somatic alterations. After candidate alterations were confirmed, the entire procedure was repeated separately on a fresh aliquot of cDNA from both the cancer and normal specimens in order to exclude amplification or technical errors due to two-stage PCR. Genomic DNA sequencing was also performed on heterozygous sequence variants to confirm that identical sequence alterations were present in genomic DNA.

Methylation-specific PCR (MSP)

Because the gene ATP50 was apparently mutated, raising the possibility that it was a tumor suppressor gene, we evaluated this gene for alternative inactivation via promoter hypermethylation. MSP primer sequences of ATP50 for the methylated reaction were: forward (5′-CGAGTGGGAGCGATTTAGGAC-3′) and reverse (5′-AACGCCAAAATTACGACACG-3′), which amplify a 94-bp product. B-actin was selected as an internal control gene, using previously published MSP primers (Eads et al. 2001). CpGenome Universal Methylated DNA (Chemicon International, Inc., Temecula, CA) was used as a positive control. The detailed MSP procedure has been previously published (Sato et al. 2002).

Microsatellite instability (MSI) assay

MSI at each locus was determined by analyses of the length of each PCR-amplified microsatellite. MSI status was confirmed by MSI assays at five consensus loci (BAT25, BAT26, D2S123, D5S346, and D17S250) according to criteria from a National Cancer Institute workshop (Boland et al. 1998). Detailed procedures were as previously described (Mori et al. 2001).

Results

Project overview

A total of 603 genes (S-Table 1) were selected based on their length (under 1,000 bp) and their predicted expression in colorectal cancers according to the UCSC database. One thousand thirty-eight primer pairs (available on request) were designed to cover the entire ORFs (total bp, 366,687) of these 603 genes. Sequence data from 862 (83.0%) of these 1,038 primer sets were successfully analyzed, meaning that approximately 304,350 total bp were successfully sequenced (all primer sets for RT-PCR and cDNA sequencing are available on request).

Sequence variants

Seventy-eight sequence variants within 50 genes were found among the 603 genes studied (Table 1) (S-Table 2 for detailed information). Thus, the frequency of sequence variants was 1 per 3,902 bp (78 total variants/304,350 total bp). Of these 78 sequence alterations, 51 were homozygous (26 synonymous, 25 non-synonymous) and 27 were heterozygous (11 synonymous, 16

GENE ID	NAME	SYMBOL	ALTERATIONS
NM_001697	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit	ATP50	T108C (Gly36Gly) A218G (Lys73Arg)

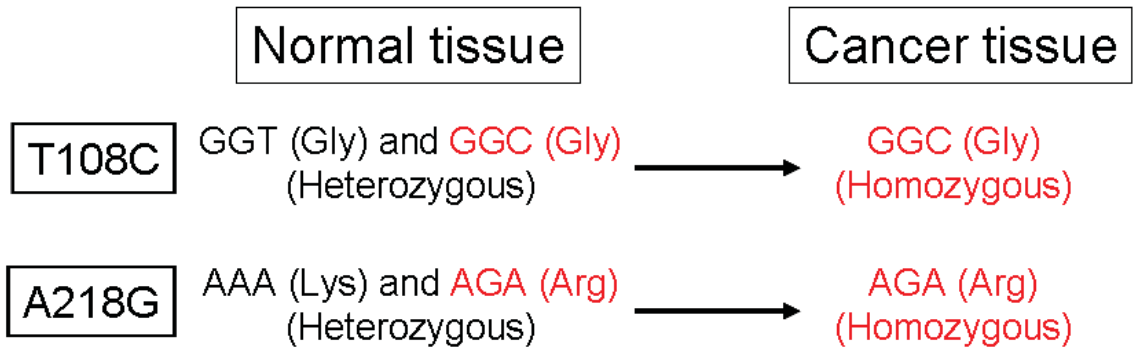

Figure 1. cDNA sequencing of ATP50. Two different alteration sites were detected. At the 108th nucleotide, colorectal cancer tissue had only a mutant cytosine nucleotide, while normal colon contained both a thymine (wild) and a cytosine (mutant). Both codons GGT and GGC encoded glycine (synonymous alteration). At the 218th mucleotide, colorectal cancer tissue had only a mutant guanine nucleotide, while normal tissue contained both an adenine (wild) and a guanine (mutant). AAA encoded lysine and AGA encoded arginine (non-synonymous alteration). *Gly*, glycine; *Arg*, arginine.

Table 1. Sequence variants

	Homozygous		Heterozygous	
	Synonymous	Non-synonymous	Synonymous	Non-synonymous
Number of alterations	26	25	11	16

non-synonymous). All sequence alterations were detected in both colorectal cancer tissue and matched normal colonic epithelium, with the exception of an alteration in the gene ATP50 (NM_001697), which manifested a unique expression mechanism (Fig. 1). Forty-four sequence alterations had been previously reported, but 34 sequence alterations were completely novel, having never been reported in the SNP database at The National Center for Biotechnology Information (NCBI).

Tumor-specific regulation of gene expression

Tumor-specific regulation of gene expression was found for NM_001697 (ATP50, Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit). The sequence alterations T108C (GGT to GGC, homozygous, Gly36Gly) and A218G (AAA to AAG, homozygous, Lys73Arg) were observed only in cancer-derived cDNA, while the alterations T108TC (CGT and GGC, heterozygous, 36Gly) and A218AG (AAA and AAG, heterozygous, 73Lys and 73 Arg) were observed in cDNA from normal epithelium. Surprisingly, both T108TC (CGT and GGC, heterozygous, 36Gly) and A218AG (AAA and AAG, heterozygous, 73Lys and 73 Arg), which were identical to the two alterations observed in normal cDNA, were observed in genomic DNA from both cancer and normal tissue (Figures 2, 3). This result implied that the cancer exhibited monoallelic expression from the variant allele of ATP50, while the normal epithelium manifested biallelic heterozygous expression, i.e. from both the reported normal allele and our discovered variant mutant allele simultaneously.

MSP

One possible mechanism for monoallelic expression observed for ATP50 was DNA methylation of its promoter region. MSP showed, however, that there was no methylation of the ATP50 promoter in colorectal cancer (S-Fig. 1).

Somatic mutations

There were no somatic mutations found among the 603 genes studied or within the p53 gene.

MSI status

MSI assays showed that there was no microsatellite instability in genomic DNA (S-Fig. 2).

Discussion

In the current study, we assumed that if a mutant protein was involved in carcinogenesis or tumor progression, this mutant would be expressed and therefore detectable in tumor mRNA. i.e. we assumed that somatic mutations involved in carcinogenesis or tumor progression would be detectable by direct cDNA sequencing. By using this strategy, we avoided the need for sequencing each exon of genomic DNA, reasoning that genes which are never expressed in normal or malignant colon probably do not participate in colorectal carcinogenesis. We discovered 78 sequence variants (44 of which had been previously reported as single-nucleotide polymorphisms, but 34 of which had never been reported) among the 603 genes (304,350 bp of ORFs) studied.

Recently, Sjoblom T. et al. performed genome-wide sequencing in breast and colorectal cancers, revealing that an average of 52 mutations occurred in each colorectal cancer(Sjoblom et al. 2006). According to the article by Sjoblom et al. the somatic mutation frequency in colon cancers was 3.2 somatic mutations/Mb, on average (Table 1 of their paper). Therefore, the probability of our finding zero somatic mutations among the 603 genes (304,350 bp) that we studied was 37.76% (please see formula below), suggesting that our findings were statistically quite consistent with Sjoblom's results:

$$Probability = \left(1 - \frac{3.2}{1,000,000}\right)^{304,350} = 0.377599353$$

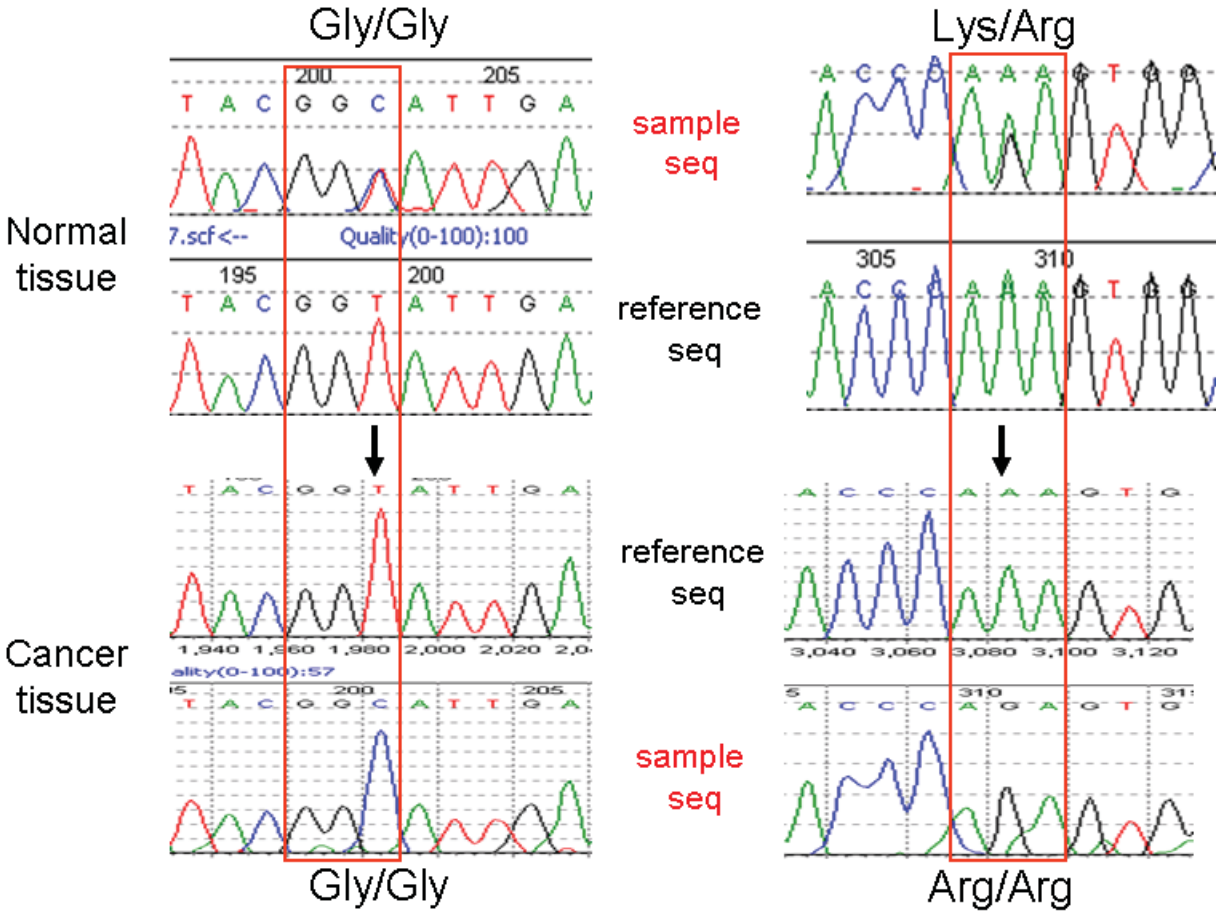

Figure 2. Representative result of cDNA sequencing of ATP50 in normal and cancer tissues. The *red box* in the *left panel* shows the 36th codon, while the *red box* in the *right panel* shows the 73rd codon. All alterations were confirmed by both forward and reverse sequencing.

The Sjoblom team also defined "CAN-genes" (candidate cancer genes) as those that were frequently mutated in colorectal cancers, and found that 69 genes could be included in this category. Although the CAN-genes KRAS, GNAS and TP53 were studied by us, no somatic mutations were found in these genes. Furthermore, in addition to the genes mentioned above, NRAS, HRAS, p16, and p27 were included in the current study, but these genes also contained no somatic mutations. Finally, results of MSI assays revealed MS-stability (MSS), implying an absence of mutations in the major DNA mismatch repair genes (although these genes were not studied due to their long ORFs). It is possible that other molecular pathogenetic pathways were involved in this colorectal tumorigenesis, such as those containing APC, MCC, DCC, or the TGF-β cascade: these genes were also not examined in the current study due to ORF length.

Approximately 24,000,000 bp among the entire genomic DNA sequence are reported as ORFs in the UCSC database. The average density of each SNP is once per 1.9 kilobases (i.e. 1,419,190 SNPs/2.7 gigabases of human genome sequence)(Sachidanandam et al. 2001). We sequenced 304,350 bp of ORFs (*viz.,* 1.26% of the total ORFs in the UCSC database: 304,350 bp/24,000,000 bp) and discovered 78 sequence variants, yielding a frequency of 1 alteration per 3,902 bp (78/304,350 bp). Our observed sequence variant distribution may provide a basis with which to estimate the number of SNPs in a single individual with colon cancer. That is, the SNPs reported above are one possible subset of the entire database; there is no guarantee that a given individual will always harbor all SNPs in the database.

The human ATP50 gene (X83218, NM_001697), encoding a 213-amino acid ATP synthase OSCP subunit, is a key structural component of the stalk of the mitochondrial respiratory chain F_1F_0-ATP synthase, which is a vital element in the cellular pathway of energy conversion (Senior,

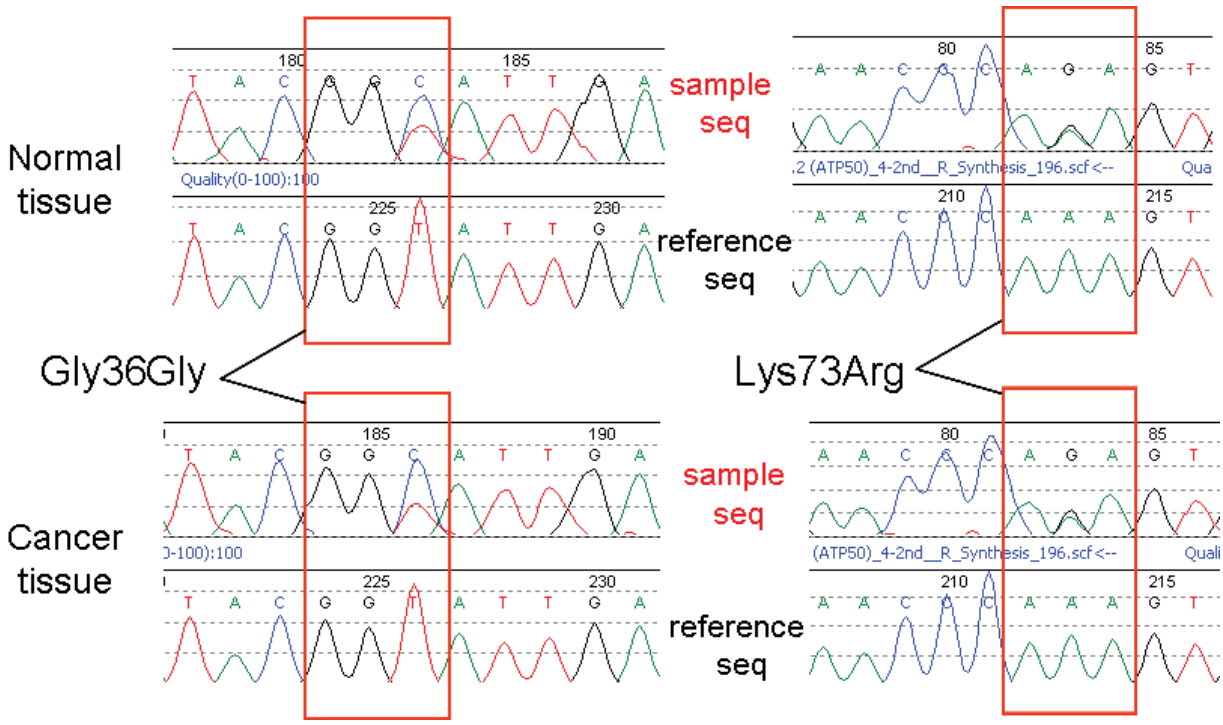

Figure 3. Genomic DNA sequencing of ATP50 in normal and cancer tissues. *Upper panels,* forward sequencing; *lower panels,* reverse sequencing. The *red boxes* show the 36th and 73rd codons. Both normal and colorectal cancer tissues contain identical heterozygosities at the 108th and 218th nucleotides.

1988). Although a mutant strain of yeast in which the delta subunit of F_1F_0-ATP synthase had been inactivated by insertional mutagenesis showed little or no ATPase activity(Giraud and Velours, 1994), and dysfunction of ATP synthase can cause a variety of degenerative diseases(Wallace, 1994), there have been no previous reports detailing a relationship between ATP synthase and tumorigenesis. We found restricted monoallelic (i.e. monoallelically silenced) expression of an altered allele from ATP50 in our colon cancer tissue, which would be expected to exert the same effect as would a somatic mutation of this gene. Genomic DNA sequencing of ATP50 revealed that this monoallelic expression was not due to LOH. We therefore studied the methylation status of the CpG island in the promoter region of ATP50 by MSP, but we found no methylation of this region. Other epigenetic mechanisms, such as histone deacetylation, might have contributed to monoallelic expression of ATP50. There was no monoallelic expression of ATP50 in 20 cancer cell lines that we examined. Although monoallelic expression of this altered ATP50 allele may be involved in a subset of colorectal cancers, further study is required to clarify the potential functional role of this gene in carcinogenesis.

This study poses several advantages as well as limitations. Firstly, it has been reported that some synonymous mutations may influence the stability of mRNA(Duan and Antezana, 2003; Chamary and Hurst, 2005) because they affect the thermodynamic stability of mRNA secondary structures (Fitch, 1974; Klambt, 1975). Nonsense-mediated mRNA decay (NMD) is also known as a surveillance pathway that rapidly degrades mRNAs containing premature termination codons(Culbertson and Leeds, 2003; Amrani et al. 2006). These mechanisms may cause instability of mRNA, accelerate the degradation of mRNA, and consequently result in difficulty in detecting sequence alterations by cDNA sequencing. Since we used cDNA as our starting material for sequencing, we may have ignored some key genes because of RNA degradation. Nevertheless, many sequence variants were detected reasonably well in the current study, suggesting that degradation of mRNA occurred rarely, if at all, as a consequence of sequence alterations. Instead, we considered it more important to increase our chances of finding sequence alterations by using cDNA rather than genomic DNA because of the lower cost, time, and labor involved in sequencing cDNA, as well

as the increased relevance of only studying genes that are expressed in the colon.

Secondly, it is conceivable that we lost some gene sequence information due to extremely low expression levels. Therefore, we employed two-stage PCR to increase our chances of successful sequencing, thereby achieving a relatively high success rate of 862/1,038 reactions, or 83.0%. Possibly, this result still may have included genes that were not expressed in our particular colorectal cancer, even though we used the UCSC database to select genes that were purportedly expressed in colorectal cancers. Our sequencing success rate appears favorable when compared to genomic DNA sequencing, where 92% of genes were successfully analyzed (Wang et al. 2004). The total number of exons sequenced in our study was 2107, implying that at least 2107 primer pairs would have been necessary to conduct this study had it been attempted by genomic DNA sequencing; in contrast, we accomplished this task using only 1038 primer sets for cDNA sequencing. This contrast demonstrates that our method is useful to explore mutations because it is not only more cost-effective, but also less demanding in time and labor.

Acknowledgements

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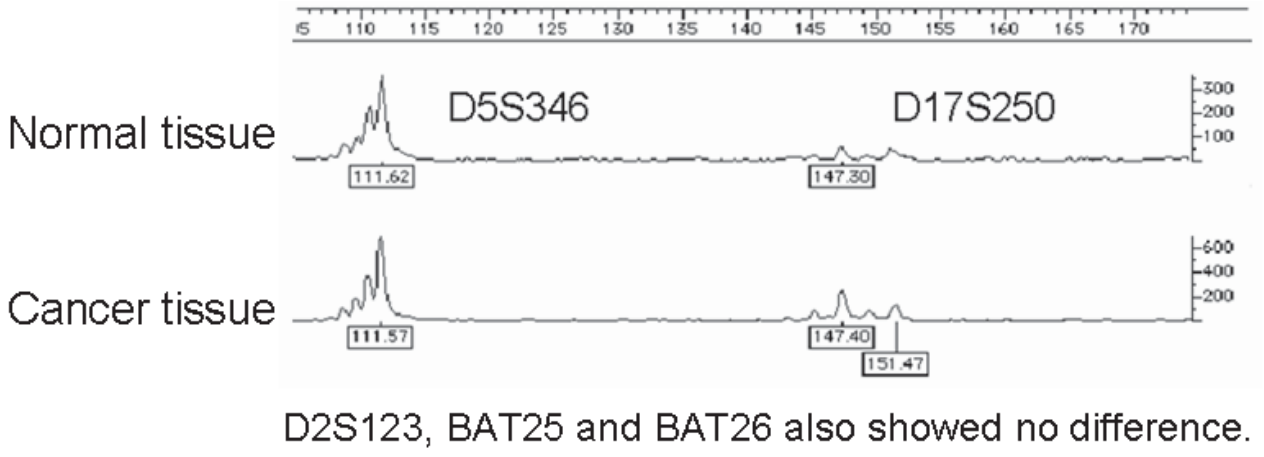
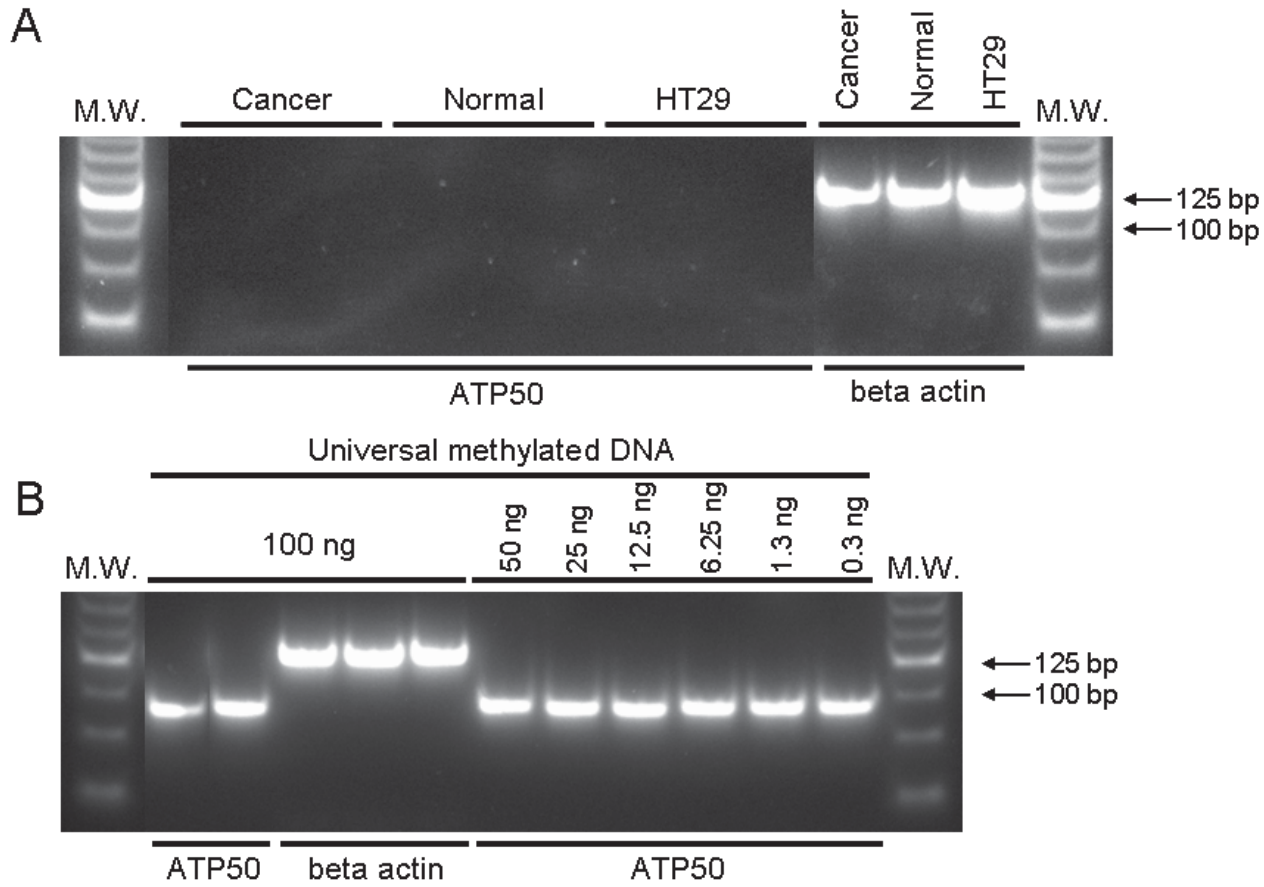
Supplement Material



S-Figure 1. Result of MSP for ATP50. (**A**) Bisulfited DNA from cancer tissue, normal tissue, and HT29 were used. The MSPs for ATP50 were triplicated. The MSP product for ATP50 was 94 bp and beta actin was 133bp. (**B**) Universal methylated DNA was used for the positive control in various amounts. M.W., molecular weight.



S-Figure 2. A representative result of MSI typing. Both cancer and normal tissue showed microsatellite stable in the marker of D5S346 and D17S250. The similar results were obtained in the marker of D2S123, BAT25, and BAT26.

S-Table-1. Gene list

GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol
NM_000100.2	CSTB	**NM_198057.1**	TSC22D3	**NM_002811.3**	PSMD7
NM_000999.2	RPL38	**NM_022652.2**	DUSP6	**NM_002812.3**	PSMD8
NM_001827.1	CKS2	**NM_021121.2**	EEF1B2	**NM_002813.4**	PSMD9
NM_001863.3	COX6B1	**NM_001412.2**	EIF1AX	**NM_170750.1**	PSMD10
NM_001866.2	COX7B	**NM_004094.3**	EIF2S1	**NM_004577.3**	PSPH
NM_001867.2	COX7C	**NM_001423.1**	EMP1	**NM_175847.1**	PTBP1
NM_002489.2	NDUFA4	**NM_001424.3**	EMP2	**NM_198974.1**	PTK9
NM_002491.1	NDUFB3	**NM_001425.1**	EMP3	**NM_002823.2**	PTMA
NM_002966.1	S100A10	**NM_207168.1**	ENSA	**NM_002824.4**	PTMS
NM_003009.2	SEPW1	**NM_001005915.1**	ERBB3	**NM_133377.1**	RAD1
NM_003063.1	SLN	**NM_001983.2**	ERCC1	**NM_153824.1**	PYCR1
NM_003095.1	SNRPF	**NM_001984.1**	ESD	**NM_000320.1**	QDPR
NM_003133.1	SRP9	**NM_001439.1**	EXTL2	**NM_004161.3**	RAB1A
NM_003498.3	SNN	**NM_001997.2**	FAU	**NM_004162.3**	RAB5A
NM_003746.1	DYNLL1	**NM_005247.2**	FGF3	**NM_002868.2**	RAB5B
NM_003860.2	BANF1	**NM_002007.1**	FGF4	**NM_198896.1**	RAB6A
NM_003945.3	ATP6V0E	**NM_002010.1**	FGF9	**NM_002870.2**	RAB13
NM_004045.2	ATOX1	**NM_023108.1**	FGFR1	**NM_183235.1**	RAB27A
NM_004485.2	GNG4	**NM_001449.3**	FHL1	**NM_201434.1**	RAB5C
NM_004541.2	NDUFA1	**NM_054014.1**	FKBP1A	**NM_198829.1**	RAC1
NM_004772.1	C5orf13	**NM_002013.2**	FKBP3	**NM_002872.3**	RAC2
NM_005274.1	GNG5	**NM_016725.1**	FOLR1	**NM_133630.1**	RAD51L3
NM_005517.2	HMGN2	**NM_004477.1**	FRG1	**NM_002881.2**	RALB
NM_005694.1	COX17	**NM_000146.2**	FTL	**NM_006325.2**	RAN
NM_005770.3	SERF2	**NM_198903.1**	GABRG2	**NM_002884.1**	RAP1A
NM_005887.1	DLEU1	**NM_000166.2**	GJB1	**NM_015646.3**	RAP1B
NM_005949.1	MT1F	**NM_024009.2**	GJB3	**NM_032626.5**	RBBP6
NM_005954.2	MT3	**NM_002061.2**	GCLM	**NM_181558.1**	RFC3
NM_005978.3	S100A2	**NM_006708.1**	GLO1	**NM_181578.1**	RFC5
NM_006156.1	NEDD8	**NM_002066.1**	GML	**NM_134427.1**	RGS3
NM_006274.2	CCL19	**NM_016592.1**	GNAS	**NM_005614.2**	RHEB
NM_006304.1	SHFM1	**NM_005301.2**	GPR35	**NM_000326.3**	RLBP1
NM_006353.2	HMGN4	**NM_002083.2**	GPX2	**NM_002938.2**	RNF4
NM_006698.2	BLCAP	**NM_002084.2**	GPX3	**NM_183045.1**	RNF6
NM_006829.2	C10orf116	**NM_203506.1**	GRB2	**NM_002946.3**	RPA2
NM_007233.1	TP53AP1	**NM_001512.2**	GSTA4	**NM_002947.3**	RPA3
NM_007281.1	SCRG1	**NM_147149.1**	GSTM4	**NM_033301.1**	RPL8
NM_012456.1	TIMM10	**NM_145871.1**	GSTZ1	**NM_033251.1**	RPL13
NM_012458.2	TIMM13	**NM_004492.1**	GTF2A2	**NM_000985.2**	RPL17
NM_012460.2	TIMM9	**NM_002095.3**	GTF2E2	**NM_000984.3**	RPL23A
NM_013332.1	HIG2	**NM_000858.3**	GUK1	**NM_000992.2**	RPL29
NM_013343.1	LOH3CR2A	**NM_005318.2**	H1F0	**NM_001001.3**	RPL36AL
NM_014041.1	SPCS1	**NM_002106.3**	H2AFZ	**NM_021029.3**	RPL36A
NM_014051.2	TMEM14A	**NM_005324.3**	H3F3B	**NM_001002.3**	RPLP0
NM_014221.1	MTCP1	**NM_005326.3**	HAGH	**NM_002949.2**	MRPL12
NM_014356.2	C6orf123	**NM_005327.1**	HADHSC	**NM_001007.3**	RPS4X
NM_014445.2	SERP1	**NM_005330.3**	HBE1	**NM_001015.3**	RPS11
NM_014624.3	S100A6	**NM_004494.1**	HDGF	**NM_001019.3**	RPS15A
NM_014792.2	KIAA0125	**NM_139011.1**	HFE	**NM_001020.3**	RPS16
NM_016096.2	ZNF706	**NM_005340.3**	HINT1	**NM_001022.3**	RPS19
NM_016305.1	SS18L2	**NM_002118.3**	HLA-DMB	**NM_001023.2**	RPS20
NM_016565.2	CHCHD8	**NM_002128.3**	HMGB1	**NM_001025.3**	RPS23
NM_020142.3	LOC56901	**NM_002129.2**	HMGB2	**NM_002960.1**	S100A3
NM_020179.1	FN5	**NM_004965.6**	HMGN1	**NM_005620.1**	S100A11
NM_020181.1	C14orf162	**NM_002131.2**	HMGA1	**NM_000664.3**	ACACA
NM_020248.1	CTNNBIP1	**NM_173158.1**	NR4A1	**NM_198970.1**	AES

(*Continued*)

S-Table-1. (*Continued*)

GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol
NM_020408.3	C6orf149	**NM_002136.1**	HNRPA1	**NM_001636.1**	SLC25A6
NM_021104.1	RPL41	**NM_031314.1**	HNRPC	**NM_001001787.1**	ATP1B1
NM_021127.1	PMAIP1	**NM_002138.3**	HNRPD	**NM_001687.4**	ATP5D
NM_021177.3	LSM2	**NM_021644.2**	HNRPH3	**NM_001002256.1**	ATP5G3
NM_023937.2	MRPL34	**NM_006896.2**	HOXA7	**NM_004047.2**	ATP6V0B
NM_031286.2	SH3BGRL3	**NM_153715.1**	HOXA10	**NM_198589.1**	BSG
NM_031287.2	SF3B5	**NM_156037.1**	HOXB6	**NM_004927.2**	MRPL49
NM_032412.2	ORF1-FL49	**NM_004502.2**	HOXB7	**NM_006136.2**	CAPZA2
NM_032574.1	LOC84661	**NM_024016.2**	HOXB8	**NM_022845.2**	CBFB
NM_032747.1	USMG5	**NM_014620.2**	HOXC4	**NM_001760.2**	CCND3
NM_052871.2	MGC4677	**NM_153693.1**	HOXC6	**NM_171827.1**	p32/CD8A
NM_052971.1	LEAP-2	**NM_006897.1**	HOXC9	**NM_001773.1**	CD34
NM_080677.1	DYNLL2	**NM_014212.2**	HOXC11	**NM_000611.4**	CD59
NM_138448.2	ACYP2	**NM_024501.1**	HOXD1	**NM_001780.3**	CD63
NM_139286.3	CDC26	**NM_134421.1**	HPCAL1	**NM_004359.1**	CDC34
NM_194327.1	GALIG	**NM_182638.1**	HPS1	**NM_058197.2**	p16/CDKN2A
NM_198835.1	ACACA	**NM_005524.2**	HES1	**NM_001280.1**	CIRBP
NM_020115.3	ACRV1	**NM_198431.1**	HSPA4	**NM_001833.1**	CLTA
NM_001124.1	ADM	**NM_001540.2**	HSPB1	**NM_022645.2**	CSH2
NM_000674.1	ADORA1	**NM_005528.1**	DNAJC4	**NM_012140.3**	SLC25A10
NM_000676.2	ADORA2B	**NM_181353.1**	ID1	**NM_148979.1**	CTSH
NM_001630.1	ANXA8	**NM_002166.4**	ID2	**NM_000396.2**	CTSK
NM_001154.2	ANXA5	**NM_174856.1**	IDH3B	**NM_001336.2**	CTSZ
NM_080649.1	APEX1	**NM_004508.2**	IDI1	**NM_001915.2**	CYB561
NM_000041.2	APOE	**NM_005533.2**	IFI35	**NM_004418.2**	DUSP2
NM_152876.1	FAS	**NM_021068.1**	IFNA4	**NM_004427.2**	PHC2
NM_000486.3	AQP2	**NM_000612.2**	IGF2	**NM_001970.3**	EIF5A
NM_053286.1	AQP6	**NM_001552.1**	IGFBP4	**NM_001419.2**	ELAVL1
NM_001659.1	ARF3	**NM_000576.2**	IL1B	**NM_198194.1**	STOM
NM_001660.2	ARF4	**NM_172200.1**	IL15RA	**NM_202001.1**	ERCC1
NM_001663.2	ARF6	**NM_005536.2**	IMPA1	**NM_023110.1**	FGFR1
NM_001664.2	RHOA	**NM_014214.1**	IMPA2	**NM_201557.1**	FHL2
NM_004040.2	RHOB	**NM_198219.1**	ING1	**NM_004468.3**	FHL3
NM_175744.3	RHOC	**NM_198337.1**	INSIG1	**NM_057092.1**	FKBP2
NM_005168.2	RND3	**NM_002198.1**	IRF1	**NM_016730.1**	FOLR1
NM_001665.2	rho G	**NM_004030.1**	IRF7	**NM_004477.1**	FRG1
NM_004309.3	ARHGDIA	**NM_181493.1**	ITPA	**NM_002032.1**	FTH1
NM_001177.3	ARL1	**NM_002228.3**	JUN	**NM_002035.1**	FVT1
NM_004311.2	ARL3	**NM_002231.2**	CD82	**NM_001487.1**	BLOC1S1
NM_004314.1	ART1	**NM_004137.2**	KCNMB1	**NM_004483.3**	GCSH
NM_032468.2	ASPH	**NM_033360.2**	KRAS	**NM_004124.2**	GMFB
NM_005171.2	ATF1	**NM_002295.2**	RPSA	**NM_000581.2**	GPX1
NM_004024.2	ATF3	**NM_005563.3**	STMN1	**NM_002085.1**	GPX4
NM_001677.3	ATP1B1	**NM_005564.2**	LCN2	**NM_147148.1**	GSTM4
NM_001679.2	ATP1B3	**NM_005566.1**	LDHA	**NM_002095.3**	GTF2E2
NM_001001977.1	ATP5E	**NM_201544.1**	LGALS8	**NM_002107.3**	H3F3A
NM_001002015.1	ATP5F1	**NM_004987.3**	LIMS1	**NM_005342.1**	HMGB3
NM_005175.2	ATP5G1	**NM_005574.2**	LMO2	**NM_002133.1**	HMOX1
NM_001002258.1	ATP5G3	**NM_002346.1**	LY6E	**NM_002134.2**	HMOX2
NM_001003701.1	ATP5J	**NM_002353.1**	TACSTD2	**NM_156036.1**	HOXB6
NM_001694.2	ATP6V0C	**NM_014220.1**	TM4SF1	**NM_024017.3**	HOXB9
NM_001697.2	ATP5O	**NM_002354.1**	TACSTD1	**NM_000194.1**	HPRT1
NM_004322.2	BAD	**NM_030885.2**	MAP4	**NM_005343.2**	HRAS
NM_053056.1	CCND1	**NM_203378.1**	MB	**NM_174856.1**	IDH3B
NM_138578.1	BCL2L1	**NM_002386.2**	MC1R	**NM_000628.3**	IL10RB
NM_004050.2	BCL2L2	**NM_182763.1**	MCL1	**NM_181431.1**	FOXK2

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S-Table-1. (*Continued*)

GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol
NM_000713.1	BLVRB	**NM_012328.1**	DNAJB9	**NM_181468.1**	ITGB4BP
NM_005180.5	PCGF4	**NM_005370.4**	RAB8A	**NM_201543.1**	LGALS8
NM_004331.2	BNIP3L	**NM_177524.1**	MEST	**NM_002359.2**	MAFG
NM_032515.3	BOK	**NM_005371.3**	METTL1	**NM_004528.2**	MGST3
NM_004332.1	BPHL	**NM_017459.1**	MFAP2	**NM_022792.2**	MMP19
NM_007306.1	BRCA1	**NM_145791.1**	MGST1	**NM_002448.1**	MSX1
NM_198590.1	BSG	**NM_002413.3**	MGST2	**NM_005962.3**	MXI1
NM_001207.3	BTF3	**NM_002414.3**	CD99	**NM_079424.1**	MYL6
NM_001731.1	BTG1	**NM_002415.1**	MIF	**NM_032104.1**	PPP1R12B
NM_007311.2	BZRP	**NM_022791.2**	MMP19	**NM_004547.4**	NDUFB4
NM_172369.1	C1QG	**NM_002434.1**	MPG	**NM_182739.1**	NDUFB6
NM_001217.2	CA11	**NM_021126.3**	MPST	**NM_005005.1**	NDUFB9
NM_000387.3	SLC25A20	**NM_012331.2**	MSRA	**NM_020529.1**	NFKBIA
NM_006888.2	CALM1	**NM_002451.3**	MTAP	**NM_002520.4**	NPM1
NM_005184.1	CALM3	**NM_015675.1**	GADD45B	**NM_002607.2**	PDGFA
NM_005185.2	CALML3	**NM_001002841.1**	(MYL4	**NM_005022.2**	PFN1
NM_001745.2	CAMLG	**NM_079423.1**	MYL6	**NM_000942.4**	PPIB
NM_001003962.1	CAPNS1	**NM_002478.3**	MYOD1	**NM_206873.1**	PPP1CA
NM_004346.2	CASP3	**NM_032103.1**	PPP1R12B	**NM_183079.1**	PRNP
NM_001755.2	CBFB	**NM_005594.2**	NACA	**NM_002765.2**	PRPS2
NM_004059.3	CCBL1	**NM_182744.1**	NBL1	**NM_145888.1**	KLK10
NM_001759.2	CCND2	**NM_014222.2**	NDUFA8	**NM_002790.2**	PSMA5
NM_199246.1	CCNG1	**NM_004548.1**	NDUFB10	**NM_152255.1**	PSMA7
NM_001239.2	CCNH	**NM_004549.3**	NDUFC2	**NM_176783.1**	PSME1
NM_001763.1	CD1A	**NM_002496.1**	NDUFS8	**NM_183236.1**	RAB27A
NM_000733.2	CD3E	**NM_181827.1**	NF2	**NM_004583.2**	RAB5C
NM_001769.2	CD9	**NM_001001716.1**	NFKBIB	**NM_133629.1**	RAD51L3
NM_005191.2	CD80	**NM_005008.2**	NHP2L1	**NM_021033.4**	RAP2A
NM_152942.1	TNFRSF8	**NM_198175.1**	NM23A/NME1	**NM_002899.2**	RBP1
NM_001244.2	TNFSF8	**NM_000904.1**	NQO2	**NM_000976.2**	RPL12
NM_001001392.1	CD44	**NM_000270.1**	NP	**NM_001016.2**	RPS12
NM_198793.1	CD47	**NM_199185.1**	NPM1	**NM_000331.2**	SAA1
NM_000560.2	CD53	**NM_006172.1**	NPPA	**NM_005981.3**	TSPAN31
NM_203330.1	CD59	**NM_002524.2**	NRAS	**NM_002970.1**	SAT
NM_004357.3	CD151	**NM_004559.2**	YBX1	**NM_006745.2**	SC4MOL
NM_001786.2	CDC2	**NM_007105.1**	SLC22A18AS	**NM_006746.3**	SCML1
NM_033534.1	CDC2L2	**NM_005602.4**	CLDN11	**NM_001037.3**	SCN1B
NM_052827.1	CDK2	**NM_175568.1**	P2RX4	**NM_003000.1**	SDHB
NM_000075.2	CDK4	**NM_175081.1**	P2RX5	**NM_183352.1**	SEC13L1
NM_078467.1	CDKN1A	**NM_002567.2**	PBP	**NM_014563.2**	TRAPPC2
NM_004064.2	CDKN1B	**NM_002573.2**	PAFAH1B3	**NM_003016.2**	SFRS2
NM_000077.3	CDKN2A	**NM_181696.1**	PRDX1	**NM_152235.1**	SFRS8
NM_078626.1	CDKN2C	**NM_000281.2**	TCF1/PCBD1	**NM_004593.1**	SFRS10
NM_005195.2	CEBPD	**NM_032403.1**	PCDHGC3	**NM_173217.1**	ST6GAL1
NM_001806.2	CEBPG	**NM_002592.2**	PCNA	**NM_170679.1**	SKP1A
NM_001809.2	CENPA	**NM_033023.1**	PDGFA	**NM_005984.1**	SLC25A1
NM_004365.2	CETN3	**NM_002608.1**	PDGFB	**NM_022875.1**	SMN2
NM_005507.2	CFL1	**NM_213612.1**	SLC25A3	**NM_004596.3**	SNRPA
NM_001817.1	CEACAM4	**NM_002642.3**	PIGC	**NM_198216.1**	SNRPB
NM_152253.1	CHKB	**NM_002648.2**	PIM1	**NM_198220.1**	SNRPB2
NM_013324.4	CISH	**NM_006224.2**	PITPNA	**NM_177542.1**	SNRPD2
NM_001281.2	CKAP1	**NM_002653.3**	PITX1	**NM_004175.3**	SNRPD3
NM_001284.2	AP3S1	**NM_000929.1**	PLA2G5	**NM_022807.2**	SNRPN
NM_001288.4	CLIC1	**NM_001005376.1**	PLAUR	**NM_000454.4**	SOD1
NM_001291.2	CLK2	**NM_021910.1**	FXYD3	**NM_006943.2**	SOX12
NM_001293.1	CLNS1A	**NM_021105.1**	PLSCR1	**NM_001047.1**	SRD5A1

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S-Table-1. (*Continued*)

GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol	GenBank Accession No.	Gene Symbol
NM_007097.2	CLTB	**NM_153321.1**	PMP22	**NM_003132.1**	SRM
NM_004368.2	CNN2	**NM_174930.2**	PMS2L5	**NM_003135.1**	SRP19
NM_007310.1	COMT	**NM_001003686.1**	PMS2L3	**NM_003144.2**	SSR1
NM_001300.3	KLF6	**NM_022716.1**	PRRX1	**NM_003155.1**	STC1
NM_001861.2	OX4I1	**NM_002696.1**	POLR2G	**NM_177528.1**	SULT1A2
NM_001305.3	CLDN4	**NM_006232.2**	POLR2H	**NM_004177.3**	STX3A
NM_001306.2	CLDN3	**NM_006233.4**	POLR2I	**NM_004604.3**	STX4A
NM_001307.3	CLDN7	**NM_021129.2**	PPA1	**NM_003164.2**	STX5A
NM_001878.2	CRABP2	**NM_203430.1**	PPIA	**NM_177534.1**	SULT1A1
NM_004379.2	CREB1	**NM_000943.4**	PPIC	**NM_003166.2**	SULT1A3
NM_001310.2	CREBL2	**NM_177951.1**	PPM1A	**NM_181491.1**	SURF5
NM_181571.1	CREM	**NM_177969.1**	PPM1B	**NM_014231.3**	VAMP1
NM_005206.3	CRK	**NM_206877.1**	PPP1CB	**NM_014232.1**	VAMP2
NM_005207.2	CRKL	**NM_002710.1**	PPP1CC	**NM_005638.3**	SYBL1
NM_001889.2	CRYZ	**NM_006241.3**	PPP1R2	**NM_006754.2**	SYPL1
NM_139014.1	MAPK14	**NM_002715.1**	PPP2CA	**NM_003187.3**	TAF9
NM_177436.1	CSE1L	**NM_178002.1**	PR 53/PPP2R4	**NM_005643.2**	TAF11
NM_022644.2	CSH2	**NM_000945.3**	PPP3R1	**NM_172208.1**	TAPBP
NM_177560.2	CSNK2A1	**NM_005399.3**	PRKAB2	**NM_134324.1**	TARBP2
NM_001320.5	CSNK2B	**NM_207578.1**	PRKACB	**NM_201437.1**	TCEA1
NM_001321.1	CSRP2	**NM_212461.1**	PRKAG1	**NM_213648.1**	TCF7
NM_000396.2	CTSK	**NM_138981.1**	MAPK10	**NM_181738.1**	PRDX2
NM_004394.1	DAP	**NM_002756.2**	MAP2K3	**NM_201443.1**	TEAD4
NM_020548.4	DBI	**NM_002764.2**	PRPS1	**NM_003201.1**	TFAM
NM_001924.2	GADD45A	**NM_139277.1**	KLK7	**NM_174886.1**	TGIF
NM_004083.4	DDIT3	**NM_002774.2**	KLK6	**NM_003255.3**	TIMP2
NM_001355.2	DDT	**NM_213633.1**	PSG4	**NM_003270.2**	TSPAN6
NM_030655.2	DDX11	**NM_203287.1**	PSG11	**NM_003271.3**	TSPAN4
NM_213566.1	DFFA	**NM_148976.1**	PSMA1	**NM_021137.3**	TNFAIP1
NM_000791.2	DHFR	**NM_002789.3**	PSMA4	**NM_000363.3**	TNNI3
NM_007326.1	CYB5R3	**NM_002791.1**	PSMA6	**NM_005079.1**	TPD52
NM_138281.1	DLX4	**NM_002794.3**	PSMB2	**NM_003287.2**	TPD52L1
NM_203316.1	DPAGT1	**NM_002801.2**	PSMB10	**NM_199362.1**	TPD52L2

S-Table 2. Sequence variants

		Homozygous alteration			Heterozygous alteration			
GenBank Accession No.	**Gene Symbol**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**
NM_000320	QDPR Leu132Leu	G396A,	rs**2597775**						
NM_000331	SAA1			C209T, Ala70Val	**rs1136743**				
				T224C, Val75Ala	**rs1136747**				
NM_001007	RPS4X Leu164Leu	G492A,	**rs7580**						
NM_001020	RPS16 Gly5Gly	C15T,	**rs17626**						
	T27G, Ser9Ser		**rs17628**						
NM_001047	SRD5A1					A309AG, Pro103Pro	**rs3822430**		
						G348GA, Ala116Ala	**rs8192186**		
NM_001320	CSNK2B Tyr46Tyr	T138C,	**rs14365**						
NM_001636	SLC25A6 Phe136Phe	T408C,	**rs7205**						
NM_001697	ATP50 Gly36Gly	T108C,	**rs17728665**	A218AG, Lys73Arg	*				
NM_001760	CCND3							T775TG, Ser259Ala	**rs1051130**
NM_001817	CEACAM4			T668A, Val223Glu.	*				
NM_001861	COX4I1							G7GA, Ala3Thr	**rs17855751**
NM_001889	CRYZ Gly18Gly	G54A,	**rs4650284**			T138C, Gly46Gly	*		
NM_002131	HMGA1 Ser2Ser	T6C,	*	G49A, Glu17Lys	*				
	G78T, Arg26Arg	*		G112A, Gly38Arg	*				
	C255A,	*		C143T,	*				

(Continued)

Table 2. (*Continued*)

			Homozygous alteration			Heterozygous alteration			
GenBank Accession No.	**Gene Symbol**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**
	Gly85Gly		Pro48Leu C217T,	*					
			Arg73Gly C236T,	*					
			A237G, Pro79Leu G286A,	*					
			Glu96Lys						
NM_002136	HNRPA1 Gly248Gly	C744T,		*					
NM_002414	CD99					C369CT, Ala121Ala	**rs4575010**		
NM_002642	PIGC Gly89Gly	T267C,	**rs2230471**						
NM_002813	PSMD9			T50C, Val17Ala	**rs2230681**				
NM_003144	SSR1			C388T, His130Tyr	*				
NM_003255	TIMP2					G303GA, Ser101Ser	**rs2277698**		
NM_004064	CDKN1B							T326TG, Val109Gly	**rs2066827**
NM_004137	KCNMB1							G193GA, Glu65Lys	**rs11739136**
NM_004175	SNRPD3 Ala101Ala	T303C,	**rs3176991**						
NM_004365	CETN3							G28GC, Val10Leu	**rs4873**
NM_004468	FHL3 Pro180Pro	G540A,	**rs7366048**						
NM_004549	NDUFC2							C136CG, Leu46Val	**rs8875**
NM_005171	ATF1					C327CT, Tyr109Tyr	**rs1129406**		
NM_005191	CD80					G135GA,	**rs2228017**		

(Continued)

Table 2. (*Continued*)

		Homozygous alteration				Heterozygous alteration			
GenBank Accession No.	**Gene Symbol**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**
					Val45Val				
NM_005301	GPR35							G85GA, Ala29Thr.	*
NM_005342	HMGB3 Asn186Lys	C558G,	*					A880AC, Ser294Arg	**rs3749172**
NM_005594	NACA					T543TA, Ile181Ile	**rs4788**		
NM_005984	SLC25A1 Lys277Lys	A831G,	*						
NM_006353	HMGN4 Gly66Gly	G198A	**rs4871**						
NM_006896	HOXA7 Ala32Ala	T96G,	**rs2301720**	G52A, Ala18Thr	**rs2301721**				
NM_007310	COMT His12His	C36T,	**rs4633**	G322A, Val108Met	**rs4680**				
NM_007311	BZRP His53Arg			A158G, Ala68Ala	**rs6971**	G204GA,	**rs6972**		
NM_012328	DNAJB9 Pro61Pro	G183A,	**rs1043615**						
NM_013332	HIG2 Glu28Glu	A84G,	*						
NM_014212	HOXC11 Ser12Ser	T36G,	**rs4759315**						
NM_014232	VAMP2			T346A, Ser116Thr	*				
NM_021068	IFNA4						His49Pro	A146AC,	*
							G178GC, Gly60Arg	*	
							T190TA, Phe64Ile	*	
							G187GC, Glu63Gln		**rs3203576**
NM_024009	GJB3						C357CT,	*	

(Continue)

Table 2. (*Continued*)

		Homozygous alteration				Heterozygous alteration			
GenBank Accession No.	**Gene Symbol**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**	**Synonymous alteration**	**NCBI SNP Database**	**Non-Synonymous alteration**	**NCBI SNP Database**
						Asn119Asn			
NM_033251	RPL13			G334A, Ala112Thr	**rs9930567**				
NM_052871	MGC4677			G28T, Ala10Ser	**rs28673896**	C12CT, Thr4Thr	*	C109CT, Arg37Cys	*
NM_058197	CDKN2A			A160T,			*		
		Arg54Gly							
NM_145888	KLK10	A318C, Gly106Gly	**rs2075688**	T347C, Leu116Pro			*		
		C336G, Thr112Thr	**rs1061368**						
		G423A, Leu141Leu	**rs2075689**						
NM_172200	IL15RA			C248T, Pro83Leu			*		
				A337C, Thr113Pro			*		
NM_172369	C1QG			G644A,			*		
		Gly215Glu							
NM_181571	CREM			T410C,			*		
		Ile137Thr							
NM_198970	TEAD4							C580CT,	*
				Pro194Leu					
NM_201544	LGALS8							A166AG,	**rs1041937**
				Met56Val.					
				G542GC, Gly181Ala	*				

* No report found.