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MASSIVE PARALLEL DNA PYROSEQUENCING ANALYSIS OF THE TUMOR SUPPRESSOR BRG1/SMARCA4 IN LUNG PRIMARY TUMORS

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MUTATION IN BRIEF

HUMAN MUTATION

Massive parallel DNA pyrosequencing analysis of the tumor suppressor BRG1/SMARCA4 in lung primary tumors

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ABSTRACT: The tumor suppressor gene, SMARCA4 (or BRG1), which encodes the ATPase component of the chromatin remodeling complex SWI/SNF, is commonly inactivated by mutations and deletions in lung cancer cell lines. However, SMARCA4 alterations appear to be rare in lung primary tumors. Ultra-deep sequencing technologies provide a promising alternative to achieve a sensitivity superior to that of current sequencing strategies. Here we used ultra-deep pyrosequencing to screen for mutations over the entire SMARCA4 coding region in 12 lung tumors without detectable BRG1 protein. While automatic-fluorescence-based sequencing detected one somatic mutation (p.K586X), the pyrosequencing revealed additional variants, thus increasing the sensitivity. One of the variants, which affected a consensus splice site, was confirmed by individual cloning of PCR products, ruling out the possibility of PCR or pyrosequencing artifacts. This mutation, confirmed to be somatic, was present at a frequency of ten percent, suggesting normal cell contamination in the tumor. Our analysis also allowed us to determine the sensitivity and to identify some limitations of the technology. In conclusion, in addition to cell lines, SMARCA4 is biallelically inactivated in a significant proportion of lung primary tumors, thereby constituting one of the most important genes contributing to the development of this type of cancer. ©2010 Wiley-Liss, Inc.

KEY WORDS: Deep sequencing, SMARCA4, BRG1, lung cancer, tumor suppressor gene, 454-Roche

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INTRODUCTION

The accurate identification of the complete set of genes mutated in cancer is becoming increasingly important. This knowledge will serve not only to guide drug design but also to select patients who will benefit from new targeted therapies. The unavoidable presence of mixed non-malignant cells in primary tumor specimens in conjunction with the relatively low sensitivity of the fluorescence-based Sanger sequencing hamper the detection of point mutations, large deletions and indels [Ahrendt et al., 1999; Blanco et al., 2009]. We previously showed that the frequency of alterations detected in cancer genes in lung cancer cell lines is twice that in lung primary tumors, suggesting the existence of masked gene mutations in the latter [Blanco et al., 2009]. Therefore, if cancer patients are to take full advantage of new targeted treatments, this obstacle requires a prompt solution. The use of high-throughput sequencers may be useful for overcoming this hurdle. These instruments perform massive parallel sequencing and simultaneously analyze samples and sequences [Shendure and Ji, 2008] and so may be able to detect minor gene variants. Massive parallel resequencing is proving to be especially useful for identifying gene variants in viruses of infected patients [Wang et al., 2007]. In cancer, deep resequencing has been used to detect low-prevalence mutations in cancer cells, including drug-resistance mutations from biopsies taken after relapse [Thomas et al., 2006], and to identify subclonal populations in leukemia patients [Campbell et al., 2008].

BRG1 is a central component of the SWI/SNF chromatin remodeling complex with a bromodomain and helicase/ATPase activity [Klochendler-Yeivin et al., 2002]. We reported a high incidence of inactivating mutations at *SMARCA4* (also known as *BRG1*) (MIM#603254), in lung cancer cell lines [Medina et al., 2008]. This is a very large gene that encodes a protein of 1,614 aminoacids. About a quarter of the cell lines screened featured *SMARCA4* inactivation, making it the fifth most frequently mutated gene in non-small cell lung cancer (NSCLC) cell lines, the most common type of lung cancer. However, these observations contrasted with the seemingly low initial rate of inactivating mutations of *SMARCA4* detected in lung primary tumors [Medina et al., 2004]. We hypothesize that many *SMARCA4* mutations went undetected due to normal cell contamination of the tumor specimens. In support of this, two earlier studies used immunohistochemical staining to evaluate the levels of BRG1 in lung primary NSCLCs, and revealed that 30 percent of the tumors had no detectable protein [Reisman et al., 2003 and Fukuoka et al., 2004]. In the present work we used a PCR-based massive parallel pyrosequencing approach to search for mutations at *SMARCA4* in lung primary tumors.

METHODS

Patient selection, tissue microarray construction and immunostaining

Formalin-fixed paraffin-embedded (FFPE) lung primary tumors and matching normal tissues were provided by the CNIO Tumour Bank Network, in collaboration with Spanish hospitals. The study was approved by the institutional review boards and ethics committees and informed consent was obtained from each patient. FFPE tissue blocks from lung primary tumors were used for tissue microarray construction, as previously described [Conde et al., 2006], and further processed for BRG1 immunohistochemical staining using the anti-BRG1 (H88) sc-10768 from Santa Cruz Biotechnology, at (1:15) dilution. The protocols used for immunostaining were those previously described [Conde et al., 2006]. Sections were counterstained with hematoxylin-eosin for quality evaluation. Finally, 12 adenocarcinomas with very low to null immunoreactivity for BRG1 upon double-blind analysis were selected for further studies. For genomic DNA extraction, freshly frozen tissue from tumors was meticulously dissected to ensure enriched material of at least 40% tumor cells. Approximately 10-20- μ m sections were collected from normal and tumor samples and placed in 1% SDS/proteinase K (10 mg/ml) at 58°C overnight. Digested tissue was then subjected to phenol-chloroform extraction and ethanol precipitation following standard procedures.

PCR, vector cloning and Sanger sequencing

All mutations or variations and their predicted effect at the protein level have been named according to the Human Genome Variation Society guidelines (<http://www.hgvs.org>). The A of the ATG translation initiation codon corresponds to position +1 according to the *SMARCA4* mRNA RefSeq EU_430759.1. For mutation screening of the *SMARCA4* gene, exons 2 to 34 (NM_003072.3) were

amplified from 30 ng of genomic DNA extracted from the 12 selected tumors. All PCR reactions were carried out with the proofreading *Phusion* High-Fidelity polymerase (Finnzymes, Finland). In order to suit the read-length requirements for 454 pyrosequencing (250 bp on each reading sense strand), exon 4 was divided into two amplicons. Also, the close proximity of exons 10 and 11 made it appropriate to merge them in a single amplicon. Thus, a total of 33 amplicons were obtained from each tumor sample. The sequences of the amplicons and the primers used are shown in the supplementary methods. The PCR products were sequenced individually by automatic Sanger sequencing and by 454-pyrosequencing (GS FLX System, Roche) (see below). Sanger sequencing of PCR products was carried out using the Big Dye Terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with an ABI PRISM 3700 DNA Analyzer (Applied Biosystems), according to the manufacturer's instructions. All the mutations found were confirmed with independent PCR products. To confirm the variants found by deep sequencing and to compare their frequencies, the purified PCR products were cloned in the pGEM-T Easy Vector System (Promega, Madison, WI), following the manufacturer's instructions. Positive colonies were picked, grown and processed using the NucleoSpin Multi-96 Plus plasmid purification kit (Macherey Nagel, Düren, Germany). The purified products were sequenced using the Big Dye Terminator cycle sequencing kit, as described above, and the resulting sequences were aligned and analyzed using the BioEdit program (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>).

Analysis of loss of heterozygosity

The microsatellite markers selected to analyze loss of heterozygosity (LOH) on chromosome 19p were, from telomere to centromere: D19S886, D19S883, D19S586, D19S583, D19S221, D19S914. The latter four microsatellites flank the *SMARCA4* locus while the former two flank *LKB1*. The PCRs were performed using fluorescent-labeled primers and carried out according to the following protocol: 4 min at 94°C for initial denaturation; 30-32 cycles at 94°C for 30 s, 55°C for 30 s and 72°C for 30 s; then at 72°C for an additional 7 min. PCR products were analyzed in an automated sequencer ABI PRISM 37130X1 (Applied Biosystems) using the GeneMapper 4.0 analytical program. For each microsatellite marker, the normal DNA sample was used to determine the allele size for the corresponding subject. Allele ratios were calculated following the manufacturer's instructions. Since incomplete allelic losses are commonly observed in heterogeneous tumor samples, ratio values ≤ 0.67 or ≥ 1.35 were considered to be unambiguously indicative of LOH. Homozygous cases were considered to be non-informative (NI) for LOH.

454 pyrosequencing

The set of 12 tumor samples was divided into two groups, called LEFT and RIGHT, each containing six tumors. Thus, all amplicons from six tumors were lumped into two pools, the LEFT pool (tumors T01 to T06) and the RIGHT pool (tumors T07 to T12). The PCR products were individually quantified using the Quant-iT PicoGreen dsDNA reagent (Molecular Probes, Invitrogen). Mutation control amplicons were generated using the same primers and added to the amplicon pools. The controls, exon 5 from the cell line NCI-H522 carrying the frameshift mutation c.805_806delCC (generating a premature stop codon) and exon 16 from the cell line NCI-H2126 carrying the nucleotide substitution, c.2990T>A (p.W764R, affecting a highly conserved aminoacid) were amplified, generating the mutant amplicons E5mut and E16mut, respectively. The amplicons were quantified by PicoGreen and spiked into the pools equimolarly according to the following guideline: E16mut was spiked equimolarly into the LEFT pool without dilution and E5mut was mixed with normal E5 to a ratio of 1:50 and spiked equimolarly into the LEFT pool. Similarly, E5mut was spiked equimolarly into the RIGHT pool without dilution and E16mut was mixed with normal E16 to a ratio of 1:50 and spiked equimolarly into the RIGHT pool. The final pools containing all the amplicons from the tumors and the control cell lines were concentrated using Microcon YM-30 centrifugal filters (Millipore) and shipped for library construction and pyrosequencing. The pyrosequencing was conducted on a GS FLX sequencing platform (454 Life Sciences, CT, and Roche Applied Sciences, Indianapolis, IN) according to the manufacturer's

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instructions and using the suggested reagents. To this end, the amplicon pools were treated as fragmented genomic DNA. Briefly, a DNA library was prepared from the PCR fragments upon addition of adaptor sequences A and B (Digitags), which were captured and purified by streptavidin-biotin binding. Single-stranded template DNA (sstDNA) was then generated and the necessary amount of sstDNA molecules per bead was determined by a titration run. Single PCR amplicon molecules captured on individual beads were amplified in a second emulsion PCR (emPCR) process using universal primers that yields about 10^7 copies of the input DNA molecule. The emulsion was then disrupted; the beads were isolated and loaded onto picotiter plates. Sequencing reactions were carried out by synthesis using ultra-deep pyrosequencing (Margulies et al., 2005) on a Genome Sequencer FLX instrument located at the Life Sequencing facility (Valencia, Spain) (www.lifesequencing.com), which provides a maximum of 100 million bases distributed in 400,000 high-quality reads.

Deep sequencing data analysis

To verify the sequencing process quality, the original 454 reads were queried (Tera-BLASTN, Decypher v7.6.2.1) against a custom database of contaminating sequences. Reads that matched with external organisms (significance threshold=0.01) were excluded from subsequent analyses. The GS Amplicon Variant Analyzer (AVA) v1.1.03 (454 Life Sciences) was used to align the deep sequencing reads against a reference made up of the sequences of all the amplicons (NM_003072.3) using the algorithm's default parameters. A total of 12 flowgrams (SFF format) were used as input. AVA software automatically trimmed the primers used to prepare the amplicon library where appropriate, combined the LEFT and RIGHT reads, and performed the alignments taking into account the base call qualities. We chose not to apply any filter value to the frequency of the reported variants to increase the number of candidates. Finally, the candidate changes were manually curated following the instructions in the program manual, taking into consideration coverage, bidirectional support for the variants, presence of homopolymers at the vicinity of the variant position and variant position in the read. All putative nucleotide changes with a frequency lower than the average noise level in the variation frequency plot were not considered. We also manually discarded variants with very low frequency (less than 1%) that showed no other convincing evidence of relevance. Finally, the flowgrams for the remaining variations were visually inspected to assess the fidelity of the change.

RESULTS**BRG1 immunostaining and fluorescence-based automatic sequencing in lung primary tumors**

We performed immunostaining for BRG1 in a panel of 122 lung primary tumors and found 46 (37%) with very low to null levels of BRG1 that were considered negative for BRG1 protein immunostaining (Fig. 1A). These tumors were candidates for the presence of a biallelic inactivating mutation and/or deletion. The frequency of negative BRG1 immunostaining distributed similarly between the two main histopathological types of lung cancer (adenocarcinomas and squamous cell carcinomas). Subsequently, we selected 12 of the lung tumors with negative BRG1 immunostaining to screen for mutations at each of the coding exons and at the intron-exon boundaries using fluorescence-based automatic Sanger sequencing. In addition to negative for BRG1 immunostaining the tumors were selected on the basis of availability for matched normal DNA. We found a single mutation at exon 10, an A>T nucleotide substitution predicting a change from the lysine at position 586 to a stop codon (Fig. 1B). Several known single nucleotide polymorphisms (SNPs) were also observed (Table 1).

In addition, we analyzed loss of heterozygosity (LOH) using six microsatellite markers spanning 10 Mb in the short arm of chromosome 19p. Four of them surrounded the *SMARCA4* locus and two were close to the *STK11* locus, another tumor suppressor gene frequently inactivated in lung cancer. The results are shown in Figure 1C. Information about gene alterations in other known cancer genes i.e. *TP53* (MIM#[191170](#)), *STK11* (MIM#[602216](#)), *KRAS* (MIM#[190070](#)) and *EGFR* (MIM#[131550](#)), is also provided for these tumors. Six (50%) of the tumors had clear LOH in the markers near *SMARCA4*, and

also had mutations at one or more cancer genes, indicating that normal cell contamination was low in these specimens. Three of the tumors, negative for LOH, exhibited allele ratios close to the cut-off values (data not shown) and no mutations at other genes, implying a masking effect due to normal cell contamination or to tumor heterogeneity. The remaining tumors did not feature LOH, but were positive for mutations at other genes. Clear LOH surrounding the *SMARCA4* locus was found in the *SMARCA4*-mutant tumor identified by Sanger sequencing (Fig. 1C). This indicates the loss of the remaining normal allele and so the homozygous nature of the mutation, thus supporting the two-hit hypothesis [Knudson 1971].

Figure 1. *SMARCA4* alterations in lung cancer. (A) Representative examples of BRG1 positive and negative immunostaining in lung cancer cell lines (a, cell line NCI-H23 harboring the nonsense mutation E1533X; b, wild type cell line NCI-H1395) and lung primary tumors (c and d, primary tumors negatively and positively stained for BRG1, respectively). Original magnification, 400x. (B) Example of the nonsense mutation (p.K586X) in a lung primary tumor (T01) compared with matched normal tissue (N) as detected by Sanger sequencing. (C) Loss of heterozygosity (LOH) on chromosome arm 19p was analyzed using the indicated microsatellite markers flanking *SMARCA4* (band 19p13.2) and *STK11* (band 19p13.3) loci in the panel of lung primary tumors used in the study. Mutation status of those tumors for other genes is also indicated. NI, non-informative. On the right are examples of the LOH analysis using the indicated microsatellites and tumor samples. The asterisk indicates the LOH.

Deep resequencing of the coding region of *SMARCA4* in lung primary tumors

We have previously reported that lung cancer cell lines bear about twice as many mutations at any known cancer gene as do lung primary tumors [Blanco et al., 2009]. The low frequency of mutations at *SMARCA4* in lung primary tumors compared with lung cancer cell lines is probably a consequence of normal cell contamination in conjunction with the low sensitivity of current sequencing approaches. To investigate the possible presence of mutations that had not been detected in the automatic Sanger sequencing analysis, we took a deep-resequencing approach using the 454-FLXs machine (Roche Diagnostics). *SMARCA4* is a very large gene, with an open reading frame of 4,845 bp distributed among 33 coding exons. We amplified each of these exons using a high-fidelity proofreading polymerase, thereby obtaining 33 different amplicons. Due to the large number of amplicons we avoided using barcodes in the primers to identify individual tumors. Instead, we pooled the amplicons to construct two independent libraries for massive parallel sequencing (see methods for details).

Figure 2. Workflow and 454 output. (A) Intron-primed, exon wise high-fidelity PCR amplification of *SMARCA4* from genomic DNA obtained from 12 selected BRG1-negative lung primary tumors (T01 to T12). PCR products were separated in agarose gels and the specific bands were cut out and purified. The purified amplicons from all of the tumors were quantified and mixed equimolarly in two independent pools (LEFT and RIGHT, containing all *SMARCA4* exons of tumors T01 to T06, and tumors T07 to T12, respectively). Each pool was spiked with control amplicons (see methods for details). The panel at the bottom shows an agarose gel separation of the final concentrated pools. (B) Plot representing the total number of reads in forward and reverse, for each of the exons within each pool of amplicons generated by 454 deep sequencing.

Each of the two pools contained the entire coding sequence and the intron-exon boundaries of the *SMARCA4* gene (8,351-bp; Supp. Methods) from six separate tumor samples. To measure the accuracy and sensitivity of the assay we included two control amplicons consisting of two known mutations from lung cancer cell lines (c.805_806delCC from NCI-H522 and c.2290T>A from NCI-H2126) previously identified in our laboratory [Medina et al., 2008]. The amplicons containing each mutation were included in the pools without prior dilution or diluted at 1:50 with normal blood DNA. A schematic representation of the approach and the length of each amplicon are depicted in Figure 2A and in the Supplementary Methods, respectively. Our approach was designed to provide 1,000 reads per individual amplicon.

The analysis yielded a total of 346,951 sequences, of which 260,000 (75%) mapped to the reference sequence of *SMARCA4* (127,247 for LEFT and 139,753 for RIGHT). The rest were products unrelated to *SMARCA4*, probably non-specifically amplified during the PCR process. About 50.5% of the reads were on forward and 49.5% were on reverse sense strands. The number of reads per amplicon in each pool was highly variable, ranging from about 130 to 37,000 (Fig. 2B and Supp. Table S1). As depicted, amplicons 2, 6 and 26 had the fewest reads (ranging from 128 to 354) followed by amplicons 4B, 8, 10-11, 15 and 19, with 283 to 618 reads. More than half of the amplicons (about eighteen) gave over 1,000 reads (six had more than 5,000) per amplicon in each pool, which ensures a sensitivity of 5% and 1%, respectively, according to the manufacture's indications for single base changes and multibase deletions.

SMARCA4 mutations and other variants identified by ultra-deep resequencing analysis

All the variants detected by Sanger sequencing were called in the 454-pyrosequencing data, including all the germ-line SNPs and a nonsense mutation (c.1756A>T) (Table 1). The controls included in the analysis without prior dilution were also evident in their respective pools. In addition to known variants, there were many small insertions and deletions, especially in homopolymeric tracts. These probably correspond to artifacts that arose during pyrosequencing; difficulties in resolving this type of sequence are well known [Campbell et al., 2008]. Other changes, mainly nucleotide substitutions, were also evident and could have been artifacts arising during the PCR/sequencing processes or true mutations.

The control mutations and the SNPs in the study allowed us to calculate the approximate sensitivity of the pyrosequencing and the distribution of the PCR products from the distinct individuals. The c.2290T>A mutation control was detected in the LEFT pool at a combined frequency of 7%, which was within the range of expected frequencies (7-14%) (Supp. Table S2). The same mutation in the RIGHT pool had an expected frequency of 0.15-0.3%, so although the observed frequency (0.14%) was very close, it was below the lower cut-off value to qualify as a mutation. Likewise, the c.805_806delCC was found at 5.3% in the RIGHT pool, close to the range of expected frequencies (7-14%) while it was not found (0%) in the LEFT pool. The observed frequencies of SNPs were close to the expected value, according to the observations from the Sanger sequencing (Supp. Table S2). Taken together, these observations imply that while the pool contains an unequal distribution of the various amplicons, the distribution of the same exons among different individuals is very similar.

We established the following arbitrary criteria to select those changes that were candidates for tumor-associated mutations: i) changes with a frequency greater than or equal to 0.5, 1 or 5%, depending on the number of reads for a given amplicon (i.e., 0.5% for amplicons with ≥15,000 reads, 1% for amplicons with ≥300 reads and 5% for amplicons with <300 reads). These thresholds were only valid for nucleotide substitutions in non-homopolymeric regions; ii) changes that appeared in both the forward and reverse reads; and iii) changes that appeared in only one of the tumor pools. All the changes that fulfilled these criteria are listed in Table 1; the variants included known polymorphisms, the mutation controls and the mutation detected by Sanger sequencing. The remaining changes were considered to be candidate mutations, some of which were explored further.

Conventional cloning of PCR products and subsequent Sanger sequencing confirmed some variants found in the 454-pyrosequencing

To determine whether the alterations identified were true mutations or artifacts arising during the PCR or the pyrosequencing process we selected some of the candidate mutations (Table 1) and subcloned the amplicons for each of the tumors within a given pool. We then performed Sanger sequencing in no fewer than 80 clones per tumor. When an alteration was confirmed, we independently repeated the PCR, in the specific tumor DNA, and subcloned again to verify the presence of the mutations and to discard any PCR errors.

Table 1. List of all the variants fulfilling our criteria, detected at *SMARCA4* using 454-pyrosequencing. The location according to mRNA Ref. Seq. EU430759.1 and the predicted change in the protein are indicated. SNPs and control mutations are also included. *Total reads* refers to the total number of reads for the indicated position in the LEFT and the RIGHT pools. % *variants* refers to the percentage of variants detected with respect to the total number of reads at that position.

Exon/Intron	LEFT		RIGHT		Nucleotide Change	Predicted effect	Status	Detected Sanger
	% variants ¹	Total reads	% variants ¹	Total reads				
I2	22.8	394	0	321	c.223-53T>C	None	SNP	yes
I3	15.7	708	0	772	c.356-14A>G	None	SNP	yes
E5	0	1162	5.3	1119	c.805_806delCC	p.P270fs	Control mutation	NA
E8	19	279	0.9	224	c.1413G>A	p.K471K	SNP	yes
E9	16.6	7389	0.04	4918	c.1509A>G	p.A503A	SNP	yes
E9	48.3	7354	20	4919	c.1524T>C	p.H508H	SNP	yes
E10	6.4	373	0	317	c.1756A>T	p.K586X	Somatic mutation	yes
I10	12.3	381	0	312	c.1761+29G>A	None	SNP	yes
I10	46	287	13	177	c.1762-37T>C	None	SNP	yes
I14	22	578	0	655	c.2123+12C>T	None	SNP	yes
E15	1.06	378	0	286	c.2141T>C	p.V714A	Candidate mutation	no
E16	7	711	0.14	732	c.2290T>A	p.W764R	Control mutation	NA
E16	1.4	733	0	733	c.2315A>G	p.N772S	Candidate mutation	no
I17	0.01	18291	1.8	28664	c.2505+2T>C	Abnormal splicing	Candidate mutation	no
E28	0.5	905	16	493	c.3954C>T	p.D1318D	SNP	yes
E29	0	478	1.2	493	c.4192C>A	p.R1398S	Candidate mutation	no
E31	6	634	0	588	c.4485C>T	p.D1495D	SNP	yes
E33	59	1341	20.5	919	c.4788T>C	p.D1596D	SNP	yes

SNP, single nucleotide polymorphism previously reported in [Medina et al., 2008] or unpublished observations. ¹Only changes found in a frequency between 0.5-5%, depending on the number of reads for a given amplicon (i.e., 0.5% for amplicons with $\geq 15,000$ reads; 1% for amplicons with ≥ 300 reads and 5% for amplicons with less than 300 reads). These thresholds were only used for nucleotide substitutions outside repetitive sequences. Small indels were not considered in our analysis.

The following changes were chosen for verification: c.2505+2T>C, c.2141T>C and c.4192C>A. Overall, we individually cloned 18 amplicons and obtained about 1,500 individual sequences. Two of the variants were confirmed in the analysis and, therefore, assigned to a given tumor. However, one of them (c.2141T>C) was not found in any of the tumors during the conventional cloning of the PCR products. We cannot rule out the possibility that this is a true inactivating mutation with a frequency below the threshold of detection of the assay. On the other hand, the other two mutations, c.2505+2T>C and c.4192C>A, were confirmed in the first cloning but only c.2505+2T>C was verified in the second, independent subcloning. This suggests that c.4192C>A arose during the PCR. Table 2 summarizes the results and Figure 3 depicts the analysis of the c.2505+2T>C mutation detected by pyrosequencing and verified by individual cloning of the PCR products (Figs. 3A-B).

Table 2. Selected candidate *SMARCA4* mutations were tested for verification by conventional cloning of PCR products in each of the tumors in the assigned pool. At least 80 clones per PCR product were sequenced. The first verification was performed with the same PCR product as used in the deep sequencing while the second is an independent PCR product.

Nucleotide change	Observed frequency in pool	Expected frequency per tumor	First verification	Second verification	Sample ID	LOH	Diagnostic
c.2141T>C	1.06%	6.36%	No	NA	NA	NA	Unknown
c.2505+2T>C	1.8%	11%	Yes (9/90, 10%)	Yes	T08	Yes	Somatic mutation
c.4192C>A	1.2%	7.2%	Yes (4/88, 4.5%)	No (0/160)	T11	Yes	PCR error

NA, not applicable, the mutation could not be confirmed in the first verification; LOH, loss of heterozygosity.

This mutation was confirmed to be present at a frequency of about 11%, which is below the reported threshold for mutation detection by fluorescence Sanger sequencing. The tumor carrying the mutation (T08) was negative for mutations at other genes (Fig. 1C) and had no clear LOH, suggesting more than 75% normal cell contamination. This mutation was not present in the matched normal DNA, thus indicating a tumor specific alteration.

Figure 3. Analysis of a nucleotide substitution found by pyrosequencing. (A) Variation plot analysis of *SMARCA4* amplicon 17, where a substitution mutation at the donor splice site of intron 17 (c.2405+2T>C) was detected at a relative allele frequency of approximately 2% (arrow). (B) Sequencing and alignment of individual bacterial clones transformed with amplicon 17 assigned the mutation to T08 and provided information about its relative frequency. (C) chromatograms confirming the c.2405+2T>C mutation during the cloning of an independent PCR product.

DISCUSSION

BRG1 is a catalytic component of the SWI/SNF chromatin-remodeling complex and regulates gene expression by disrupting histone-DNA contacts in an ATP-dependent manner [Roberts and Orkin 2004]. Several lines of evidence attest to the importance of BRG1 in tumor development. *Brg1*-heterozygous mice have a marked predisposition to develop tumors [Bultman et al., 2000], BRG1 induces cell-growth arrest after ectopic expression in BRG1-deficient tumor cells alone or after co-expression with p16 or RB [Reisman et al., 2002; Kang et al., 2004] and it binds to significant tumor suppressor proteins, including BRCA2 [Bochar et al., 2000]. More recently, *SMARCA4* germ-line mutations have been associated with a cancer prone disease, the rhabdoid tumor predisposition syndrome [Schneppenheim et al., 2010], further demonstrating that *SMARCA4* is a *bona fide* tumor suppressor. Wong et al. reported the presence of *SMARCA4* homozygous intragenic deletions and a concomitant absence of protein in some cancer cells lines [Wong et al., 2000] and we also found that 30 percent of the lung cancer cell lines carry a *SMARCA4* biallelic inactivation. In spite of the high frequency of *SMARCA4*-inactivating mutations in lung cancer cell lines, in lung primary tumors we reported a rare presence of *SMARCA4* mutations [Medina et al., 2004]. Detection of somatic mutations in primary tumors has mostly been based on fluorescence-

based Sanger sequencing but the sensitivity of this approach is clearly poor (it rarely detects variants frequencies of <20%) [Wang et al., 2007].

Here we show that massive parallel sequencing increases the rate of mutation detection in *SMARCA4* in lung primary tumors by at least eight percent relative to automatic fluorescence-based methods. Consistent with our present observations, Thomas and collaborators used deep pyrosequencing to screen for *EGFR* mutations in lung primary tumors and reported a 15 percent increase in the number of mutations compared with those identified by Sanger sequencing [Thomas et al., 2006]. They also detected the presence of mutations in low tumor content lung cancer biopsies, detecting a mutation with a prevalence of two percent in approximately 137,000 reads. This implies that, despite selecting specimens for high tumor cell content, normal cell contamination hinders mutation detection. In our study we selected specimens with a tumor cell content of over 40%. However, tumor cell content evaluation performed microscopically cannot be extrapolated to the tumor cell content in the same specimen subjected to DNA and RNA extraction.

Overall we found inactivating and biallelic mutations at *SMARCA4* in our selected group of lung primary tumors, confirming that *SMARCA4* is a major tumor suppressor gene in lung cancer. These are likely inactivating mutations because predict truncated proteins and lead to the absence of BRG1 protein, undetectable by immunostaining. Nevertheless, we expect to have underestimated the frequency of *SMARCA4* gene alterations. Large intragenic deletions are undetectable by this approach but account for about 20% of the alterations at *SMARCA4* in lung cancer cell lines [Medina et al., 2008]. Moreover, mutations in homopolymeric tracts, a type of alteration commonly found in lung cancer cell lines, were excluded from our analysis because they are highly prone to pyrosequencing artifacts [Campbell et al., 2008]. This type of alteration may also have been missed. Another limitation of our approach is the unequal depth of coverage among different exons. This is a general problem when pooling different amplicons for deep sequencing, as noted by others [Qin et al., 2010; Rohlin et al., 2009], and is attributable to amplification bias affecting product yield. These drawbacks need to be carefully considered when using pyrosequencing to search for mutations, especially in tumor suppressor genes, since these are often altered through large deletions and indels within repetitive sequences. An additional limitation to increased sensitivity is the generation of mutations by DNA polymerases during the PCR [Campbell et al., 2008], although this could be solved using exon-capturing approaches that avoid prior PCR. Exon-capturing has other advantages such as its amenability to simultaneously selecting multiple exons of various genes, which facilitates rapid screening for mutations [Choi et al., 2009; Lalonde et al., 2010]. Even so, these new strategies will not serve to detect the presence of large intragenic deletions in tumor suppressor genes, for which additional screening methods will be required.

In the present analysis we performed deep sequencing of a pool of PCR products from 400 individual amplicons containing the entire coding region of *SMARCA4*. The enhanced efficacy of pooling PCR products over prior pooling of genomic DNA was demonstrated by Out and collaborators [Out et al., 2009], who compared the reliability of deep sequencing after pooling 287 genomic DNAs from tumor samples and subsequently performed long-range PCR of the pooled sample and compared the results with those from a PCR of 88 individual samples whose products were then pooled. They observed that the correlations of the allelic frequencies with the results of the Sanger sequencing were significantly weaker in the former, probably due to differences in the integrity of the genomic DNA in the individual tumor samples. Here we found that the control mutations and the SNPs were within the range of expected frequencies, indicating that the identical PCR products from different individuals were similarly distributed in the pool. This indicates that within the conditions of our experimental design, the pooling of identical amplicons does not affect the sensitivity of the approach, as compared to the use of bar-coded amplicons. As well as identifying novel mutations undetected by Sanger sequencing, pyrosequencing analysis was able to identify all the variants detected by fluorescence Sanger sequencing, including the germ-line polymorphisms and the mutation controls.

In conclusion, the present work is one of only few to report the use of deep sequencing to search for low-prevalence mutations in human cancer. We demonstrated that, like in lung cancer cell lines, homozygous *SMARCA4* mutations are present in lung primary tumors. The use of massive parallel pyrosequencing was more sensitive for detecting mutations than fluorescence Sanger sequencing and is, therefore, a promising tool for mutation screening in mixed populations.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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Supplementary Table S1. Read number in forward and reverse for each amplicon within each pool provided by the 454 sequencing platform. The expected number of reads per amplicon per sample is also provided

Amplicon	LEFT			RIGHT			Expected No. reads per sample	
	FORWARD	REVERSE	TOTAL	FORWARD	REVERSE	TOTAL	LEFT	RIGHT
E-2	100	125	225	54	74	128	38	21
E-3	394	364	758	321	266	587	126	98
E-4A	569	458	1027	465	390	855	171	143
E-4B	355	260	615	154	129	283	103	47
E-5	858	441	1299	632	543	1175	186	168
E-6	157	197	354	50	94	144	59	24
E-7	412	302	714	431	434	865	119	144
E-8	243	226	469	150	177	327	78	55
E-9	3763	4560	8323	1716	3598	5314	1387	886
E-10/11	285	333	618	164	204	368	103	61
E-12	517	509	1026	675	648	1323	171	221
E-13	12046	10157	22203	19309	18065	37374	3701	6229
E-14	378	234	612	348	328	676	102	113
E-15	378	0	378	288	4	292	63	49
E-16	37	864	901	7	799	806	129	115
E-17	5708	13089	18797	9477	19260	28737	3133	4790
E-18	878	786	1664	1336	1141	2477	277	413
E-19	454	0	454	284	0	284	76	47
E-20	3636	4697	8333	5042	4048	9090	1389	1515
E-21	562	540	1102	468	715	1183	184	197
E-22	515	531	1046	613	567	1180	174	197
E-23	2837	3638	6475	3125	6144	9269	1079	1545
E-24	25964	12944	38908	17911	9776	27687	6485	4615
E-25	544	653	1197	724	713	1437	200	240
E-26	105	100	205	72	90	162	34	27
E-27	775	745	1520	743	885	1628	253	271
E-28	530	491	1021	437	94	531	170	89
E-29	466	439	905	413	156	569	151	95
E-30	501	529	1030	603	645	1248	172	208
E-31	343	320	663	285	320	605	111	101
E-32	933	1178	2111	788	756	1544	352	257
E-33	895	869	1764	647	385	1032	294	172
E-34	536	0	536	580	0	580	89	97
Total	66674	60579	127253	68312	71448	139760	21209	23293

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Supplementary Table S2. Comparison of observed frequencies, obtained by deep sequencing, of SNPs and control mutations and their respective expected frequencies, according to observations from Sanger sequencing. Since tumor samples may have different amounts of normal cell contamination, aneuploidy and LOH, the exact expected frequency for each SNP and mutation control cannot be calculated. Thus, we have calculated the maximum (all samples have LOH, except tumors homozygous for a given SNP) and minimum (no samples have LOH, except the tumors homozygous for the SNP) expected frequencies.

Nucleotide change	Status	Left Pool					Right Pool				
		Tumors with variants (Sanger)	MEF	MAF	% variants	Total reads	Tumors with variants (Sanger)	MEF	MAF	% variants	Total reads
c.222-53T>C	SNP	T4 ¹ , T5 ¹	20	50	22.8	394	-	0	0	0	321
c.356-14A>G	SNP	T4, T5	17	33.3	15.7	708	-	0	0	0	772
c.805_806delCC	Control mutation	H522 cells (1/50)	0.15	0.3	0	1162	H522 cells	7	14	5.3	1119
c.1413G>A	SNP	T4, T5	17	33.3	19	279	-	0	0	0.9	224
c.1509A>G	SNP	T4, T5	17	33.3	16.6	7389	-	0	0	0.04	4918
c.1524T>C	SNP	T1, T2 ¹ , T4, T5 ¹	40	75	48.3	7354	T9, T11 ¹	17	33.3	20	4919
c.1761+29G>A	SNP	T4, T5	17	33.3	12.3	381	-	0	0	0	312
c.1762-37T>C	SNP	T1, T2 ¹ , T4, T5 ¹	40	71	46	287	T11 ¹	9	29	13	177
c.2123+12C>T	SNP	T4, T5	17	33.3	22	578	-	0	0	0	655
c.2290T>A	Control mutation	H2126 cells	7	14	7	711	H2126 cells (1/50)	0.15	0.3	0.14	732
c.3954C>T	SNP	-	0	0	0.5	905	T7	8.3	27	16	493
c.4485C>T	SNP	T1	8.3	17	6	634	-	0	0	0	588
c.4788T>C	SNP	T1, T2, T4, T5 ¹ , T6	42	85	59	1341	T9 ¹ , T11	18	43	20.5	919

¹Indicates that the tumor is homozygous for the polymorphism. Given the presence of normal cell contamination in all tumor specimens, the homozygous or heterozygous nature of a SNP can be determined. T, Tumor identification. MEF, Minimum expected frequency; MAF, Maximum expected frequency.

Supplementary Methods 1. List of *SMARCA4* amplicons obtained by intron-primed high-fidelity PCR of tumor genomic DNA. Amplicon names are designated by the name of the exons (E, exon number), and their size is indicated in base pairs (bp). Letters in bold indicate the priming sequences used for amplification. Exon 4 was split into two amplicons, A and B; exons 10 and 11 were amplified together.

E2		325 bp				
1	ccagactgac	caggactgtc	ttccagCAGG	AGGCCACTGT	CTGCAGCTCC	
51	CGTGAAGATG	TCCACTCCAG	ACCCACCCCT	GGGCGGAAC	CCTCGGCCAG	
101	GTCCTTCCCC	GGGCCCTGGC	CCTTCCCCTG	GAGCCATGCT	GGGCCCTAGC	
151	CCGGGTCCCT	CGCCGGGCTC	CGCCACAGC	ATGATGGGGC	CCAGCCCAGG	
201	GCCGCCCTCA	GCAGGACACC	CCATCCCCAC	CCAGGGGCCT	GGAGGGTACC	
251	CTCAGGACAA	CATGCACCAG	ATGCACAAGg	tagggatccc	tgtgcccgcc	
301	tcgcac ctgc	ggcctctgcc	cacta			
E3		304 bp				
1	tcgagcttct	ctcgggcagc	gcatagctgc	gctgccacct	cacgttccac	
51	atgctgaccc	tgccttgcca	tggtccctct	cgcagCCCAT	GGAGTCCATG	
101	CATGAGAAGG	GCATGTCGGA	CGACCCGCGC	TACAACCAGA	TGAAAGGAAT	
151	GGGGATGCGG	TCAGGGGGCC	ATGCTGGGAT	GGGGCCCCCG	CCCAGCCCCA	
201	TGGACCAGCA	CTCCAAGgt	acagaactgc	gttccttctc	gccttgtgtt	
251	tgtcatactc	cagagtctct	agatcatttt	cctc ccctgg	gttcccacag	
301	gatg					
E4A		261 bp				
1	atgctgccga	gtgaccagt	ggctgacctt	tctctgcagG	TTACCCCTCG	
51	CCCCCGGGTG	GCTCTGAGCA	TGCCTCTAGT	CCAGTTCCAG	CCAGTGGCCC	
101	GTCTTCGGGG	CCCCAGATGT	CTTCCGGGGC	AGGAGGTGCC	CCGCTGGATG	
151	GTGCTGACCC	CCAGGCCTTG	GGGCAGCAGA	ACCGGGGCCC	AACCCCATTT	
201	AACCAGAAC	AGCTGCACCA	GCTCAGAGCT	CAGATCATGG	CCTACAAGAT	
251	GCTGGCCAGG	G				
E4B		266 bp				
1	AGCTCAGAGC	TCAGATCATG	GCCTACAAGA	TGCTGGCCAG	GGGGCAGCCC	
51	CTCCCCGACC	ACCTGCAGAT	GGCGGTGCAG	GGCAAGCGGC	CGATGCCCGG	
101	GATGCAGCAG	CAGATGCCAA	CGTACCTCC	ACCCTCGGTG	TCCGCAACAG	
151	GACCCGGCCC	TGGCCCTGGC	CCTGGCCCCG	GCCCGGGTCC	CGGCCCGGCA	
201	CCTCCAAATT	ACAGCAGGCC	TCATGgtaag	actggctgcc	ctggcc ctca	
251	ggtgtctcag	agcgaa				
E5		183 bp				
1	ctgggcgcag	gcataaacct	gggacgcact	gttttctctt	ttgtttctcc	
51	ctacatgtag	GTATGGGAGG	GCCCAACATG	CCTCCCCCAG	GACCCTCGGG	
101	CGTGCCCCCC	GGGATGCCAG	GCCAGCCTCC	TGGAGGGCCT	CCCAAGCCCT	
151	GGCCTGAagg	tga gctccct	cttctatggt	ggt		
E6		381 bp				
1	gcagctcagc	agctttccat	ttccagccc	ggatggggccc	cagagctcaa	
51	catgacgccc	tggccccttg	ccttctccca	gGACCCATGG	CGAATGCTGC	
101	TGCCCCCAGC	AGCACCCCTC	AGAAGCTGAT	TCCCCCGCAG	CCAACGGGCC	
151	GCCCTTCCCT	CGCGCCCCCT	GCCGTCCCAC	CCGCCGCCTC	GCCCGTGTATG	
201	CCACCGCAGA	CCCAGTCCCC	CGGGCAGCCG	GCCCAGCCCG	CGCCCATGGT	
251	GCCACTGCAC	CAGAAGCAGA	GCCGCATCAC	CCCCATCCAG	AAGCCGCGGG	
301	GCCTCGACCC	TGTGGAGATC	CTGCAGGAGC	GCGAGTACAG	gtgagggcgg	
351	ggccca gttg	ccaaggtcac	tgccctgtgt	c		
E7		230 bp				
1	tgccctggca	accctctcac	cgcctttgct	ccttgtctct	gtccccaggC	
51	TGCAGGCTCG	CATCGCACAC	CGAATTCAGG	AACTTGAAAA	CCTTCCCGGG	
101	TCCCTGGCCG	GGGATTGCG	AACCAAAGCG	ACCATTGAGC	TCAAGGCCCT	
151	CAGGCTGCTG	AACTTCCAGA	GGCAGgtggg	tgctggcatg	gccgcagctt	

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201 tccgaaaagg **gcctttgtca ccaacactgc**

E8 281 bp

1 **tgccacagag ctgtgcagtg** cgcgggcttg tctctctccc tcttacagCT
 51 GCGCCAGGAG GTGGTGGTGT GCATGCGGAG GGACACAGCG CTGGAGACAG
 101 CCCTCAATGC TAAGGCCTAC AAGCGCAGCA AGCGCCAGTC CCTGCGCGAG
 151 GCGCCATCA CTGAGAAGCT GGAGAAGCAG CAGAAGATCG AGCAGGAGCG
 201 CAAGCGCCGG CAGAAGCACC AGgtacgtc cggtagggccc aaggccctgc
 251 agccccgcca **cctggctgccc tggcttgtcc a**

E9 270 bp

1 **agatgtgtcc accatgctgc** tgaagggtca gccttctctt ttgtgctttc
 51 ctgcaggAAT ACCTCAATAG CATTCTCCAG CATGCCAAGG ATTTCAAGGA
 101 ATATCACAGA TCCGTCACAG GCAAAATCCA GAAGCTGACC AAGGCAGTGG
 151 CCACGTACCA TGCCAACACG GAGCGGGAGC AGAAGAAAGA GAACGAGCGG
 201 ATCGAGAAGG AGCGCATGCG GAGGCTCATG gtatggctct gccttcttga
 251 **cgtgcgctct tctacatgtg**

E10/11 409 bp

1 **tgtgccacca cattgcagta acccccatgc** tttttagtagGC TGAAGATGAG
 51 GAGGGGTACC GCAAGCTCAT CGACCAGGAA GAAGGACAAG CGCCTGGCCT
 101 ACCTCTTGCA GCAGACAGAC GAGTACGTGG CTAACCTCAC GGAGCTGGTG
 151 CGGCAGCACA AGGCTGCCCA GGTCGCCAAG GAGAAAAAGA AGAAAAAGAA
 201 AAAGAAAGtg tgctgggctt ggcattggtgc ccgcccgggg tgggatggga
 251 gcagccgtct tcacgtgtgt ggctcagcc ttgtgggtca gggcctgacc
 301 gtgtctctct ctatttccag AAGGCAGAAA ATGCAGAAGG ACAGACGCCT
 351 GCCATTGGGC CGGATGCCGA Ggtgaggaag cagggtttct **tgtggaagta**
 401 **tcaagctag**

E12 201 bp

1 **cagtgtcaca cgaatgactc** tttttcagCC TCTGGACGAG ACCAGCCAGA
 51 TGAGCGACCT CCCGTGAAG GTGATCCACG TGGAGAGTGG GAAGATCCTC
 101 ACAGGCACAG ATGCCCCCAA AGCCGGGCAG CTGGAGGCCT GGCTCGAGAT
 151 GAACCCGGGg tgagttgggc cttgcattcc **agatgcagtg gggatccaag**
 201 **t**

E13 147 bp

1 **gctggctctg agcagatttg** tatgaaagcc cttacatttt ttctagGTAT
 51 GAAGTAGCTC CGAGGTCTGA TAGTGAAGAA AGTGGCTCAG AAGAAGAGGA
 101 AGAGgtaaga gtgcatttcc tggctttcaa **ggctctcagt gcccact**

E14 198 bp

1 **ggatgaactg aggtgacatg** ggcttgtctc ttggttagGAG GAGGAGGAAG
 51 AGCAGCCGCA GGCAGCACAG CCTCCCACCC TGCCCGTGGA GGAGAAGAAG
 101 AAGATTCCAG ATCCAGACAG CGATGACGTC TCTGAGGTGG ACGCGCGGCA
 151 CATCATTGAg taaggggtcc cgacacaggt **tgttctgtgc cagcttcc**

E15 271 bp

1 **ttaccgggca cctccatctc** actcccagGA ATGCCAAGCA AGATGTCGAT
 51 GATGAATATG GCGTGTCCCA GGCCCTTGCA CGTGGCCTGC AGTCCTACTA
 101 TGCCGTGGCC CATGCTGTCA CTGAGAGAGT GGACAAGCAG TCAGCGCTTA
 151 TGGTCAATGG TGTCTCAAA CAGTACCAGg tgaggtaggg ggtggggagg
 201 ccaccggccac gtagctgcct cgggtgcagggt gttcccagggt ggtgccccgt
 251 **tcagacctca ggacagacctg a**

E16 247 bp

1 **aggaccctct ggtgtccgac** ccggccttca gtcctggcgt ggccgcatct
 51 gtccttgacg ATCAAAGGTT TGGAGTGGCT GGTGTCCCTG TACAACAACA
 101 ACCTGAACGG CATCTGGCC GACGAGATGG GCCTGGGGAA GACCATCCAG
 151 ACCATCGCGC TCATACGTA CCTCATGGAG CACAAACGCA TCAATGGGCC
 201 CTTCTCATC ATCGTGCCTC TCTCgtgagt **accgctgcc agcaaca**

E17 135 bp

1 **gactctcatt tccttgttcc** atcagaACGC TGTCCAAC TGCGTACGAG
 51 TTTGACAAGT GGGCCCCCTC CGTGGTGAAG GTGTCTTACA AGgttaggtca
 101 cagccactga ggtttcctct **ctt gctacgg aggtg**

E18 180 bp

1 **ccggtctcaa aagccgagct** gtgcatectg cttcccttgc agGGATCCCC
 51 AGCAGCAAGA CGGGCCTTTG TCCCCAGCT CCGGAGTGGG AAGTTCAACG
 101 TCTTGCTGAC GACGTACGAG TACATCATCA AAGACAAGCA CATCCTCGCC
 151 AAGgtaacgt **gtccctgtgg gaaatgccag**

E19 324 bp

1 **tgccctgatt gccactctg** gggcccgcag ATCCGTTGGA AGTACATGAT
 51 TGTGGACGAA GGTACCCGCA TGAAGAACCA CCACTGCAAG CTGACGCAGG
 101 TGCTCAACAC GCACTATGTG GCACCCCGCC GCCTGCTGCT GACGGGCACA
 151 CCGCTGCAGA ACAAGCTTCC CGAGCTCTGG GCGCTGCTCA ACTTCTGCT
 201 GCCCACCATC TTCAAGAGCT GCAGCACCTT CGAGCAGTGG TTTAACGCAC
 251 CCTTTGCCAT GACCGGGGAA AAGgtgggtt tgcccagctg tgcccagctg
 301 gacg**gttcca ggtgcggctg gctt**

E20 229 bp

1 **cccacatccg caccttctag tgagacctct** gtcgcoctcc tttggaggta
 51 acgcttgctt ctctgtctt gggggttcc agGTGGACCT GAATGAGGAG
 101 GAAACCATTC TCATCATCCG GCGTCTCCAC AAAGTGCTGC GGCCCTTCTT
 151 GCTCCGACGA CTCAGAAGG AAGTCGAGGC CCAGTTGCC GAAAAGgtga
 201 **tgaggtttt aggggagcca ccagtgaag**

E21 246 bp

1 **accttggggc ctctgtgagca ttatgtgtcc** cctgcagGTG GAGTACGTCA
 51 TCAAGTGC GA CATGTCTGCG CTGCAGCGAG TGCTCTACCG CCACATGCAG
 101 GCCAAGGGCG TGCTGCTGAC TGATGGCTCC GAGAAGGACA AGAAGgtggg
 151 ccccagctc ccccaactgc attccccact ggtgttccaa ggccggcagc
 201 gtggcagcca gagcagagcg **tgctctgacc atcgggtcat gatctg**

E22 151 bp

1 **ccatttgggt ccctctcatc** tgccttcag GGCAAAGGCG GCACCAAGAC
 51 CTGTATGAAC ACCATCATGC AGCTGCGGAA GATCTGCAAC CACCCCTACA
 101 TGTTCAGCA CATCGAGgtg agcccgcgc ggtg**ggacg gctcaggccc**
 151 **t**

E23 144 bp

1 **cctgtcactg accctctct ccttgccctg** cagGAGTCCT TTTCCGAGCA
 51 CTTGGGGTTC ACTGGCGGCA TTGTCCAAGG gtgagaagct tcccaactgg
 101 atgggggtgg caggtg**gtcc acccagaggt tttctgtcgt tttg**

E24 248 bp

1 **gtttaacatc ctgcgccttc tctcctgect** cctccacact ccagGCTGGA
 51 CCTGTACCGA GCCTCGGGTA AATTGAGCT TCTTGATAGA ATTCTTCCCA
 101 AACTCCGAGC AACCAACCAC AAAGTGCTGC TGTCTGCCA AATGACCTCC
 151 CTATGACCA TCATGGAAGA TTAATTTGCG TATCGCGGCT TAAATACCT
 201 CAGGCTTGAT Ggtgagtatg ag**ccagtgag gcgtttctta cagggtt**

E25 330 bp

1 **gtccccactc taccctgag gtcaccccgc** tgaccctgtt ctctctgtg
 51 cccgtcagGA ACCACGAAGG CGGAGGACCG GGGCATGCTG CTGAAAACCT
 101 TCAACGAGCC CGGCTCTGAG TACTTCATCT TCCTGCTCAG CACCCGGGCT
 151 GGGGGGCTCG GCCTGAACCT CCAGTCGGCA GACACTGTGA TCATTTTGA
 201 CAGCGACTGG AATCCTCACC AGgtaaaagc gggccgggccc ccaggtcag
 251 gagaaggaag ggggtgcctg caaaacctcg aggagacggc cctggcttga
 301 gggtc**ctcca gcctcctcca cattgtctt**

E26	327 bp				
1	tttatgacct cctgggctcc	tttgggactg	actggcacct	cttccccag	
51	GACCTGCAAG CGCAGGACCG	AGCCCACCGC	ATCGGGCAGC	AGAACGAGGT	
101	GCGTGTGCTC CGCCTCTGCA	CCGTCAACAG	CGTGGAGGAG	AAGATCCTAG	
151	CTGCAGCCAA GTACAAGCTC	AACGTGGACC	AGAAGGTGAT	CCAGGCCGGC	
201	ATGTTTCGACC AGAAGTCCTC	CAGCCATGAG	CGGCGCGCCT	TCCTGCAGGC	
251	CATCTTGGAG CACGAGGAGC	AGGATGAGgt	gagcccagca	ccggccccga	
301	ccccctccca gcgtgaatgg tggacgc				
E27	199 bp				
1	cctcctcagc ggcactgaca	gtttgcaatc	ttatagGAGG	AAGACGAGGT	
51	CCCCGAGCAC GAGACCGTCA	ACCAGATGAT	CGCCCGGCAC	GAGGAGGAGT	
101	TTGATCTGTT CATGgtaagc	gctgcaggct	ggatggggca	gttcaggcat	
151	cccactctgc tgccaccagg	ag caaagcag	acgtcctagt	gccccaggt	
E28	286 bp				
1	tgcctgcatg ctgatgcctc	tcccgttgcc	tccctgcccc	ccagCGCATG	
51	GACCTGGACC GCAGGCGCGA	GGAGGCCCGC	AACCCCAAGC	GGAAGCCGCG	
101	CCTCATGGAG GAGGACGAGC	TCCCCTCGTG	GATCATCAAG	GACGACGCGG	
151	AGGTGGAGCG GCTGACCTGT	GAGGAGGAGG	AGGAGAAGAT	GTTCCGCCGT	
201	GGCTCCCGCC ACCGCAAGGA	GGTGGACTAC	AGCGACTCAC	TGACGGAGAA	
251	GCAGTGGCTC AAGgtacatg	ctggagaggc	ccagca		
E29	414 bp				
1	cgacctgggt gctggctgtc	ctattttact	actattgacc	ctgaagGCCA	
51	TCGAGGAGGG CACGCTGGAG	GAGATCGAAG	AGGAGGTCCG	GCAGAAGAAA	
101	TCATCACGGA AGCGCAAGCG	AGACAGCGAC	GCCGGCTCCT	CCACCCGAC	
151	CACCAGCACC CGCAGCCGCG	ACAAGGACGA	CGAGAGCAAG	AAGCAGAAGA	
201	AGCGCGGGCG GCCGCTGCC	GAGAAACTCT	CCCCTAACCC	ACCCAACCTC	
251	ACCAAGAAGA TGAAGAAGAT	TGTGGATGCC	GTGATCAAGT	ACAAGGACAG	
301	gtaagcgagg aggcggggag	ggcgggggct	gtaggggtcc	ccgtgggagc	
351	aggcctggca tctgcactct	gactctgcac	actcaggctt	gggccgctca	
401	ctcttttcaact catc				
E30	165 bp				
1	tagccgcccgg ttctgccttg	cagCAGCAGT	GGACGTCAGC	TCAGCGAGGT	
51	CTTCATCCAG CTGCCCTCGC	GAAAGGAGCT	GCCCCAGTAC	TACGAGCTCA	
101	TCCGCAAGCC CGTGGACTTC	AAGAAGATAA	AGgtaaccct	gacgtt gtac	
151	ctgcgccccg catgt				
E31	182 bp				
1	tcctgaggta agacctgctc	ctcccgtcca	ctgcagGAGC	GCATTTCGAA	
51	CCACAAGTAC CGCAGCCTCA	ACGACCTAGA	GAAGGACGTC	ATGCTCCTGT	
101	GCCAGAACGC ACAGACCTTC	AACCTGGAGG	GCTCCCTGgt	gagggcaccg	
151	ctgggggttg gg gatgggccc	actcccacag	ct		
E32	303 bp				
1	catccactca agcccctggt	gtctctgccc	agATCTATGA	AGACTCCATC	
51	GTCTTGCAGT CGGTCTTCAC	CAGCGTGCGG	CAGAAAATCG	AGAAGGAGGA	
101	TGACAGTGAA GGCAGGAGAG	GTGAGGAGGA	GGAAGAGGGC	GAGGAGGAAG	
151	GCTCCGAATC CGAATgtgag	tcccgggggg	gttcaggacg	ccgggggttca	
201	cgtggyccc agagccccca	aggccccagc	ttttcacagc	cctcccggct	
251	cccagacgcc ccttgctgtg	gggtgctgc	attcccagag	ctcaaggctg	
301	tct				

E33 318 bp

1 **cattcccaga gctcaaggct gtctttccct** cccggtcccc tccagCTCGG
51 TCCGTCAAAG TGAAGATCAA GCTTGGCCGG AAGGAGAAGG CACAGGACCG
101 GCTGAAGGGC GGCCGGCGGC GGCCGAGCCG AGGGTCCCGA GCCAAGCCGG
151 TCGTGAGTGA CGATGACAGT GAGGAGGAAC AAGAGGAGgt gaggccgggc
201 ccccgagcag gcagagctgg catgtggcag gaggcataccc ggggccctga
251 tgggacagcc ctgtggggcg gtgctcccac gccccacgc tgcagggtggg
301 **aaagctgagg cttgacct**

E34 196 bp

1 **cggcccacat cagtgttttc** ttgagcaagt ttattttaag tttggcaggt
51 ccctggcaag gtgccctggc aggggtggcc aacgcacact ctctcctcct
101 gtccctctc cagGACCGCT CAGGAAGTGG CAGCGAAGAA GACTGAgccc
151 cgacattcca gtctcgacc cgagcc**cctc gttccagagc tgagat**