Zama_Uzumcu, Supplemental Material

Techniques used in epigenetic analyses

When examining tissues for potential epigenetic alterations, methylation has been primarily analyzed as a first step, [1; 2] since it is preserved in genomic DNA after multiple extraction and purification steps, whereas histone associations with DNA are more labile and dependent on the conditions of extraction. However, in light of the fact that in some cases, histone modifications lead the way for the establishment of epigenetic marks (see Section 2.2 in the main manuscript), techniques to detect these modifications should be performed in tandem. Numerous articles are available for detailed information on techniques used to study methylation and histone modifications to which the readers are referred [3; 4; 5]. Here we summarize, in brief, the options available for choosing an appropriate technique (see Figure 1 in the main manuscript).

1. Bisulfite treatment and sequencing

The bisulfite-sequencing technique has remained the gold standard analysis, to obtain detailed gene-specific CGI methylation information [6]. Bisulfite conversion refers to the chemical reaction where all cytosine residues are converted to uracils by deamination. This is followed by the conversion of uracil to thymine in double stranded DNA (dsDNA), after PCR. Methylated cytosines are protected by the actions of bisulfite and therefore remain as cytosines in the dsDNA. Subsequent direct sequencing of the PCR product or cloning and sequencing and comparison to the original DNA sequence identify the cytosines that were methylated vs unmethylated in the genomic DNA. More

sophisticated sequencing techniques now allow for high-throughput analysis with either typical Sanger sequencing [7] or pyrosequencing [8; 9] or mass spectrometry [10].

While detailed cancer epigenome studies have been conducted for many years [3; 11], recent environmental exposure studies have necessitated the examination of the whole epigenome and not just individual genes. Genome-wide studies are needed to identify new networks/pathways that are potentially altered due to environmental exposures. These involve either restriction enzyme (RE) digestions at methylation sensitive sites or the immunoprecipitation of methyl cytosine residues (e.g. MeDIP) followed by genomic arrays or high throughput sequencing. Combinations of these techniques alongside chromatin immunoprecipitation (ChIP) assays have given rise to a wide range of choices that can be employed to study epigenetic alterations due to environmental exposures.

2. Global methylation assays

2.1. Restriction enzyme (RE) digestion-based methods

Restriction landmark genomic scanning (RLGS), methylation sensitive restriction fingerprinting (MSRF) and methylation sensitive arbitrarily-primed PCR (AP-PCR) are RE digestion based techniques that are used to explore genome-wide methylation changes. These assays can be conducted in-house or on comparative genomic arrays as described in section 2.3.1.

RLGS. A RE digestion is conducted with the methylation sensitive NotI whose recognition site is found in nearly 2400 sequences across the genome, predominantly near CpG rich regions [12]. *NotI* digests only non-methylated CpG recognition sites, but does not digest methylated CpG sites. Following further digestion with *HinfI* and *EcoRV*, 2-D

gel electrophoresis, and autoradiographic detection, the status of methylation in the specific RE products from different samples, is characterized by presence/absence of spots and their intensity, between the samples. After the changes are documented, the RE products can be sequenced and matched to genome databases for identification [13; 14; 15]. The advantage of using RLGS is its ability to identify thousands of landmarks in a single run, in a copy number specific manner. However, the use of *NotI* RE site alone as an identifying parameter restricts the potential candidates to be only in CpG rich regions and only visually identifies hypomethylated sites. On the other hand the latter can be an advantage since most other assays demonstrate the presence of hypomethylated sequences by the absence of a signal, for which there can be a myriad of confounding technical reasons.

MSRF. In MSRF, genomic DNA is subjected to *MseI* digestion, which does not cut in CpG rich regions but allows for genomic DNA to be further digested. Subsequently, *BstUI* (a methylation-sensitive enzyme) RE digestion is conducted on an aliquot of the *MseI* digested sample. Since *BstUI* will digest only those sites that are not protected by methylation, a differential display of products is possible after PCR and polyacrylamide gel electrophoresis (PAGE). Potential candidates are then isolated followed by reamplification of the samples, sequencing, and database searches that reveal the identities of the candidate genes [1].

AP-PCR. AP-PCR is very similar to MSRF except that a pair of restriction enzymes that are isoschizomers are employed. There are various combinations of methylation-sensitive and insensitive enzymes that can be used, the most common ones being *HpaII* and *MspI*,

respectively (another example: methylation-sensitive *SmaI* and methylation-insensitive *XmaI*). In brief, genomic DNA is digested with an RE (global cutter) in combination with methylation-sensitive *HpaII* that cuts only if both cytosines in the site are unmethylated, or methylation–insensitive *MspI* enzymes that only cuts at the outer cytosine regardless of the methylation status [16]. This is followed by PCR using degenerate primer sets designed to amplify methylation sites PCR products are separated using PAGE and visualized by SYBR green nucleic acid staining. Hypo- or hypermethylation is determined by the relative band intensity or presence/absence of bands between the various samples. The differentially amplified products are isolated, reamplified, and sequenced. The identity and chromosomal location is determined by sequence alignment to known genome databases [2; 17].

Variations of RE digestion based methods. HpaII tiny fragment enrichment by ligation-mediated PCR (HELP) and differential methylation hybridization (DMH) are variations on the technique employing *HpaII* and *MspI*, except that the RE-digested DNA is amplified, ligated to adapters, and labeled with fluorescent dyes or radioactive moieties such as ^{32}P, and used as probe(s) on high density genomic arrays (HELP) or low density arrays (DMH) [18; 19; 20].

2.2. Immunoprecipitation-based methods

2.2.1. Methylated DNA immunoprecipitation (MedIP) and methyl binding domain affinity purification (MAP)

In MedIP, an anti-methylcystosine antibody (usually a monoclonal antibody) is used to immunoprecipitate methylated DNA providing an unbiased means to enrich methylated genomic DNA. This method can provide a 90% enrichment of the methylated DNA in a dose-dependent, sequence-independent manner. Immunoprecipitated DNA and input DNA are labeled differentially and competitively hybridized to genomic arrays (see section below, [21; 22]).

Alternatively, in MAP, an affinity column that contains the methyl-CpG binding domain of the MeCP2 protein is prepared that allows for the isolation of CGIs from genomic DNA [23]; this principle is being used extensively in epigenome analysis [24]. Similar to MeDIP, the MAPed DNA and the input DNA are then labeled with different dyes and hybridized to the genomic arrays to determine differentially methylated sequences.

2.3. Detection methods

2.3.1. Array-based technologies–comparative genomic arrays/CGI arrays

The techniques mentioned in section 2.1 provide potential candidates that are isolated, sequenced and then identified by matching to genome databases which is time consuming and low-coverage. With microarray technology, the platform is widened to encompass large-scale genome analysis with multiple regions of the same gene or the whole genome arrayed on a chip. For example, in Affymetrix GeneChip arrays, methylation of a single gene or limited number of genes that span short regions of the genome can be examined. Multiple short oligonucleotides that are designed to anneal to the methylated or unmethylated versions of the queried sequence are synthesized and arrayed on a chip. RE-digested, immunoprecipitated DNA or bisulfite-treated DNA from different samples

are labeled with a fluorescent dye. After hybridization with the oligonucleotide array, the signal intensity at each spot is compared between the methylated and unmethylated CGIs, which is then expressed as a percentage for the whole gene. Since this method provides good coverage of each queried sequence, resolution of 2-3 CpGs can be obtained [25]. However, these arrays require single channel hybridization i.e., running each sample separately and conducting at least three repeats. On the other hand, there are recently available commercial arrays from Nimblegen and Agilent that employ dual channel (two samples) hybridization: the treated DNA vs the native input DNA are labeled with different fluorescent dyes and hybridized simultaneously thus reducing inter-array variations and need for technical replicates. These rely on arrays with longer oligonucleotide sequences (~60 mers vs the ~20 mers used in Affymetrix arrays) covering the promoter region CGIs alone or CGIs both upstream and downstream of the transcription start site or the whole genome. Furthermore, custom arrays are available with these platforms making them more attractive for examining whole pathways that could be queried at one time [5; 18; 26].

2.3.2. Deep sequencing/pyrosequencing

Multiple generations of high throughput methylation arrays have now been more empowered with the advent of these very sophisticated sequencing techniques. Massively parallel sequencing of thousands to millions of DNA sequences at a time using variations of the classical Sanger technique or pyrosequencing are now available. These allow for very extensive coverage and higher resolution and also bypassing any pretreatments or hybridization to arrays.

Platforms are now available to conduct deep sequencing, using pyrosequencing (for example, 454 Sequencing, from Roche) that involves RE digestion with methylation sensitive and insensitive isoschizomers and subsequent polymerase reactions. As the nucleotides are added, inorganic pyrophosphate (PPi) is released and converted to ATP by ATP-sulfurylase and adenosine-5-phosphosulfate. This reaction is coupled to the conversion of luciferin to oxyluciferen by luciferase and ATP to generate a proportional amount of visible light, which is quantified by a charge-coupled-device camera in the pyrosequencer. Since the restriction digests have differential number of cut sites and overhangs, the signal intensities corresponding to individual nucleotides are representative of the methylated cytosine vs the unmethylated cytosine. This method is able to provide up to 400,000 reads of over 100 bases per run [8]. An alternative system (e.g., Illumina Genome Analyzer, generally referred to as Solexa sequencing system) employs bead arrays, which provide shorter runs of 25-35 bases but up to 40 million reads. Shotgun sequencing is conducted after genomic DNA is fragmented, ligated to PCR primer adapters, and bisulfite converted [27; 28; 29].

2.4. Detection of histone modifications

2.4.1. Chromatin immunoprecipitation (ChIP)

Analysis of histone modifications is usually conducted with ChIP analysis in addition to the widely employed immunohistochemistry [24]. In brief, ChIP involves the isolation of chromatin from cells that are treated with crosslinking reagents to covalently link the DNA-binding proteins to chromatin. After the cells are lysed, the genomic DNA is isolated and sonicated to obtain sheared chromatin. Immunoprecipitation is conducted

with an antibody against the protein of interest and the DNA fragments isolated. This DNA can then be used in quantitative real time PCR (QPCR) to quantify the target DNA or on other cutting edge platforms such as ChIP–chip or ChIP-seq. The former involves hybridization to a tiling array and the latter involves high throughput /deep sequencing of the DNA fragments [30; 31].

Micro-RNA studies employ classical mRNA detection methods such as Northern hybridizations and microarray analysis. We refer the readers to a recent article by Hunt and colleagues on new, more sensitive and reliable techniques being developed to detect and study micro-RNAs [32].

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