Loss of heterozygosity analyzed by single nucleotide polymorphism array in cancer

Hai-Tao Zheng, Zhi-Hai Peng, Sheng Li, Lin He

INTRODUCTION
Cancer arises from the accumulation of inherited polymorphism (i.e. SNPs) and mutation and/or sporadic somatic polymorphism (i.e. non-germline polymorphism) in cell cycle, DNA repair, and growth signaling genes[1]. Neoplastic progression is generally characterized by the accumulation of multiple somatic-cell genetic alterations such as the tumor progresses to advanced stages[2,4]. The classic mechanism of tumor suppressor gene inactivation is described by the two-hit mode in which one allele is mutated (or promoter hypermethylation or a small intragenic deletion) and the other allele is lost through a number of possible mechanisms, resulting in the loss of heterozygosity (LOH) at multiple loci[16,17]. Loss of heterozygosity is the most common molecular genetic alteration observed in human cancers. In the model of colorectal tumorigenesis, mutational inactivation of tumor suppressor genes predominates[18].

Loss of heterozygosity and studying methods
LOH is caused by a variety of genetic mechanisms, including physical deletion of chromosome nondisjunction, mitotic nondisjunction followed by republication of the remaining chromosomes, mitotic recombination, and gene conversion. The mechanisms of LOH are remarkably chromosome-specific. Some chromosomes display complete loss. However, more than half of the losses are associated with the loss of only a part of the chromosome rather than the whole chromosome[13]. LOH is also a common form of allelic imbalance and the detection of LOH has been used to identify genomic regions that harbor tumor suppressor genes and to characterize different tumor types, pathological stages, and progression[4,19].

In addition to the inherited and sporadic polymorphisms, many tumors exhibit aneuploidy and chromosomal instability in which the diploid structure of the genome is corrupted. A modest increase in copy number (such as trisomy for a region) would not give rise to allelic imbalance in the SNP assay. Allelic imbalance in the SNP assay should thus usually indicate true LOH, except in the case of extreme amplification[10].

Global patterns of LOH can be analyzed through allelotyping of tumors with polymorphic genetic markers from each chromosomal arm[17]. Most investigations have concentrated on defining the minimal regions of loss of specific chromosomes in various cancers in an effort to identify the putative tumor suppressor genes targeted by the losses. Two allele RFLPs and Southern analysis give way to simple sequence length polymorphisms such as PCR-based microsatellite, and both have been proved to be reliable genetic markers for studying LOH[18]. RFLP markers have low heterozygosity rates and are available in small number, gel-based microsatellite assay is difficult to automate and not readily scalable[19]. Microsatellite markers are reliable genetic markers for studying LOH, but only
a modest number of SSLPs are used in LOH studies because the genotyping procedure is rather tedious and difficult to automate and are not readily scalable.

As a result, most genome-wide scans for LOH have been conducted at low resolution with a relatively small number of polymorphic markers. Previous allelotyping analysis of cancer by many groups was restricted to particular chromosomal regions or arms, or else used a relatively low density of markers. For example, an average of 120 microsatellites has been used to determine the allelotype of multiple different human neoplasms in a series of studies since 1995, and the highest density microsatellite allelotype is ~280 polymorphic markers before the year 2000.[12,13]

We conducted a genome-wide LOH study of 83 tumor samples obtained from Chinese patients in sporadic colorectal cancer. We employed 400 fluorescence-labeled microsatellite marker primers to amplify the corresponding loci of genomic DNA and then electrophoresed the polymerase chain reaction products and analyzed the fluorescent signals. The LOH frequencies were high (>35%) but not associated with the tumor stage and progression in 20 loci. Loss of other loci, including two narrow regions on chromosome 2, was related to the tumor stage.[15,16] In some loci, we performed detailed deletion mapping to narrow the loss region.

SNPs are the most common form of sequence variation in human genome, occurring approximately in every 1 200 bp.[17] SNPs may occur at more than 2 million sites in the genome, thus making it possible to place SNPs at high density along the genome.[18]

High-throughput polymorphism detection technologies hold great promise for the characterization of complex diseases including cancer. High-density mapping of genetic losses reveals potential tumor suppressor loci and might be useful in the clinical classification of individual tumors. SNP array has been introduced recently for genome-wide screening of chromosome imbalance.

Higher density SNP array can be used effectively to detect small regions of chromosomal changes and provide more information regarding the boundaries of loss regions. In addition, more markers increase confidence in a detected event. If multiple adjacent SNPs show a consistent change, the confidence in the call is much higher than when it is based on a single SNP.[19]

HuSNP chip (the first generation of SNP chip), an array of oligonucleotide probes for 1494 SNP loci, is distributed in all human chromosomes with an average of 2.57 cm between each SNP markers. A recent study using microarray has demonstrated a 97% accuracy on the basis of the collected hybridization signals using Affymetrix HuSNP 3.1 software. Tumor and normal samples are allelotyped on separate chips. For each patient's tumor, each SNP allele is scored as LOH, retention of heterozygosity, uninformative, or uncertain by comparing the genotype calls for tumor and normal samples.

The DNA sample is subjected to 24 multiplex PCR reactions, the resulting products are pooled, hybridized to the SNP array, stained with streptavidin-phycoerythrin, and assayed by fluorescence detection. Briefly, the detector for each SNP locus contains four rows of 25-mer oligonucleotides, two of which contain oligonucleotides that perfectly match either SNP allele A or SNP allele B, whereas the other two contain single-base mismatches at various positions. The allelotype at a locus is determined by fluorescence intensity ratios in an automated fashion. Affymetrix HuSNP mapping system is used to determine tumor and normal allelotypes.

A general scanner scans chips and genotyped “call” is made from the collected hybridization signals using Affymetrix HuSNP 3.1 software. Tumor and normal samples are allelotyped on separate chips. For each patient's tumor, each SNP locus is scored as LOH, retention of heterozygosity, uninformative, or uncertain by comparing the genotype calls for tumor and normal (autologous) pairs. The possible SNP calls made by Affymetrix genotyping software are A, B, AB, AB_A (i.e,
AB or A), AB_B (i.e., AB or B), and "no call". "no call", AB_A, AB_B calls are considered to be noninformative[33].

Both amplified and unamplified DNA give similar results in terms of SNP call and LOH[30]. LOH can be established or inferred from 10K SNP array data using only amplified tumor DNA with the Affymetrix Genechip chromosome copy number tool.

**SNP array application in LOH detection**

Using SNP detection array, Wang et al.[14] found that breast cancer is highly heterogeneous, with the proportion of LOH ranging widely from 0.3% to >60% of heterozygous markers.

The call rate is 74.9-83.2% over all samples, yielding 1 120–1 205 SNPs scored per sample using HuSNP array[14,34,37,38]. The median of heterozygous loci is 341-349 with an average coverage of one SNP per 7.9-8.7cm[8,37]. Using 10K SNP array, the call rate is 91.1% over all samples[9]. In lung and breast cancer, the average call rate does not vary significantly between the lymphoblastoid and tumor cell lines[14,37].

**LOH result comparison between SNP array and microsatellite**

Very few reports have presented allelotyping data on multiple sites in the same tumor using two different methods, LOH between SNP array and microsatellite is concordant in the majority of analyzed kinds of cancer samples[14,30,34]. Most affected LOH regions are consistent with those in previous LOH studies, lending validity to both the method and results[10].

The range of consistency between two methods in different loci varies from 50% to 100% in bladder cancer[30]. Moreover, when the two methods are compared by chromosome arms, the concordance is very robust[14]. In osteosarcoma assay, 14 of 18 microsatellite markers have associated SNPs with LOH[14].

By comparing the microsatellite results in selected areas of several chromosomes with SNP array-based detection of allelic imbalance, in 69 sites, 60 microsatellite markers correlate, but nine microsatellite markers do not correlate with adjacent HuSNP markers[33].

Janne et al.[8] found that neither HuSNP nor SSLP is perfect. Using two methods together, the combined informative rate is 84%, and both methods provide calls for loci that were not informative by the other methods. However, a combined analysis is unlikely to be practical for future studies.

The comparison shows that, given a sufficient number of polymorphic markers, the SNP array can be used to screen both small and large chromosomal losses. But neither technique is currently infallible in identifying LOH[14].

**LOH conflict between SNP array and microsatellite**

Lindblad-Toh et al.[14] examined a number of instances of apparent conflict between SSLPs and SNP-based analysis by repeating the analysis and found that discordance is slightly more often due to the errors in SSLP rather than in SNP genotyping. SNP genotyping thus appears to be at least as accurate as the SSLP approach.

SNPs associated with the remaining four microsatellite markers do not show any LOH[30]. Allelic imbalance has been detected in microsatellite analysis but not detected by the SNP, which is probably caused by a no-signal genotype call either in the tumor or in normal DNA or in both. This problem can be solved by increasing the number of SNPs for the specific loci and by developing a more sensitive method for the generation of calls[44].

**Possible reason of discrepancy**

Because of the lower average heterozygosity rate of SNPs (0.33) compared to microsatellite, approximately a threefold SNPs is required for an equivalent resolution.

It is difficult to determine whether the apparent discordance is due to the technical limitation or if the microsatellite markers recognize a smaller region with a different allelic loss pattern compared to the adjacent regions scored by SNP[30].

The possible reasons are as follows: limitation of mapping data; differences in resolution, amplification efficiency, and differential sensitivity between microsatellite and SNP, technical limitations such as a genotype call by the Affymetrix softwares, the presence of bad SNPs in the array[14,36].

**Cancer classification by LOH pattern using SNP array**

Finding unique LOH pattern by SNP array in different groups of breast cancer, in part defined by expression signature, adds confidence to newer schemes of molecular classification. Furthermore, exclusive association between biological subclasses and restricted LOH event provide rationale to search for targeted genes[37]. Janne et al[8] demonstrate that clustering of LOH data can distinguish SCLC from NSCLC with reasonable accuracy.

**Advantage of SNP array**

SNP array assay is accurate, automatic, and readily adaptable to the clinical setting and high-density mapping. Analysis of genetic alterations with HuSNP assay saves considerable time over microsatellite analysis. The assay involves multiplex amplification and other methods that can be completed in one day. The SNP array method is also a molecular technique that allows the detection of chromosomal imbalance in tumor DNA. A minimal quantity (120–135 ng) of sample DNA is needed for each SNP assay. The amplification step makes it possible to use only a small amount of genomic DNA, which is often essential when working with limited clinical materials[14,33].

The 10K array also provides calls (either LOH or retention) for 71.7% and 22.3% of the loci identified as non-informative by HuSNP and SSLP, respectively. The proximal and distal ends of the deletion are clearly identified and single LOH events identified using SSLP fall within these regions. The mapping 10K array can identify more than twice the number of LOH regions compared to SSLP or HuSNP. The minimum, mean, and median sizes of these regions are substantially smaller by the mapping 10K array than by the other two methods. The maximum
size of the LOH regions is similar by the three methods\[^8\]. Disadvantage of SNP array is

SNP array is difficult to distinguish all polymorphisms and to detect low level polymorphism and requires PCR amplification.

The average proportion of LOH informative markers out of callable markers is 31–33\%, which is a considerably lower heterozygosity rate than that of SSLPs (typically 70\%), but can readily increase to about 50\% by selecting SNP with higher heterozygosity\[^15,16\].

High false positive rate (11–21\%) and false negative rates (19.9\%) have been observed with this technology, limiting its utility in both SNP and tumor analysis\[^8\],\[^10,11\].

Array-based methods of SNP detection may have a certain degree of inaccuracy ("noise"), and moreover, the precise genomic mapping of each SNP is still not completely stable. Thus, “true” regions of LOH can be interrupted by apparently false positively “retained” SNP alleles. Conversely, true regions of retention of heterozygosity may be interrupted by false LOH calls\[^15\].

In summary, with the increasing number of SNPs available and technical progression\[^11\], it is possible to probe the entire genome, and specific regions at much higher resolution. SNP array hybridization is an accurate and efficient method for evaluating genome-wide tumor LOH at present.

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